AN ABSTRACT OF THE THESIS OF

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Title: Isolation and Characterization of DNA-damage-repair/toleration Genes from *Arabidopsis thaliana*

Abstract approved: Redacted for privacy
John B. Hays

Arabidopsis thaliana was used as a model green plant for studies of DNA damage and repair following UV irradiation. Research results indicated the following: (1) UV (254 nm) fluences that result in measurable cyclobutane pyrimidine dimer (CBPD) levels (about one CBPD per 2 X 10^4 nucleotides) have significant physiological effects; (2) photoreactivation is the predominant pathway of UV-induced damage repair in *Arabidopsis*; (3) *Arabidopsis* photolyase levels are increased significantly by UV-B irradiation; (4) *Arabidopsis* photolyase is markedly temperature-sensitive, both *in vitro* and *in vivo*.

A genetic technique was developed to isolate *Arabidopsis* cDNAs encoding putative plant DNA-damage-repair/toleration (DRT) activities by partial complemetation of *E. coli* DNA-repair-deficient mutations. The initial work yielded six unique cDNAs, probably encoding four distinct classes of

activities: (1) *DRT100* increased resistance of mutant bacteria to UV irradiation, mitomycin C and methyl methanesulfonate (MMS) treatments, supported the growth of λ*red*⁻ *gam*⁻ and P1 phages, and promoted conjugational recombination in RecA⁻ bacteria; (2) the activities encoded by *DRT101* and *DRT102* appear to be UV-specific, since they fail to provide resistance to DNA damage by mitomycin C and MMS; (3) Drt103 conferred UV resistance in a light-dependent manner, but its amino acid sequence shows only marginal similarity to those of microbial photolyases; and (4) *DRT111* and *DRT112* increased resistance of *E.coli* resolvase-deficient mutants to UV, mitomycin C, MMS and nitroquinoline oxide, and partially restored the ability of these mutants to carry out conjugal recombination.

The transcript levels of the *Arabidopsis DRT* genes were examined in response to various DNA-damaging agents. Levels of *DRT100* mRNA increased in plants treated with UV-B light, mitomycin C and MMS. The transcript levels of *DRT101*, whose activity is specific for UV-induced DNA damage in *E.coli*, became elevated in response not only to UV-B irradiation but also to mitomycin C treatment.

Isolation and Characterization of DNA-damage-repair/toleration Genes from *Arabidopsis thaliana*

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Ap:	ampicillin
AP:	apurinic/apyrimidinic
Ap^R:	ampicillin-resistant
Ap^S:	ampicillin-sensitive
CBPD:	*cis syn*-cyclobutane pyrimidine dimer
cDNA:	complementory DNA
CFU:	colony-forming unit
CHS:	chalcone synthase
Cm:	chloramphenicol
CTAB:	cetyltrimethylammonium bromide
DNA:	deoxyribonucleic acid
DRT:	DNA-damage repair/toleration
EDTA:	ethylenediaminetetraacetic acid
ESS:	endonuclease-sensitive site
IPTG:	isopropyl-thio-B-D-galactoside
Kn:	kanamycin
Kn^R:	kanamycin-resistant
kDa:	kilodalton
LRM:	leucine-rich motif
miniprep:	crude small-scale plasmid preparation
MMS:	methyl methanesulfonate
mRNA:	message RNA
MSS:	Murashige-Skoog salt
MW:	molecular weight
ng:	nanogram (1 gram X 10^{-9})
NQO:	4-nitroquinoline-N-oxide
ORF:	open reading frame
PCR:	polymerase chain reaction
PFU:	plaque-forming unit
RNA:	ribonucleic acid
Tris:	2-amino-2(hydroxymethyl),1,3-propanediol
Tc:	tetracycline
Tc^R:	tetracycline-resistant
UV:	ultraviolet
µg:	microgram (1 gram X 10^{-6})
µl:	microliter (1 liter X 10^{-6})
X-gal:	5-bromo-4-chloro-3-indoyl-β-D-galactoside
XP:	xeroderma pigmentosum

ISOLATION AND CHARACTERIZATION OF DNA-DAMAGE-REPAIR/TOLERATION GENES FROM *ARABIDOPSIS THALIANA*

I. INTRODUCTION

DNA is subject to damage produced by environmental agents,such as radiation and reactive chemicals, and by endogenous events occurring naturally in the cellular environment. The survival of an organism depends on the exceptionally precise duplication of its genome. To achieve this precision, the DNA polymerase must synthesize DNA with extraordinary accuracy. Equally important, a variety of repair mechanisms are needed to remove DNA lesions ahead of the replication fork and to correct mistakes left behind by the replication mechinery.

The molecular basis of DNA repair is best understood in bacteria, partly because of the well-defined genetic systems. Insights derived from bacteria are applicable to eukaryotes. Repair activities that recognize various alterations in DNA, and also other important forms of damage, such as the most common radiation-induced lesions, presumably appeared very early during evolution, as they seem to be universally distributed in living cells. When DNA repair enzymes catalyzing relatively uncomplicated steps are isolated from

higher organisms, their mechanisms of action are similar or identical to their bacterial counterparts. The more complicated multi-enzyme nucleotide excision pathway in *Escherichia coli* acts on DNA damaged by a spectrum of damaging agents so as to yield so-called bulky adducts. Although a similar system to that in *E.coli* has yet to be identified in higher cells, both *E. coli uvr* mutants and DNA repair-deficient human cell lines (e.g. Xeroderma pigmentosum) show similar sensitivities to the same spectrum of bulky adduct damage (Grossman, *et al.*,1988).

With the advent of recombinant DNA technologies, DNA repair genes and their products have been identified, mapped, isolated, sequenced and examined in great detail in numerous biological systems, including bacteria, yeast, *Drosophila*, rodent and human cells. On the other hand, DNA repair mechanisms in plants have been less readily demonstrated, and only in the last year have a few plant repair genes been cloned, sequenced, and functionally characterized.

A. Mechanisms of DNA Repair

1. Direct Reversal of DNA Damage

Photoreactivation. The repair of DNA has been extensively examined in relation to damage induced by ultraviolet (UV) light, partially because a certain UV spectrum is a component of solar radiation and thus a naturally occurring DNA-damaging agent. Although there are a

variety of other photoproducts in UV-irradiated DNA, the *cis, syn* cyclobutane pyrimidine dimer (CBPD) is thought to be one of the most biologically significant lesions. CBPDs result when the double bonds of the C^5 and C^6 carbon atoms of two adjacent pyrimidines become saturated, resulting in the formation of four-membered ring. The two pyrimidines are thus covalently linked (Varghese and Wang, 1967). The biological importance of CBPDs has long been demonstrated by the fact that the transforming activity of UV-irradiated *Haemophilus influenzae* DNA increases following the monomerization of these lesions by nonenzymatic photoreversal (Setlow and Setlow, 1962). Also, the removal of CBPDs from DNA by treatment with the photoreactivating enzyme, photolyase, which is only effective on this type of lesion, reduces the lethal effects of UV (see sections below). Another DNA photoproduct of UV thought to be biologically significant is pyrimidine-(6-4)-pyrimidone photoproduct (6-4 photoproduct). This lesion, in which the C^6 position of a 5′ pyrimidine is covalently linked the C^4 position of an adjacent 3′ pyrimidine, occurs at TC, CC, infrequently at TT, and not at CT, sequences in UV-irradiated DNA (Franklin *et al.*, 1982). Although it does not occur as frequently as CBPDs, the 6-4 photoproduct may be more important biologically. It is thought to be closely involved with the mutational effects of UV irradiation. It is known that UV-induced mutations at TC and CC sequences in *E.coli* DNA closely coincided with sites of 6-4 photoproducts (Brash and Haseltine, 1982).

The lethal and mutagenic effects of UV-induced CBPDs can be photoreversed by subsequent exposure of damaged cells to specific wavelengths of visible light (Husain and Sancar, 1987). Photoreactivation (Fig. 1) is mediated by the enzyme photolyase. It specifically binds to CBPD-containing DNA, then obsorbs photons of a specific frequency unique to each enzyme, and finally breaks the covalent bond attaching the two pyrimidines in the cyclobutane ring (Sancar and Sancar, 1987). Photolyases from *E. coli* (Sancar and Sancar, 1984), *Saccharomyces cerevisiae* (Sancar *et al.*, 1987), *Anacystis nidulans* (Eker *et al.*, 1990), *Methanobacterium thermoautotrophicum* (Kiener *et al.*, 1989), and *Scenedesmus acutus* (Eker *et al.*, 1988) have been purified to near-homogeneity; all have molecular weights from 50000 to 60000. The genes for the *E. coli* (Sancar *et al.*,1984), yeast (Sancar, 1985), and *A. nidulans* (Yasui *et al.*, 1988) enzymes have been cloned and sequenced. The proteins of these cloned genes have also been overproduced.

Photolyases bind to CBPD-containing DNA with high affinity. The in vitro experiments showed that photolyases from *E. coli, M. thermoautotrophicum* and *S. cerevisiae* interact with DNA in the same manner; the phosphodiester bond 5′ and the three or four phosphodiester bonds 3′ to the dimer are contacted (Husain *et al.*, 1987, Kiener *et al.*, 1989, Baer and Sancar, 1989). All the significant contacts made by the three photolyases are on the damaged strand; single- and

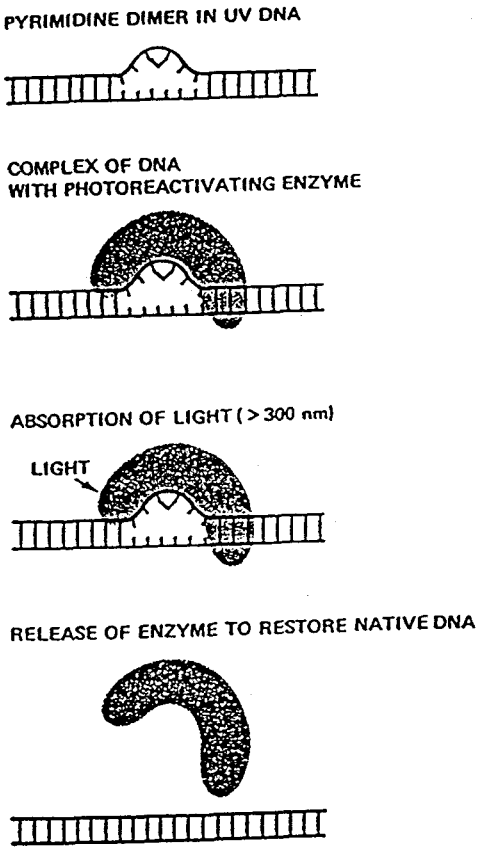

Fig.1. Model for enzyme-catalyzed photoreactivation. Photolyase binds to CBPDs in a light-independent manner, absorbs a photon, reverses the dimer, and dissociates from the DNA. Reproduced, with permission, from Friedberg, E.C. (1985).

double-stranded DNAs are repaired with equal efficiency. Binding of photolyase to CBPDs in DNA makes these lesions more accessible to *E. coli* ABC excinuclease (Sancar *et al.*, 1984), and increases the efficiency of excision repair in *E. coli* (Yamamoto *et al.*, 1983, Hays and Lee, 1985). Similarly, yeast photolyase appears to stimulate yeast nucleotide excision repair *in vivo* (Sancar and Smith, 1989).

All photolyases studied thus far contain two chromophores (Sancar and Sancar, 1987), and each enzyme falls into one of two classes according to its chromophore composition. The folate class enzymes, including those from *E. coli* and *S. cerevisiae* (Johnson *et al.*, 1988), contain $FADH_2$ and folate, whereas the deazaflavin enzymes, of which *A. nidulans*, *Streptomyces griseus* (Eker *et al.*, 1981), *S. acutus* (Eker *et al.*, 1988), and *M. thermoautotrophicum* (Kiener *et al.*, 1989) are members, contain FADH and deazaflavin. The most effective wavelengths for photoreactivation of dimers *in vivo* in *E. coli* are 365-400 nm (Samson *et al.*, 1988). Action spectra for photoreactivation obtained with purified yeast photolyase I exhibit a single peak at 365-380 nm (Madden and Werbin, 1974), while those for *S. griseus* (Eker *et al.*, 1986) and *A. nidulans* (Eker *et al.*, 1990) photolyases are at 443 and 440, respectively. The presence of $FADH_2$ in *E. coli* and yeast enzymes suggests that it plays a crucial role in photolysis, and electron donation to the dimer is a cental common feature of the action mechanism. The second chromophores (pterin or

deazaflavin derivatives) are the major light-absorbing cofactors and may function by transfer of energy to $FADH_2$ or may directly transfer an electron to the dimer.

It should be noted that photoreactivation may also occur in the absence of enzymes, and can be promoted by tryptophan-containing peptides (proteins) (Sutherland and Griffin, 1980). Thus it is important to distinguish between photolyases, which catalyze the process efficiently, and proteins that promote the reaction in a relatively unspecific manner (Lindahl, 1982).

Transmethylation. DNA is a strong electophile and, therefore is subject to modification by many alkylating agents, such as methyl methanesulfonate (MMS), nitrosourea, nitroso-guanidine, and S-adenosyl-methionine (Barrows and Magee, 1982). Alkylation damage is repaired by direct removal of the alkyl group as well as by the excision of the modified base or neighboring nucleotides (Samson *et al.*, 1988). In *E. coli*, the methylated DNA is directly repaired by two DNA methyltransferases, the Ada and Ogt proteins. The *ada* gene has been cloned and sequenced and its product greatly overproduced (Demple *et al.*, 1985, Bhattacharyya *et al.*, 1988); the protein is a monomer, molecular weight(MW) of 38,000, which has a strong preference for dsDNA containing O^6-mGua. The Ogt protein is a monomer of 171 amino acids with a MW of 19000. The *ogt* gene has also been cloned and sequenced (Potter *et al.*, 1987). The Ogt protein shows about 40% identity over its

C-terminal half with the corresponding region of the C-terminal fragment of the Ada protein. The *ogt* gene is expressed constitutively, and the activity of the enzyme does not increase upon treatment with alkylating agents (Rebeck *et al.*, 1988).

2. Base Excision Repair

Treatment of cells with physical or chemical agents yields various damaged bases in DNA which can be excised by DNA glycosylases; an apurinic/apyrimidinic (AP) site thus produced is repaired by sequential reactions catalyzed by AP endonuclease, exonuclease, DNA polymerase and ligase. This process, called base excision repair, functions not only for damaged bases, but also for abnormal bases, such as a uracil, in DNA.

DNA Glycosylases. DNA glycosylases can be defined as enzymes which catalyze hydrolysis of an N-glycosylic bond linking a base to the deoxyribose-phosphate backbone of DNA. They can be classified into two groups, one possessing only a glycosylase activity and the other carrying both glycosylase and AP endonuclease activities. Uracil-DNA glycosylases and 3-methyladenine-DNA glycosylases are examples of the former class, while T4 endonuclease V and *E. coli* endonuclease III are representatives of the latter class.

Some enzymes contain both DNA glycosylase and AP endonuclease activities in the same polypeptide. The

bacteriophage T4 endonuclease V and the *Micrococcus luteus* UV endonuclease are specific for UV-induced CBPDs. They work by the same mechanism: (1) cleavage of the glycosylic bond of the 5′ pyrimidine of the dimer and (2) cleavage of the phosphodiester bond 3′ to the apyrimidinic sugar. The two enzymatic activities of both T4 and *M. luteus* endonucleases can be easily uncoupled (Liuzzi *et al.*, 1987). When the enzyme reaction is carried out at high pH (8.0-8.5), substantial uncoupling occurs; that is, molecules accumulate with a cleaved glycosylic bond and intact AP site, and the AP endonuclease activity can be completely inhibited by modification of the AP site with methoxyamine. The gene for T4 endonuclease V (*denV*) has been cloned and sequenced, and overproduced in *E. coli* (Valerie *et al.*, 1984, Recinos *et al.*, 1986, Chenevert *et al.*, 1986); it encodes a protein of 138 amino acids with an MW of 16078. But this enzyme, which is extremely specific for UV-induced CBPDs, has no homology to another dimer-specific enzyme, DNA photolyase. Recently, Hamilton *et al* (1992) reported that a yeast anolog, redoxyendonuclease, recognizes and cleaves CBPDs and AP sites in a manner similar to T4 endonuclease V.

Some other DNA glycosylase-AP endonucleases have broad substrate specificities. These enzymes have a similar catalytic mechanism. They release damaged bases by DNA-glycosylase action, and then cleave the phosphodiester bond 3′ to the abasic sugar. Among them, *E. coli* endonuclease III is

the best characterized. The enzyme, which has been purified to homogeneity (Cunningham and Weiss, 1985), is a monomeric protein of MW of 23,546. Its crystal structure has recently been resolved to 2.0 Å resolution (Kuo *et al.*, 1992), and may provide a structural basis for studying mechanisms of damaged-DNA recognition and of the glycosylase-AP endonuclease. It has an absolute requirement for dsDNA, but has no cofactor requirement and is active in the presence of EDTA (Weiss and Cunningham, 1985). Substrates for the enzyme are generated by UV, ionizing radiation, and some chemical oxidants such as OsO_4 and permanganate (Asahara *et al.*, 1989). In contrast with dimer-specific DNA glycosylase-AP endonucleases, the glycosylase and AP endonuclease functions of endonuclease III are tightly coupled; AP sites do not accumulate during the reaction of the enzyme with DNA containing pyrimidine dimers. Similar enzymes from yeast (Gossett *et al.*, 1988), and mammalian cells (Doetsch *et al.*, 1987, Kim and Linn, 1989) have been purified, and well characterized. These enzymes share common properties: MW of 25,000-47,000, a strict requirement for dsDNA, and no cofactor or divalent metal ion requirement.

AP Endonucleases. Another class of endonucleases respond to the presence of AP sites in DNA that have been generated by the action of DNA glycosylases, or by spontaneous, or physical or chemical agent-induced, depurination and depyrimidination. These enzymes hydrolyze the

phosphodiester bond either 5′ or 3′ to the AP site, generating 3′-hydroxyl and 5′-phosphoryl, or 3′-phosphoryl and 5′-hydroxyl termini (Myles and Sancar, 1989). Two of the most extensively investigated AP endonucleases are *E. coli* exonuclease III and endonuclease IV. Exonuclease III is the major *E. coli* AP endonuclease, accounting for 85-90% of total AP endonuclease activity in cell-free extracts (Kow, 1989). It is encoded by the *xth* gene, which has been cloned and sequenced (Saporito *et al.*, 1988). The *nfo* or *xth* single mutants are only marginally sensitive to UV and ionizing radiation; however, the combination of *xth* and *nfo* mutations makes cells extremely sensitive to these agents, suggesting that the two enzymes constitute a backup system for dealing with damage caused by radiation (Cunningham *et al.*, 1986). The *xth nfo uvrA* triple mutation is lethal (Saporito *et al.*, 1989).

AP endonucleases have been purified from yeast (Armel and Wallace, 1984), *Drosophila* (Spiering and Deutsch, 1986), bovine (Sanderson *et al.*, 1989) and human (Kane and Linn, 1981) cells. These proteins act in a manner similar to that of *E. coli* exonuclease III. Recently, the gene for human AP endonuclease (*HAP1*) has been cloned and sequenced (Robson and Hickson, 1991). The protein shows only about 20% identity with *E. coli* exonuclease III, but corrects DNA repair and mutagenesis defects in *E. coli xth* (exonuclease III) mutants.

3. Nucleotide Excision Repair

This is the primary mechanism for repair of such DNA adducts as CBPDs, 6-4 photoproducts, psoralen-thymine adducts, and other DNA lesions that cause major helical distortion and which are lethal and/or mutagenic. Also, recent studies suggest that nucleotide excision repair may also play a backup role in repairing DNA lesions which do not distort the helix extensively and which are ordinarily repaired by methyltransferases (Voigt *et al.*, 1989) and glycosylases (Van Houten and Sancar, 1987). This repair mechanism does not, however, repair mismatches or extrahelical loops (Thomas *et al.*, 1986). Nucleotide excision repair is thought to involve four steps: (1) a specific endonuclease activity is responsible for incision of the DNA phosphodiester backbone on both sides of the lesion; (2) the resulting gap is filled in following synthesis of new DNA by a DNA polymerase, originating at the 3′-hydroxyl terminus of the incision and using the opposite strand as a template; (3) the lesion-containing fragment is displaced during this process until it is finally excised; (4) the repair process is then completed by a ligase which joins the newly synthesized DNA to the phosphodiester backbone of the parental DNA.

Nucleotide Excision Repair in E. coli. The UvrA, UvrB, and UvrC proteins, encoded by three genes, *uvrA*, *uvrB*, and *uvrC*, respectively, are responsible for nucleotide excision repair in the bacterium. Mutations in any one of the three

genes abolish excision repair *in vivo* (Sancar and Sancar, 1988). The genes have been cloned and sequenced, and all the three proteins have been purified to homogeneity (Thomas *et al.*, 1985). The UvrA protein is a dimer (monomer MW = 103,874), is an ATPase, and has two "zinc finger" DNA binding motifs (Doolittle *et al.*, 1986). The UvrB protein (MW = 76118) also has the consensus ATPase sequence but contains no detectable ATPase activity, and binds weakly to DNA. It makes a $(UvrA)_2(UvrB)_1$ complex with UvrA dimer, however, in an ATP-dependent reaction (Oh *et al.*, 1989; Van Houten, 1990). The UvrC protein has a molecular weight of 66,038, and binds to single- and double-stranded DNA (both damaged and undamaged) with low affinity. In the absence of DNA, UvrC does not interact with UvrA, UvrB, or the $(UvrA)_2(UvrB)_1$ complex (Orren and Sancar, 1989). The incision reaction includes the following steps (see Fig 2): (1) UvrA associates with UvrB to form a $(UvrA)_2(UvrB)_1$ complex in an ATP-dependent manner; (2) the complex binds to the damaged site on DNA, resulting in the formation of a UvrB-DNA complex and the dissociation of $(UvrA)_2$ from the complex. (3) UvrC interacts with the damaged DNA-UvrB complex and mediates the dual incisions. The main incision pattern is hydrolysis of the eighth phosphodiester bond 5′ and the fourth phosphodiester bond 3′ to the adducted nucleotide, which results in the release of a 12-mer containing the damaged nucleotide (Sancar and Rupp, 1983). The incision complex is composed of only the UvrB and UvrC subunits; therefore, UvrA functions as a damage recognition

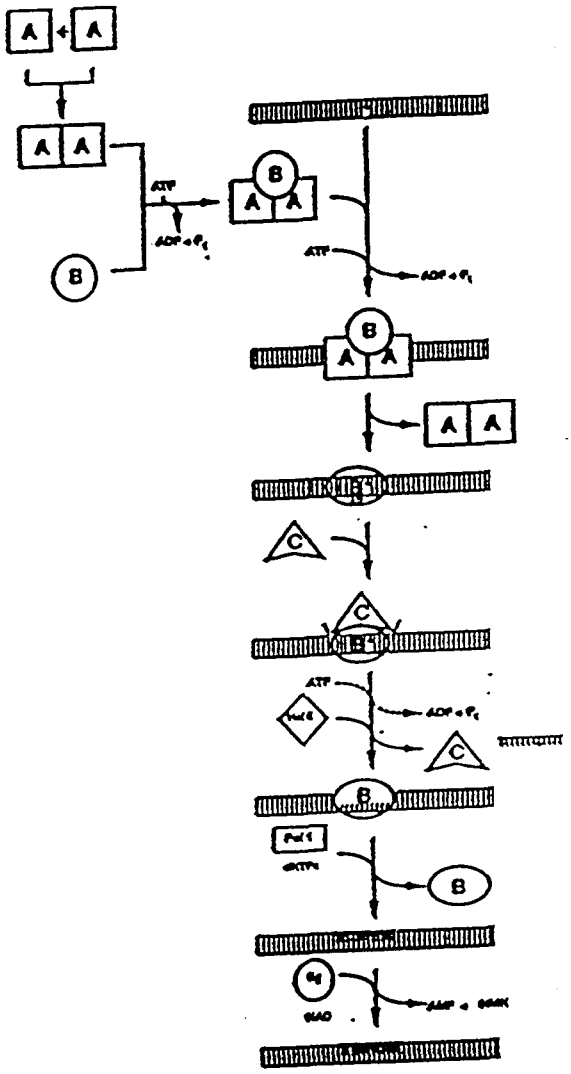

Fig.2. Model for nucleotide excision repair in *E. coli*. Incision by the UvrB-UvrC complex requires ATP binding; the incision sites are shown by small arrows. Displacement of UvrC and excised oligomer by helicase II (*helII*) probably requires ATP hydrolysis (indicated with broken arrows). Reprinted with permission from Orren *et al.*, (1992).

and UvrB delivery protein. (4) The UvrB-UvrC-DNA complex is recognized by helicase II (*uvrD* gene product) and DNA polymerase I, and is dissociated from the incision site along with the excised oligomer (Orren *et al.*, 1992). The released UvrC protein can engage in new rounds of incisions. (5) Finally, the repair process is finished by functions of DNA polymerase I and DNA ligase.

Nucleotide Excision Repair in Yeast. In yeast *S. cerevisiae*, at least 95 different genetic loci have been identified that are involved with cellular resistance to radiation and/or chemicals (Rubin, 1988). Mutations in DNA repair have been classified into epistasis groups; two mutants are classified into the same epistasis group if a strain carrying a mutation is no more sensitive to UV irradiation than the most sensitive of the single mutant strains. Based on this rule, yeast mutants sensitive to UV irradiation have been classified into three epistasis groups: (1) members of *RAD3* group are defective in excision repair; (2) *RAD6* mutants are abnormal with regard to mutagenesis; (3) the *RAD52* group is defective in recombinational repair. Mutants at five of the loci in the *RAD3* epistasis group, *RAD1* (Reynolds *et al.*, 1987), *RAD2* (Madura and Prakash, 1986), *RAD3* (Reynolds *et al.*, 1985), *RAD4* (Gietz and Prakash, 1988) and *RAD10* (Reynolds *et al.*, 1985), are completely defective in the incision step of excision repair. To date genes complementing all five mutants have been cloned and analyzed, revealing a number of

interesting characteristics. All five genes lack introns, and while the *RAD10* has a coding region of only 630 bp, the other four have significantly larger open reading frames of approximately 2.2-3.3 kb in length. The *RAD1* gene encodes a protein of 1100 amino acids with a molecular weight of 126360. Comparison of the amino acid sequence with other DNA repair proteins shows no sequence homologies. *RAD1-lacZ* fusion experiments indicate that the gene is weakly expressed, and that its expression is not increased by exposure to DNA damaging agents (Nagpal *et al.*, 1985). *RAD2* encodes a protein of 117,700 MW, and is inducible by DNA damage (Robinson *et al.*, 1986). The *RAD3* gene encodes a protein with MW of 89,700. The amino terminus of Rad3 has a region of homology with other known ATPase; and it also has, in its C-terminal half, a region of homology to the DNA binding helix-turn-helix motif (Walker *et al.*, 1982). Rad4 is a protein of 87,173 MW and has a short stretch of 24 amino acids which is homologous with an N-terminal portion of Rad10 (Friedberg, 1988). Rad4 is toxic to *E. coli* because only plasmids carrying certain mutations in *RAD4* gene that inactivate the Rad4 function in *S. cerevisia* can be propagated in *E. coli* (Fleer *et al.*, 1987). The *RAD10* gene has also been cloned (Reynolds *et al.*, 1985). Sequence analysis of the *RAD10* gene revealed a coding region of 630 bp which encodes a protein of 210 amino acids. Although the *RAD10* gene has been transcribed and translated in *E. coli,* it does not complement the UV sensitivity of either *uvrA, uvrB* or *uvrC* *E. coli* mutants(Weiss and Friedberg 1985).

Nucleotide Excision Repair in Mammalian Cells. Significant advances in the study of the human nucleotide excision repair pathway have been made in the past few years. Five of the human genes correcting rodent UV complementation groups have now been cloned, and efforts to characterize these genes have proceeded well. The cloning of the first human nucleotide excision repair gene, *ERCC-1*, was reported by Westerveld *et al* (1984). This gene encodes a protein of 297 amino acids with a MW of 32562. It shows homology to the yeast Rad10 (30% identity), and to part of the *E. coli* UvrA protein (31% identity over a region of 42 amino acids)(van Duin *et al.*, 1986). *ERCC-1* confers UV and mitomycin C resistance to CHO complementation group 1 mutants. Northern analysis of poly(A) RNA at various times after UV irradiation showed no evidence for UV inducibility of the *ERCC-1* gene (Van Duin *et al.*, 1988). Weber and co-workers (1988) reported that the *ERCC-2* gene corrected the nucleotide excision repair defect in XP (*xreoderma pigmentosum*) group D and CHO complementation group 2 cells. *ERCC-2* encodes a protein of 760 amino acids, and is the human homolog of the yeast RAD3 gene, which codes for an ATP-dependent helicase. The *ERCC-3* gene, which has been also cloned and sequenced (Myles and Sancar, 1989), encodes a protein of 782 amino acids with a Walker nucleotide binding consensus sequence (Hanawalt, 1989). The *ERCC-5* and *ERCC-6* cDNAs were cloned and characterized (Lehmann *et al.*, 1992). ERCC-6 is a 1493-aa protein with 8 putative helicase

functional domains, suggesting that it may be a third helicase involved in nucleotide excision repair, in addition to ERCC-2 and ERCC-3.

Both damage and repair of DNA are likely to be influenced by the macromolecular association of DNA and proteins in the nucleus. Distribution of damage can vary; for instance, 6-4 photoproducts and some types of chemical adducts occur more in linker (or nucleosome-free) DNA than within the nucleosomes, whereas CBPDs are distributed equally between linker and nucleosome (Lehmann *et al.*, 1992). The chromatin structure of expressed genes causes these sequences to be highly sensitive to digestion by DNAase I, whereas nonexpressed DNA sequences exist in tightly condensed chromatin structures. It has been suggested that the accessibility of certain genomic regions to repair enzymes may in part be responsible for their preferential repair (Hanawalt *et al.*, 1979). Evidence indicates that DNA is repaired more rapidly in actively transcribed genes than in inactive genes (Mellon *et al.*, 1986), and that the transcribed strand is often repaired more rapidly than the non-transcribed strand (Vrieling *et al.*, 1989).

4. Recombinational Repair - Postreplication Repair

Since the discovery of the *recA* gene of *E. coli* by Clark and Margulies (1965), the molecular mechanisms of recombination and postreplication repair in bacteria have

become increasingly well-defined. The RecA protein of *E. coli* is central to the related processes of recombinational DNA repair and homologous genetic recombination. It is also involved in the cellular SOS response to DNA damage (Roca and Cox, 1990). In a few cases, molecular processes that might ensure cell survival without removal of DNA damage have been characterized in the yeast *S. cerevisiae* (Cole *et al.*, 1989), and in human cells (Hsieh *et al.*, 1986). However, our knowledge of eukaryotic recombinational repair is still very limited.

In *E. coli,* two separate repair events, recombinational repair and cross-link repair, employ some of the same proteins and share many of the same intermediates. In both cases, genetic information in a homologous duplex is used to restore the integrity of a duplex that has a lesion involving both strands. When a replication fork encounters a DNA adduct that blocks replication, synthesis of DNA on the undamaged strand may continue; on the damaged strand, however, DNA polymerase appears to be blocked at the adduct and to reinitiate synthesis further along the chain, leaving a several-hundred-base gap (Fig 3). The daughter strand is therefore believed to be terminated with a 3′-OH at the position of the adduct. The resulting single-stranded DNA is thought to activate the RecA protein, which is believed to promote autolysis of the LexA repressor and thus to induce the bacterial DNA-damage-response ("SOS", see sections below). The SOS response would cause

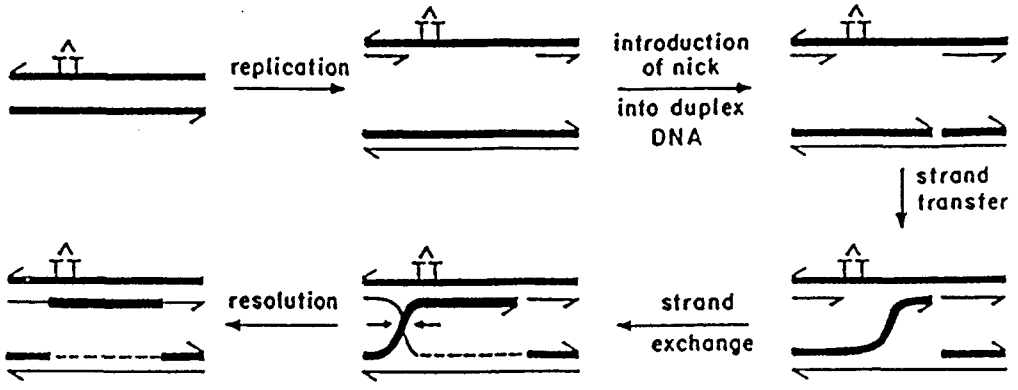

Fig.3. Model for repair of a post-replication gap by RecA-mediated sister strand exchange. RecA protein binds at the post-replication gap promoting homologous pairing. The 3′ terminus of the nicked strand is transferred by RecA protein into the gap, and strand exchange produces a crossed molecule which is cut by resolvase leading to completion of the repair. Reproduced with permission from Walker, G.C. (1985).

increased synthesis of RecA protein in readiness for pairing the intact sister duplexes. Pairing might occur at the site of the gap, with the single-stranded region of the gapped molecule wound in the major groove of the duplex. Once homologous contacts were established, a cutting-*in-trans* enzyme might nick the intact sister duplex at a site opposite the gap. The nick would enable RecA protein to transfer the 3′-OH terminus of the nicked strand into the gap, thus providing an intact strand complementary to the one containing the adduct. The strand exchange reaction would proceed, as RecA protein drives branch migration and heteroduplex formation past the adduct. The 3′-OH terminus of the gapped strand would also be transferred, producing a crossed strand exchange. The gap left on both duplexes could finally be filled in by DNA polymerase I and ligase. Completion of repair would permit the release of RecA protein and terminate the SOS response. This is actually a toleration rather than a repair process. That is, the final structure would retain the adduct opposite an intact complementary strand, so the adduct could be repaired by the Uvr excision system or an appropriate DNA glycosylase. Even in the absence of base or nucleotide excision repair, recombinational repair constributes to cell survival because it maintains the integrity of the duplex; if the lesion is not in an essential gene, it is eventually diluted out by successive rounds of replication and cell division (West *et al.*, 1981).

5. Regulation of DNA Repair - Inducible Repair

Inducible Repair in E. coli. The molecular mechanisms of regulation of the expression of genes in response to a variety of environmental stresses have been extensively investigated in bacterium *E. coli*. Treatment of bacterial cells with UV light or with agents that introduce bulky adducts in DNA causes coordinate induction of about 20 genes that are members of the SOS regulatory network (Walker, 1985). The SOS system is regulated by the LexA and the RecA proteins. In the absence of DNA damage, the LexA repressor binds to the operator sequences of the SOS genes and turns off their expression. Upon treatment of cells with DNA damaging agents, the RecA protein becomes an active protease and cleaves the LexA repressor, resulting in increased transcription of the SOS genes. Activation of RecA apparently occurs by its binding to the single-stranded DNA left during replication. The products of SOS inducible genes include those of the *uvrA*, *uvrB*, and *uvrD* genes, required for nucleotide excision repair, of the *recA* gene for recombinational repair, of the *umuC* and *umuD* genes, required for mutagenesis, and of the *sfiA* gene, required for inhibition of cell division in response to DNA damage.

Another regulatory network controlling the induction of repair processes in *E. coli* is the adaptive response. When *E. coli* cells are first exposed to low concentrations of

methylating agent such as N-methyl-N'-nitro-N-nitroso-guanidine (MNNG), they become more resistant to the mutagenic and lethal effects of a subsequent challenge with a higher dose of the same or similar alkylating agents (Samson and Cairns, 1977). This induced resistance is the result of a set of induced repair processes which remove DNA lesions introduced by methylating and ethylating agents. Once DNA is alkylated, the Ada protein, which is itself an O_6-mGua DNA alkyltransferase, transfers methyl groups to its Cys-69 residue and becomes a positive regulator. It binds to the so-called "Ada box" upstream of promoters of the *ada, alkA,* and *aidB* genes and induces their expression. The elevated levels of Ada increase repair of O_6-mGua and thus prevent mutagenesis; the increase in *alkA* gene product, 3-mAde DNA glycosylase II, results in increased capacity to repair 3-mAde and 3-mGua, which block DNA replication and are lethal. The function of the AidB protein is not known.

Inducible Repair in Yeast and Mammalian Cells. In contrast to *E. coli*, the regulatory responses of DNA repair genes to DNA damage are not well defined in eukaryotic systems. Of the DNA repair genes in yeast, *PHR1* (Sebastian *et al.*, 1990), which encodes DNA photolyase, and the *RAD2* (Robinson *et al.*, 1986), *RAD6* (Madura *et al.*, 1990), *RAD7* (Jones *et al.*, 1990), *RAD18* (Jones *et al.*, 1991), *RAD23* (Madura *et al.*, 1990), and *RAD54* (Cole *et al.*, 1989) genes have been reported to be induced by UV and other DNA-damaging

agents. The *RAD2, RAD7* and *RAD23* genes function in excision repair, the *RAD6* and *RAD18* genes are required for postreplication repair and for DNA damage induced mutagenesis, and the *RAD54* gene is required for the repair of DNA double strand breaks and for recombination. In mammalian cells, O_6-mGua DNA methyltransferase activity can be increased up to 100 fold after treatment of whole animals or rodent and human cell lines with alkylating agents (Lehmann *et al.*, 1992). However, evidence for molecular mechanisms that control the induced expression of these genes in response to DNA damage has been lacking, and there is as yet no sound evidence that these inducible proteins participate in repair processes that enhance cell survival.

B. Repair and Toleration of DNA Damage in Plants

Organisms have evolved mechanisms to repair and tolerate numerous types of DNA damage; while these DNA repair systems have been well characterized in bacteria and to a lesser extent in yeast and mammalian cells, surprisingly little is known about repair and toleration of potentially harmful DNA lesions in plants.

1. Photoreactivation in Plants

Early studies showed that there was a light-dependent decrease in the content of CBPDs from the DNA of UV-irradiated cells of *Nicotiana tabacum* (Trosko and Mansour, 1968), *Ginkgo biloba* (Trosko and Mansour, 1969) and *Daucus carota* (Howland,

1975), of seedings of *Lathyrus sativus* (Soifer and Tsieminis, 1977), and of intact plants of *Wolffia microscopia* and *Spirodela polyrhiza* (Degani *et al.*, 1980). Such demonstrations have been interpreted as evidence for the enzyme-catalysed monomerization of CBPDs. Indeed, photoreactivating enzyme has been detected and partially purified from maize pollen (Ikenaga *et al.*, 1974) and from several types of bean (Saito and Werbin, 1969). The action spectrum of the maize enzyme showed a broad peak around 385-405 nm which corresponded to the observed optimum of 405 nm for photoreactivation *in vivo* (Ikenaga *et al.*,). Interestingly, the photoreactivating activity in the lima bean *Phaseolus lunatus* and the pinto bean *P. vulgaris* showed a differential tissue distribution: 95% of the activity was equally distributed between the plumule and hypocotyl, with the remaining 5% in the cotyledons and none in the radicle. As the shoots developed, the enzyme activity declined, with 2-week-old leaves having only 50% and 3 to 6-week-old leaves no more than 5% of the specific activity of the young shoots. It is surprising that no more detailed studies of photoreactivation in plants have been reported, and no plant photolyase genes have been cloned thus far. Plants should prove particularly fruitful in studies of the evolution of photoreactivation and photolyase, since plants must have developed effective means for limiting the genetically destructive effects of solar UV light at an early stage in evolution.

2. Excision Repair in Plants

The first attempts to find excision repair in plants, using tobacco (Trosko and Mansour, 1968) and Ginkgo (Trosko and Mansour, 1969) cells, were unsuccessful. Negative results were also obtained in *Chlamydomonas* (Swinton and Hanawalt, 1973), and in *Vicia faba* (Wolff and Cleaver, 1973). It was therefore suggested that plants either had not acquired excision repair mechanisms during evolution or had lost them. Using improved experimental techniques, the excision of CBPDs from a UV-irradiated plant was first shown in the grass pea, *Lathyrus salivus* (Soifer and Tsieminis, 1974). This was followed by the discovery of excision repair of UV-induced CBPDs in the DNA of wild carrot protoplasts (Howland, 1975). In the grass pea, CBPDs were removed from the DNA of irradiated seedlings at a constant rate of 2-3 X 10^4/h for up to 6 h, after UV doses which had caused up to 0.12% dimerization of available thymine residues. The excised dimers appeared in an acid-soluble form and represented 29% of the total CBPDs induced in the DNA (Soifer and Tsieminis, 1977a). In *Daucus* protoplasts, virtually all of the dimers induced by a dose of 10 J/m^2 of 254-nm radiation were removed during a 24-h post-irradiation incubation in the dark (Eastwood and McLennan, 1985). However, only 6% of those induced by a dose of 42 J/m^2 were excised in the same period. At a dose below 100 J/m^2 the maximum average rate of excision was about 25000 CBPDs/cell/h, representing 30% of the total dimers, but this

rate fell dramatically at dose above 100 J/m^2 to an average rate of 4000 CBPDs/cell/h. Excision repair has also been shown in whole irradiated duckweed (Degani *et al.*, 1980), in which 40% of the dimers were removed within 3 h in the dark, but the initial rate was not maintained and a residual 20% persisted even after 50 h. All these later observations imply that there are two basic reasons for the negative results obtained in the early experiments investigating excision repair in plants: (1) the level of incorporation of radioactive precursors into plant DNA was very low, and (2) high doses of irradiation of plants or cells completely inhibited the excision repair activities. Recently, two excision repair enzymes have been partially purified from the leaf tissue of *Brassica oleracea*. A UV-specific endonuclease (Gallagher et *al.*, 1991) and a 3-alkyladenine DNA glycosylase (Groft *et al.*, 1991) were detected by nitrocellulose filter-binding assays, using damaged PM2 DNAs as the substrates. The precise mechanisms of action of the two enzymes, however, require further investigation. To date no excision repair genes have been cloned from plants.

3. Recombinational Repair in Plants

Rosen and co-workers (1980) first reported evidence for recombinational repair in *Chlamydomonas reinhardtii*. Posttreatment of UV-irradiated recombination-proficient strains with caffeine increased survival, indicating an enhancement of recombinational repair. A DNA recombinase has

been purified to near homogeneity from broccoli (Tissier *et al.*, 1991). Sequence information has been obtained from the purified protein, and polyclonal antibodies are being raised against it in order to clone the corresponding gene (Tissier *et al.*, 1991). Recently a bacterial RecA-like protein has been partially purified from pea chloroplasts (Cerutti and Jagendorf, 1991). The protein crossreacts with *E. coli* RecA antibody, and its synthesis induced by UV irradiation, mitomycin C, and nalidixic acid. Band shift assays showed that it binds single-stranded DNA and is involved in recombination reaction *in vitro* (Cerutti *et al.*, 1992). This suggests that it is a RecA analog presumably involved in chloroplast DNA recombination or repair. The corresponding *Arabidopsis* cDNA clone has been isolated using a bacterial *recA* probe (Cerutti *et al.*, 1992); it encodes a protein highly homologous to eubacterial RecA protein, with a chloroplast transit peptide at its amino terminus.

II. PROJECT REVIEW

A. Research Objectives

This thesis work aimed to characterize the DNA repair responses to UV irradiation of a model green plant, *Arabidopsis thaliana*, which has many advantages for molecular genetic studies (Meyerowitz, 1987), and to study DNA-damage repair and tolerance mechanisms in plants. My specific approaches to these questions were the following:

1. I wanted to develop assays for determining UV-DNA repair *in vivo*. Through quantitative measurement of CBPD removal from the DNA of UV-irradiated plants, I was able to identify two major repair pathways in *Arabidopsis*, namely photoreactivation and dark repair (presumably excision repair).

2. The finding that *Arabidopsis* possesses a light-dependent UV-damage DNA repair pathway led to the analysis of photolyase activity *in vitro* and to the measurement of effects of UV-B treatment on the enzyme activity.

3. The major part of this thesis work focused on isolation and cloning of *Arabidopsis* genes that complement repair-deficient mutations in *E. coli*, characterization of their complementation phenotypes, and analysis of DNA sequences of the cDNA clones.

4. In order to investigate regulation of the expression of the *DRT* genes and of DNA repair activities in *Arabidopsis*, the responses of plant genes corresponding to the cloned DNA-damage repair/toleration (*DRT*) cDNAs, to UV irradiation and chemical treatments were determined by Northern hybridyzation analyses.

B. Rationale and Significance

1. Solar UV-B and Plant Responses

An important component of the stratosphere is the ozone (O_3) layer, which absorbs solar UV-B radiation (280-320 nm) with increasing effectiveness at shorter wavelengths and prevents essentially all radiation below 295 nm from penetrating through the atmosphere to the earth. However, the release of certain industrial pollutants, such as chlorofluoromethanes, into the atmosphere is predicted to reduce the equilibrium ozone column thickness in the coming decades (National Academy of Sciences, 1982). This reduction in the ozone layer will increase the UV-B irradiance reaching the earth, and also shift the terrestrial solar spectrum toward shorter, more biologically damaging wavelengths. The increased solar UV-B radiation represents, therefore, a significant environmental stress for terrestrial plants.

The effect of UV-light on plants, as on other biological systems, is highly wavelength dependent. Its capability to

induce photochemical injury in organisms usually increases logarithmically with decreasing wavelength over a range from about 260 to 360 nm. This is considered to be due to the absorption spectra of nucleic acids and proteins. The shortwave solar UV radiation flux (<280 nm) that prevailed during the development of early land plants may have been an important factor in higher plant evolution and development of UV protection and repair mechanisms (Lee and Lowry, 1980). Although this intense shortwave UV radiation is no longer present in the terrestrial environment today, solar UV-B radiation is increasing due to ozone depletion, and is sufficiently actinic to damage plant tissue and physiological processes of sensitive plants. A recent report indicated that CBPDs could be induced in the DNA of intact alfalfa seedlings by UV wavelengths as long as 365 nm (Quaite *et al.*, 1992). The study of plant response to solar radiation flux thus has significance both with respect to the natural latitudinal gradient of solar UV-B, and the consequences of an enhanced UV-B climate due to the ozone reduction.

Plant adaptations that temper the effects of solar UV-B radiation can include both protective and repair mechanisms. Leaf inclination may provide some protection from solar UV-B radiation, but is of limited value because a relatively large proportion of the solar UV-B flux reaches the ground as diffuse radiation (Caldwell *et al.*, 1980). Selective absorption of solar UV-B by flavonoid compounds in the outer

tissue of leaves has been shown to be an effective protective mechanism, and induction of the synthesis of flavonoid biosynthetic enzymes by solar UV-B in some plant species has been well documented (Thompson and White, 1991). Although these protective systems are effective, absorption may not be able to prevent damage in environments where UV-B flux is high and continuing to increase. Therefore, damage in nucleic acids induced by excess solar UV-B will ultimately require effective DNA repair mechanisms. It is of fundamental importance, therefore, to study DNA repair in plants in order to evaluate repair-based UV-resistance strategies.

2. *Arabidopsis* as a Model Plant for Repair Studies

In the last several decades plant geneticists have traditionally chosen economically important species such as maize, tomato, pea and barley as their experimental material. Unfortunately, these species are in several respects not ideal for genetic studies: the generation times are long, and relatively large amounts of space are required for cultivation. In addition, the genomes of these plants are large and contain large amounts of dispersed repetitive DNA, which makes it very difficult to perform such procedures as genomic blot analysis with genomic clones and chromosome walking. In contrast, the small crucifer *Arabidopsis* possesses a number of advantages that make it particularly well-suited as a model species for genetic studies (Meyerowitz, 1987): for classical genetics, its small size (typically about 30

centimeters in height at maturity), short generation time (six weeks), high seed set (about 10,000 seeds per plant), and ease of mutagenesis make it easier and faster to induce, select, and characterize new mutations in *Arabidopsis* than in other plant species; for molecular genetics, *Arabidopsis* has the smallest, simplest genome of any flowering plant; its genome, unlike those of other angiosperms, is all but devoid of repeat sequences. These important features, together with the growing availability of recombinant DNA technology, provide tools for finding and cloning genes of interest, and determining their roles in plant growth and development. Since many of these genes are common to all flowering plants, it is possible that the roles they play in agronomically significant species may ultimately emerge.

All these characteristics, together with our recent finding that *Arabidopsis* seedlings readily take up ^{3}H-thymidine and label the plant DNA, make *Arabidopsis* a very attractive model for studies of DNA repair in higher plants. I hoped, through my initial studies, to obtain more information about DNA repair in plants, and to determine whether repair mechanisms are of physiological importance in plants.

3. Importance of the Research Objectives

It is reasonable to begin with characterization of photoreactivation because (1) photoreactivation is the

simplest but most efficient UV-repair response in terms of CBPDs, the major DNA damage induced by UV irradiation; (2) only a single polypeptide enzyme is involved in photoreactivation, compared with excision repair enzymes that are mostly multi-subunit, which makes research goals easier to achieve; (3) increasing expression of photolyase gene(s) when needed, by developmental control and sunlight inducibility, may be an important strategy of plant resistance to solar UV light.

Isolation and cloning of the *Arabidopsis* DNA damage repair/toleration genes is an important step towards gaining an understanding of the molecular nature of these repair and toleration processes in plants. The sequences of the cloned *DRT* genes will provide clues to structure-function relationships and regulatory mechanisms, and detailed *in vitro* studies using their overexpressed products will provide methodology for investigations with economically important plants, and for tests of protection strategies.

Biological stress causes extensive characteristic changes in the pattern of protein synthesis that are related to the expression of specific defense responses. Although light-stimulated expression of a number of plant genes, including those concerned with biosynthesis of phenylpropanoids, has been under intensive study (Thompson and White, 1991), little is known about the regulation of DNA repair/toleration genes in plants. It is of great interest to determine whether, after

treatment with UV-B or other DNA-damaging agents, there are any changes in the mRNA levels of *DRT* genes, and whether there are any relationships between these changes and the transcription rates of these genes. I hope that my initial studies on regulation of plant repair gene transcription in response to DNA damage will provide clues for further investigations of the organization and structure of these genes in relation to activation by biological stresses, for analyses of the molecular mechanisms involved in signal perception and transduction underlying specific regulation of plant repair gene activity by environmental factors, and thus for strategies for enhancing plant resistance to solar UV by gene manipulation.

III. EXPERIMENTAL MATERIALS AND APPROACHES

A. Bacterial, Yeast, and Phage Strains

The bacterial and yeast strains, and the bacteriophages used in this work are listed in Table 1.

B. Buffers, Media and Chemicals

TE is 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0). TM is 10 mM Tris-HCl (pH 7.4), 10 mM $MgSO_4$. 1X TBE is 89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 8.0). PX-buffer is 100 mM Tris-HCl (pH 7.4), 2 mM EDTA (pH 8.0), 20 mM β-mercaptoethanol, 20% (v/v) glycerol, 10 mM dithiothreitol. PHR buffer is 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 10 mM NaCl, 10 mM dithiothreitol. TES is 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 1 M NaCl. UVE buffer is 10 mM Tris-HCl (pH 7.6), 20 mM EDTA (pH 7.6), 50 mM NaCl. TBY broth contains 1% tryptone, 0.5% NaCl, 0.5% yeast extract. TBMM broth contains 1% tryptone, 0.5% NaCl, 10 mM $MgSO_4$, 0.2% maltose and 0.0001% thiamine. YPD broth contains 2% peptone, 1% yeast extract. LB plates contain 1% tryptone, 0.5% yeast extract, 1% NaCl and 1.5% agar. TCMB plates contain 1% trypticase, 0.5% NaCl, 10 mM $MgSO_4$, and 1.1% agar. H-plates contain 1% tryptone, 0.8% NaCl, and 1.5% agar. YPD plates contain YPD broth plus 2% agar. TA (top agar) contains 1% tryptone, 0.5% NaCl, 10 mM $MgSO_4$, and

Table 1. *E. coli*, yeast strains and bacteriophages

Designation	Genotype	Source(reference)
A. *E. coli*		
AB1157	F⁻ *thi-1 his-4* Δ*(gpt-proA) argE3 thr-1 leuB6 kdgK1 rfbD(?) ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 supE44 rpsL31*	Bachmann (1972)
C600	F⁻ *thi-1 thr-1 leuB6 lacY1 supE44*	Bachmann (1972)
CS85	As AB1157, but *ruvC53 eda-51::Tn10*	Lloyd (1991)
EG333	HfrC *pyrA::Tn10 mstB cysG303* Δ*(lac-pro)XIII*	Laufer *et al.*, (1989)
FD2565	As C600, but Δ*(uvrB-chlA) Δ(kdp-phr)214*	Hays *et al.*, (1990)
FD2566	As FD2565, but Δ*(recA-srl) ::Tn10*	Hays *et al.*, (1990)
N99	*galK2 rpsL200*	Bachmann (1972)
N3398	As AB1157, but *recG258::Tn10 ruvC53 eda-51*	Lloyd (1991)
QP2895	As TS11, but Δ*(kdp-phr)214*	This laboratory
QP2897	As QP2895, but *recA::cat*	This laboratory
QP2898	As QP2897, but F′(*proA*⁺ *proB*⁺ *lacI*ᵠ *lacZ M15 Tn10*)	This laboratory
SM2266	As N99, but Δ*(lac-pro)pyrA::Tn10*	This laboratory
TS11	As N99, but *uvrC34*	Smith and Hays (1985)
XL-1 Blue	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* {F′*proAB lacIq lacZ M15 Tn10* (Tc^R)}	Bullock et al., (1987)

Table 1. (Continued)

B. *Saccharomyces cerevisiae*

DBY747	*MATa his3- 1 leu2-3 leu2-112 trp1-289 ura3-52 RAD*$^+$	Higgins *et al.*, (1984)
LN2-1I1	*MATa rad2-1 ura3-52*	Naumovski *et al.*, (1984)

C. Bacteriophage strains

B221	*bio11 red*$^-$*gam*$^-$ *c(ts) imm*^{21}	This laboratory
KC	p*lac-cre c*$^+$ kn^R	Elledge *et al.*, (1991)
W1721	p*lac-cre xis1 red3 cI(ind-)* kn^R	This laboratory
W1742	*xis1 red3 cI(ind-)*	This laboratory
Y1730	p*lac5 lacZ118 red3 cI857*	This laboratory

0.7% agar. MSS-agar plates contain 0.43% Murashige-Skoog salt mixture (pH 5.7), 1% sucrose, 0.001% nicotinic acid, 0.01% pyridoxine-HCl, 0.004% glycine, 0.0001% thiamine-HCl, 0.01% myo-inositol, and 0.8% agar. X-Gal plates contain 1% trypticase, 0.5% NaCl, 10 mM $MgSO_4$, 0.0034% X-Gal (5-bromo-4-chloro-3-indoyl-β-*D*-galactoside), 0.034 mM IPTG (isopropyl-β-*D*-thiogalactoside), and 1.1% agar. M9-minimal plates contain 0.6% Na_2HPO_4, 0.4% KH_2PO_4, 0.05% NaCl, 0.1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.2% glucose, and 1.5% agar. MacConkey-lactose plates contain 4% MacConkey base agar, and 1% lactose. Ampicillin (Ap), chloramphenicol (Cm), kanamycin (Kn), and tetracycline (Tc) were used at 50 μg/ml, 34 μg/ml, 50 μg/ml, and 12.5 μg/ml, respectively in liquid media and agar plates.

All restriction endonucleases, DNA polymerase I Klenow fragment, phage T4 DNA ligase, and RNase A were obtained from Bethesda Research Laboratories, New England Biolabs, or United States Biochemical Corp. All enzymes were used as recommended by the manufacturer's instructions. Phage T4 endonuclease V, *E. coli* photolyase, and *Anacystis nidulans* photolyase were obtained from Dr. Stephen Lloyd (Vanderbilt University), Dr. Aziz Sancar (University of North Carolina), and Dr. A.P.M. Eker (Delft University), respectively. Methyl methanesulfonate (MMS) was obtained from Fluka Chemical Co. Mitomycin C and 4-nitroquinoline-N-oxide (NQO) were from Sigma. All radioactive isotopes, including α-^{32}P-dTTP, ^{35}S-dATP, ^{3}H-thymidine and ^{14}C-thymidine, were from New England Nuclear.

C. Growth, UV and Chemical Treatments of Plants

Arabidopsis thaliana ecotype Columbia or its C-10 subline (from Dr, David Mount, University of Arizona) were used in all the experiments. Plants, grown to about eight-leaf stage on MSS-agar containing 2 μCi/ml of ^{3}H-thymidine at 22°C in a growth chamber, were irradiated with 254-nm UV light at a rate of 10 J/m^2/s, typically to 1000 J/m^2, harvested after postincubation for various times and under different conditions, and frozen at -80°C for further use. For measurement of temperature effects, plants were shifted from 22°C in a growth chamber to 30 or 37°C growth chambers or to a greenhouse for 1 h to bring agar to desired temperatures (34°C in the greenhouse), irradiated to 1000 J/m^2 and incubated at the elevated temperatures for various times. For determination of the UV-B effect on photolyase levels, plants were grown in a growth chamber at 22°C to about eight-leaf stage, then irradiated with UV-B light to 280 J/m^2. After 7-h irradiation, plants were returned to a 22°C growth chamber for 2 h, and then harvested and frozen at -80°C for extract preparation. For determination of effects of UV-B irradiation and chemical treatments on *DRT* mRNA levels, plants were grown, in 10 X 2.5 cm plastic petri dishes containing 45 ml of MSS-agar, to 10 to 12-leaf stage in a growth chamber at 22°C. Plants were then placed under 4 UVB-313 lamps (Q-panel Co.)shielded with 0.13-mm cellulose-acetate film to block UV-C radiation, and received irradiation at dose rate of 0.36 J/m^2/s for 0.5, 1,

2, 3, 4 and 6 h. Control (unirradiated and dark) plants were shielded with Mylar film or wrapped in foil for 6 h. Plants were harvested immediately after irradiation, or postincubated under continuous cool-white light illumination for 3 and 6 h, or under photoperiodic light for 24 h. For chemical treatments, plants were grown on 45-ml MSS-agar to about 12-leaf stage, then 5 ml of solutions of mitomycin C or methyl methanesulfonate (MMS) were injected, with a 5cc syringe and a 21G1/2 needle, into the agar to desired final concentrations. Agar in which control plants were grown received the same amount of sterile water. Plants were postincubated for 24 h to allow absorption of the chemicals before being harvested and frozen for subsequent RNA preparation.

D. Assays of UV Damage and Repair in Arabidopsis

Radiolabeled DNA was extracted from the irradiated plants by the CTAB procedure described by Rogers and Bendich (1985). This DNA had a molecular weight of about 50 kb, as estimated by sedimentation in neutral sucrose gradients. It was treated with phage T4 endonuclease V, which nicks specifically at CBPD sites, and subjected to alkaline-sucrose-gradient sedimentation. The radioactivity in fractions was determined, and profiles were analyzed to determine the weight-average molecular weight and thus the average frequencies of UV-endonuclease-sensitive sites (ESS) per nucleotide. These were estimated from the formula $ESS/nt = 2[1/X_w - 1/X_o]$, where X_o

and X_w are the weight-average numbers of nucleotides in single-stranded DNA before and after UV endonuclease treatment, respectively. These assays were designed to demonstrate whether DNA damage is an important part of UV effects on plants, to identify major DNA repair pathways, and to describe the time courses of DNA repair in *Arabidopsis*.

E. Assays for Arabidopsis Photolyase

After light-dependent DNA repair had been demonstrated in the assays described above, a sensitive and reproducible assay for photoreactivation in *Arabidopsis* protein extracts was developed. Photolyase extracts prepared were incubated with UV-irradiated plasmids under photoreactivating light (365 nm). Measurement of repair activity by the enzyme extracts was achieved by transforming the extract-treated plasmids into an *E. coli* triple mutant deficient in photoreactivation, excision repair, and recombinational repair (*recA uvrB phr*). These mutant cells can not be transformed with plasmids containing even a single photoproduct. The average number of lethal lesions per plasmid remaining unrepaired (p) can be estimated by the equation: $p = -\ln(t)$ where t is the relative transformation efficiency, defined as the ratio of the transformation efficiency of irradiated to unirradiated plasmid. The levels of photolyase activity at different growth stages, the effects of temperature, and the effect of UV-B treatment of plants on levels of photolyase activity were also measured by the *in vitro* photolyase assays as described above.

F. Isolation of Arabidopsis DRT Genes

Most DNA repair genes in evolutionarily distant biological systems show poor homology, especially at the level of nucleotide sequences (Myles and Sancar, 1989). Therefore, instead of screening DNA libraries, I took an alternative gene isolation approach: selection for *Arabidopsis DRT* genes that complement *E. coli* DNA-repair-deficient mutations. This was facilitated by the availability of an *Arabidopsis* cDNA library constructed in a versatile phage vector, λYES (from Dr. Ron Davis, Stanford University). The vector (Fig.4; Elledge *et al.*, 1991) includes a portion that is flanked by the phage P1 site-specific recombination *lox* sequences. Thus this region is excised as a plasmid when YES infects bacteria lysogenized with λKC expressing P1 Cre, the *lox*-specific recombinase. The excised plasmids contain an ampicillin-resistant gene (Ap^R) and replication origin from pBR322 (*ori*) for selection and propagation in *E. coli*, and a *URA3* gene, *ARS1* and *CEN4* sequences for selection and propagation in yeast. The *Arabidopsis* cDNA inserted at the unique *Xho*I site can be transcribed from either P*lac* in *E. coli* or the *GAL1* promoter in yeast, depending on their orientation.

1. Selection by Complementing RecA⁻UvrC⁻Phr⁻ Mutants.

To prevent λ phages from induction by UV and other DNA-damaging agents which would be employed in selection and

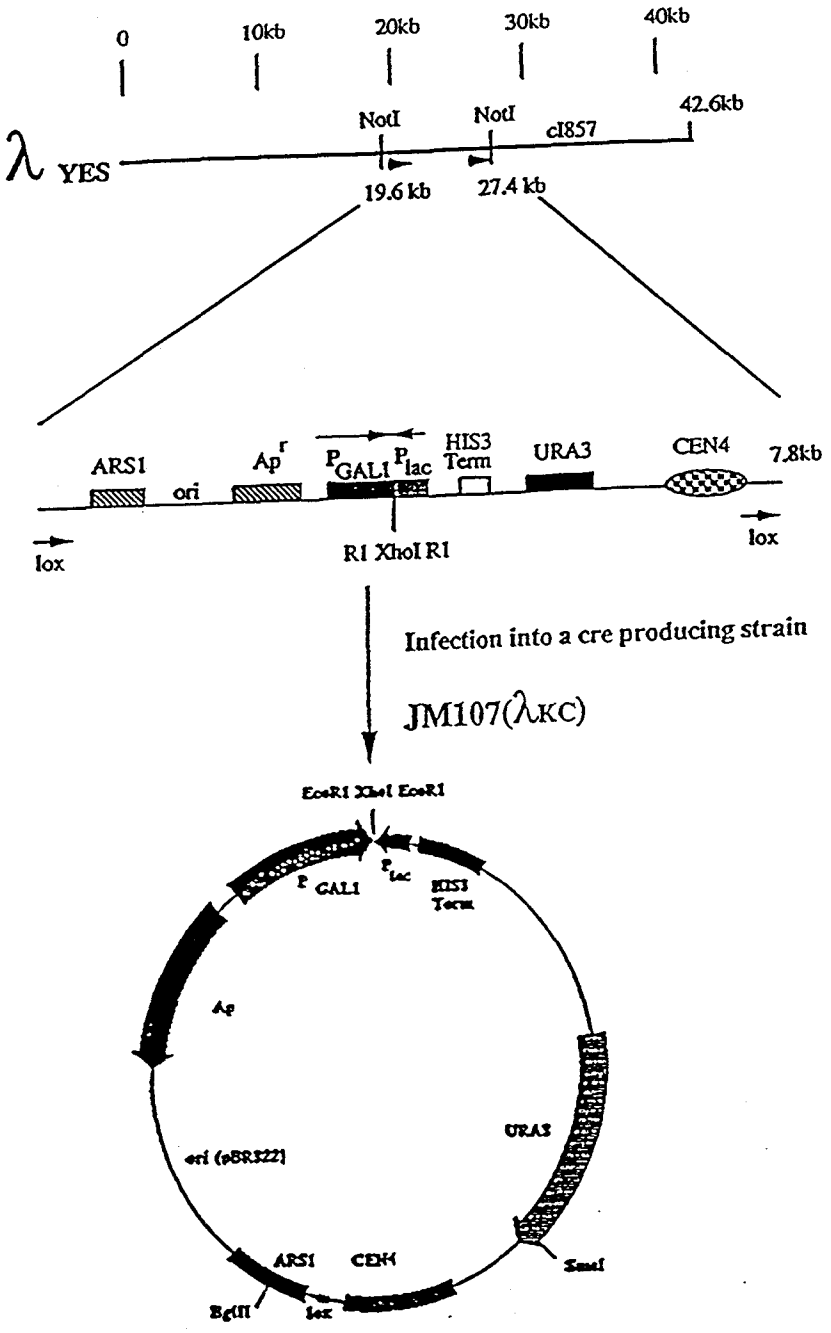

Fig.4. Schematic representation of expression vector λYES and *cre-lox*-mediated automatic subcloning. Reproduced with permission from Elledge *et al.*, (1991).

further characterization, I constructed an *cI(ind⁻)* derivative of λKC to supply *in trans* the Cre protein for excision of the plasmid region of λYES. This non-inducible phage (W1721) was produced by introducing the *cI(ind⁻)* mutation into phage λKC by a standard genetic cross between λKC and λ*xis1 red3 cI(ind⁻)* phages. Phage W1721 were then introduced into the *E. coli* triple mutant QP2898 (*recA uvrC34 phr*); the resultant lysogens were tested for UV inducibility by exposure to 0.5 J/m^2 of 254-nm UV irradiation and measurement of the concentrations of phage released from the irradiated lysogens. To make QP2898 (λW1721) lysogens, the bacteria were grown in 5-ml TBMM broth at 38°C to about 10^8 cells/ml. One ml of this culture with 10^8 W1721 phages was incubated at 38°C for 15 min without shaking, diluted the mixture into 4 ml of TBY broth, grew the cells for 30 min at 38°C, then diluted and spread them onto LB plates containing 50 μg/ml of kanamycin (LB-Kn plates), and then incubated them at 38°C for 18 h. To test whether the plasmid part of the vector could be efficiently looped out of the lambda phage DNA, the Kn^R lysogens were infected with vector phage λYES-R (without cDNA inserts), grown non-selectively for 30 min in TBY broth, plated on LB-Ap plates, and incubated overnight at 38°C. Small-scale crude plasmid preparation ("mini-preps") from eight Ap^R colonies showed that each plasmid was the product of precise excision of the DNA between the *lox* sites, leaving a plasmid (pSE936) of 7.8 kb. I also found that infection of QP2898 (λW1721) with λYES-R resulted in the production of Ap^R colonies with an

efficiency of 26% relative to plaque-forming units (PFU), compared to 11% for the *recA uvrB phr* triple mutant FD2566 (λW1721).

The details of UV selection for *Arabidopsis DRT* cDNAs are as follows. The QP2898 (λW1721) lysogens were grown in TBY broth containing kanamycin (50 μg/ml), IPTG (10 mM) and maltose (0.2%) to about 10^9 cells/ml, and resuspended in 10 mM $MgSO_4$. Then 5 X 10^9 phages from the cDNA library were incubated with 10^9 cells for 20 min at 38°C. The infection mixture was diluted in 50 ml of TBY broth, grown for 30 min at 38°C, resuspended in 10 ml of 10-mM $MgSO_4$, spread onto LB-Ap plates and incubated for 8 h at 38°C. The Ap^R colonies (about 2 X 10^8 total) were washed off the plates with TBY-Ap broth when barely visible. About one-third of the cell suspension was diluted in 100 ml of TBY-Ap broth plus 2-mM IPTG, and grown at 38°C for 2 h (final density about 5 X 10^8 cells/ml). Cells were harvested by centrifugation (6500 X g at 4°C for 10 min), resuspended in 85 ml of 10 mM $MgSO_4$ to a density of about 10^9 cells/ml, pipeted into 10 petri dishes (10 X 1.5 cm), and irradiated, with swirling, with a 15-W germicidal lamp (output principally at 254 nm) to 0.5 J/m^2. The irradiated cells were placed under two 20-W GE Black Lights (F20T-12-BL, peak intensity at 365 nm) at a distance of 15 cm, through one 3-mm glass plate to screen out far-UV wavelengths, for a 40-min photoreactivation period. Finally cells were concentrated 10-fold, spread on LB-Ap plates, and incubated at 38°C for

overnight. Of the approximately 1500 survivors, I screened 840 for heritable UV resistance, by growing them in TBY-Ap broth containing 2 mM IPTG, streaking across LB-Ap plates, and irradiating to 0.05, 0.10, 0.15 and 0.20 J/m^2, using aluminum-foil shields to limit doses. Plasmids from the six heritably resistant isolates were used to transform both QP2898 and FD2566 bacteria. The transformants all showed increased UV resistance. The six clones proved to correspond to four unique cDNAs, as determined from restriction analyses and from sequences (about 200 nucleotides) at each end of the inserts. The cDNAs were designated *DRT100, 101, 102* and *103*; and the four plasmids were called pQP1000, pQP1010, pQP1020 and pQP1030, respectively.

2. Selection by Complementation of ruvC recG Mutations

E. coli ruvC recG cells (N3398) (Lloyd, 1991) were lysogenized with λW1721, and tested for immunity and their ability to carry out plasmid excision upon infection by λYES-R, as described above. The N3398 (λW1721) lysogens were grown in TBY-Kn broth containing 10 mM IPTG and 0.2% maltose to about 10^9 cells/ml, and 10 ml of these cells were harvested by centrifugation (6500 X g at 4°C for 10 min). Cells were resuspended in 5 ml 10 mM $MgSO_4$, and incubated with 5 X 10^{10} phages from the *Arabidopsis* cDNA library at 30°C for 30 min without shaking. Then the infected cells were diluted in 100 ml TBY broth, and grown nonselectively at 30°C for 1 h. At this point total Ap^R bacteria were about 5 X 10^9, as determined

by plating a small aliquot on LB-Ap plates. To this culture Ampicillin and IPTG were added to 50 μg/ml and 2 mM, respectively, and the cells were grown for another 3 h to a density of about 10^9 cells/ml. These Ap^R bacteria were harvested and resuspended in 10 ml TBY. The entire resuspension was spread onto LB-Ap plates containing 0.06% methyl methanesulfonate(MMS) and 2 mM IPTG, and incubated at 30°C for 40 h. While all of 10^{11} control bacteria containg vector (pSE936) were killed by 0.06% MMS, the cDNA-library-containing culture yielded 25 survivors. These were further tested for resistance to 5, 10, 15 and 20 J/m^2 of UV(254 nm) light. Four of the cDNAs conferred UV resistance; the plasmids prepared from them were transformed into naive RuvC$^-$RecG$^-$ bacteria and tested for MMS and UV sensitivity. When digested with *Eco*RI endonuclease, one active plasmid released insert fragments of about 1.2 and 0.3 kb, and was designated *DRT111*; the other three plasmids showed the same 0.8-kb insert size, and were identical as vertified by DNA sequencing; these were designated *DRT112*. The respective plasmids were called pQP1110 and pQP1120.

G. Characterization of the Isolated DRT Genes

1. Measurement of Resistance to DNA-damaging Agents

E. coli strains harboring plasmids with or without *DRT* cDNAs were grown overnight in 5 ml TBY-Ap broth and inoculated into 20 ml fresh broth. After 3-h growth, IPTG was added to 2

mM final concentration, and bacterial growth continued for 2 h (to late log phase). The cells were collected by centrifugation, and resuspended in 10 mM $MgSO_4$ to 5 X 10^8 colony-forming units (CFU) per ml. 8.5 ml of these cells were irradiated with UV light(254 nm), in 10-cm-diameter petri dishes with gentle swirling. Samples were then serially diluted and spread on triplicate LB-Ap plates to determine CFU. For chemical treatments, the cells in 1 ml 10 mM $MgSO_4$ were starved by incubation at 38°C for 30 min. MMS or mitomycin C was added to desired final concentrations, and the mixtures were incubated at 38°C for another 30 min, rinsed five times with distilled water, diluted and plated on LB-Ap agar. Experiments were also performed by simply diluting the cells and directly spreading them onto LB-Ap plates containing different concentrations of the chemical agents. In each case the data represent the mean value of at least two independent experiments. To measure complementation of yeast repair-deficient mutation by *DRT* activity, a wild type yeast strain (DBY747) containing vector pSE936, and a *rad2* mutant strain (LN2-1I1) containing *DRT* plasmids or pSE936, were grown at 30°C in YPD broth containing 1% glucose and 1% galactose to about 5 X 10^7 cells/ml, harvested by centrifugation (1000 X g for 5 min at 0°C), resuspended in an equal volume of distilled water, irradiated with 254-nm UV light, spread on YPD plates, and incubated in the dark at 30°C. Survival curves were determined as described for *E. coli*.

2. Determination of Bacteriophage Plating Efficiencies

Irradiated Phages. About 5 X 10^7 λ*plac5 lacZ118 red3 cI857* (strain Y1730) phages in 1 ml TM buffer were irradiated at 254 nm in a small petri dish to desired fluences, diluted where it was necessary, and incubated at 38°C for 15 min with 0.3 ml of QP2898 bacteria harboring either pUC19 or *DRT*-cDNA-containg plasmids, previously grown in TBMM broth plus 2 mM IPTG. Mixtures were diluted with 3 ml TA, spread onto TCMB plates with or without IPTG, and concentrations of plaque-forming units (PFU) were determined after incubation of the plates overnight at 38°C.

Recombination-deficient red⁻gam⁻ Phages. $RecA^+$ control bacteria [FD2565 (pUC19) or C600 (pUC19)] and $RecA^-$ bacteria (QP2898 or DJI with pUC19 or *DRT* plasmids) were grown in TBMM broth plus 2 mM IPTG to about 10^8 cells/ml. About 0.3 ml of these cells were mixed with 3 ml TA, and layered onto TCMB plates with or without IPTG. After the mixtures solidified, 10 ul of phage aliquots of various dilutions were spotted onto the bacterial lawns. The plates (three or more each sample) were incubated overnight at 38°C, and phage concentrations estimated by counting plaque in spots. Phage P1 plating efficiencies were determined essentially the same as described above, except that the bacteria were grown in TBY broth with 5 mM $CaCl_2$ plus 2 mM IPTG, and cell lawns were made on R-plates instead of TCMB plates.

3. Measurement of Conjugal Recombination Frequencies

The procedures of Miller (1972) were employed with minor modifications. The Hfr donor strain EG333, derived from Hfr Cavalli, transfers the bacterial chromosome counter-clockwise, beginning at about 12 min; it is *pyrA::Tn10* Δ*(pro-lac)XIII* and *leu*$^+$*thr*$^+$, and therefore transfers linked tetracycline-resistance (Tc^R), Lac$^-$ and Leu$^+$Thr$^+$ markers. To test whether *DRT* cDNAs affected cell mating efficiencies, I also measured, in a parallel experiment, the efficiencies of F′ transfer by strain XL-1 Blue, whose F′ episome confers a Tc^R determinant (Bullock *et al.*, 1987). All recipients contained plasmids conferring Ap-resistance; but both donors were Ap-sensitive. Overnight cultures of the donor strains, grown in TBY broth containing tetracycline (12.5 μg/ml), and cultures of the recipient strains (with various plasmids), grown in TBY-Ap broth plus 2 mM IPTG, were subcultured in fresh broth to about 10^9 cells/ml, mixed at ratios of 5, 3, or 0.2 donors to one recipient, and incubated at 38°C for 30, 60, or 90 min. The mixtures then were stirred, diluted where necessary, and spread on LB-Ap plates to score total recipient CFU, on LB-Ap-Tc plates to score Ap^{R}Tc^R recombinants (HfrXF$^-$ cross) or transconjugants (F′ transfer), or on M9-minimal plates containing ampicillin (50 μg/ml), histidine (0.5 mM), arginine (0.06 mM), and proline (2 mM), to score Ap^R Leu$^+$Thr$^+$ recombinants. Aliquots from HfrXF$^-$ crosses were also spread on X-Gal plates plus tetracycline (12.5 μg/ml) to score Tc-

resistant Lac$^+$ colonies. These, presumably the result of *Tn10* transposition from Hfr DNA onto recipient Lac$^+$ chromosomes or integration of *pyrA::Tn10* only, were about 0.1% as frequent as Tc-resistant Lac$^-$ recombinants.

4. Determination of RecA-dependent SOS Induction

Prophage Induction. The RecA$^-$ strain DJ1 was lysogenized with a wild-type λphage (*cI*$^+$), and transformed with various plasmids of interest. These transformants were grown in 5 ml of TBY-Ap broth plus 2 mM IPTG to about 10^9 cells/ml, collected by centrifugation, resuspended in an equal volume of 10 mM $MgSO_4$, and irradiated with 254-nm UV light to 10 J/m^2. Immediately after irradiation, 0.5 ml of the irradiated cells were diluted in 4.5 ml of TBY broth (pre-warmed to 38°C), grown with shaking for 4 h at 38°C. Then 0.1 ml chloroform was added to the cultures. Mixtures were mixed vigorously for 30 seconds, and cell debris was removed by centrifugation (6000 X g for 10 min at 4°C). The phage concentrations were estimated by spotting 10 ul of phage aliquots of various dilutions, as described above.

UV Mutagenesis. RecA$^-$ bacteria (strain DJ1) were transformed with plasmids of interest, grown in TBY-Ap broth plus 2 mM IPTG to 10^9 cells/ml, harvested by centrifugation, resuspended in 10 mM $MgSO_4$, and irradiated with 254-nm UV light. Mutation to rifampicin resistance was assayed by using a triple overlay technique (Sedgwick and Goodwin, 1985):

aliquots containing about 5 X 10^8 cells were mixed with 3 ml of 0.6% LB agar (pre-warmed to 45°C), and mixtures were poured onto LB-plates containing 25 ml of agar. After the first layer solidified, a second 3-ml layer was poured and the plates were incubated at 37°C. Total numbers of surviving cells were scored. After 3-h incubation, mutagenesis plates received a third layer of 3 ml of 0.6% LB agar containing 100 μg/ml rifampicin. Rifampicin-resistant CFU were scored ater 3-day incubation at 37°C.

SOS induction. For measurement of induced expression of the SOS gene *sfiA*, a derivative of strain SM2266 (deleted for the *lac* operon) was constructed that had been made RecA⁻ (*recA::cat*) by P1 transduction, and had been lysogenized with two non-UV-inducible phages, λ*cI*(*ind*⁻) *red3 xis1* and λ *cI*(*ind*⁻) *sfiA::lacZ* (Huisman and D'Ari, 1983). The existence of the two phages was identified by their white plaques on X-Gal plates, but blue plaques on X-Gal plates plus mitomycin C(1 μg/ml). This double lysogen was transformed with various plasmids of interest, and assayed for induction of *sfiA::lacZ* expression (B-galactosidase synthesis) upon treatment with 254-nm UV light or mitomycin C. The specific activities of β-galactosidase were calculated as described by Miller (1972).

5. PCR amplification of DRT cDNAs

Since pSE936 is a low-copy-number expression vector, to amplify *DRT* cDNAs for further subcloning and

restriction mapping, polymerase chain reactions (PCRs) was performed. DNA from bacteria harboring *DRT*-cDNA-encoding plasmids was used as templates, and as primers rightward- and leftward-priming oligonucleotides corresponding respectively to the *GAL1* promoter (5′-ACTTTAACGTCAAGGAG-3′) and *lac* promoter (5′-TGTGGAATTGTGAGCGG-3′) regions of vector pSE936 that flank the cDNA inserts. I used the thermostable DNA polymerase of *Thermus aquaticus*, and standard protocols (Ausubel *et al.*, 1987) with minor modifications. Reaction mixture included 100 ng template DNA, 10 µl of 2 mM dNTPs, 10 µl of 10X reaction buffer, 2 ul of each 50 µM primer, and 2.5 unit *Taq* polymerase in a total volume of 100 ul. 35 cycles of the reactions were accomplished by denaturing at 94°C for 1 min, annealing at 60°C for 2 min, and elongating at 72°C for 5 min in a DNA-thermal cycler (Perkin-Elmer Cetus Instruments).

6. Plasmid DNA Preparation, Restriction Mapping, Cloning, and Deletion Construction

Large-scale plasmid extraction and purification were as described (Maniatis *et al.*, 1982), with minor modifications. Bacteria containing plasmids were grown overnight in 500 ml of TBY-Ap broth, and harvested by centrifugation. Cell pellets were subjected to a lysozyme- (alkaline lysis)-(isopropanol precipitation) procedure, and plasmid DNA was purified by equilibrium sedimentation in CsCl plus ethidium bromide. The alkaline-lysis method of Ausubel *et al*(1987) was employed for small-scale crude preparations("minipreps") of plasmid DNA. In

later experiments, a proprietary DNA purification system, "Magic Minipreps", was used in accordance with instructions provided by the manufacturer (Promega).

Restriction maps of the *DRT* cDNAs were determined by multiple-endonuclease digestions, as described by Ausubel *et al* (1987). All restriction enzyme reactions were performed at 37°C for 1 h, using appropriate buffers provided by the manufacturers. DNA lagations were performed at 16°C for 18 h, using T4 ligase. For cloning of *DRT100*, a 1.4-kb *Eco*RI restriction fragment of the PCR product was inserted into the *Eco*RI site of vector pUC19 (Yanisch-Perron *et al.*, 1985) in both orientations, yielding pQP1001 and pQP1002. Because of the infidelity of PCR amplification, I also excised the *DRT100*-encoding *Eco*RI fragment directly from the plasmid(pQP1000), originally isolated by UV selection in QP2898, and inserted it into pUC19 in both orientations, yielding pQP1003 and pQP1004. For cloning *DRT101, DRT102* and *DRT103*, the *Eco*RI fragments from the originally isolated plasmids pQP1010, pQP1020, and pQP1030, were ligated into the *Eco*RI site of pUC19 in each orientation, yielding respectively pQP1011 and pQP1012, pQP1021 and pQP1022, and pQP1031 and pQP1032. These constructed plasmids were obtained by transformation of appropriate *E. coli* bacteria to ampicillin resistance as described elsewhere (Hays *et al.*, 1990). Orientation of inserts was determined by restriction digestion and gel electrophoresis of "miniprep" plasmid DNA.

Partial-deletion derivatives of *DRT100* and *DRT101* were constructed by digesting plasmid pQP1003 and pQP1011 with restriction endonucleases, electrophoretic purification of the 5′ cDNA portions, and insertion into the appropriate sites of pUC19. In all cases, *DRT* cDNAs were deleted from 3′ end, and the remaining 5′ sequences were aligned for transcription initiated at the *lac* promoter. Structures were verified by restriction analyses of the constructs. The restriction fragments used to construct the deletion plasmids, and their corresponding designations follow [For bp locations see Fig.16(*DRT100*), Fig.18(*DRT101*.)] *DRT100* derivatives: pQP1003N, *Hin*dIII(pUC19 polylinker)-*Nae*I(bp 420) fragment from pQP1003 inserted into *Hin*dIII site of pUC19; pQP1003D, *Hin*dIII(pUC19 polylinker)-*Dra*I(bp 482) fragment from pQP1003 inserted into *Hin*dIII and *Hinc*II sites of pUC19; pQP1003A, *Pst*I(polylinker)-*Ava*II(made blunt by treatment with *E. coli* DNA polymerase I Klenow fragment and dNTPs, bp 721) fragment of pQP1003 subcloned into the *Pst*I and *Hinc*II sites in pUC19; pQP1003B, *Hin*dIII(polylinker)-*Bam*HI(bp 941) fragment of pQP1003 subcloned between the *Hin*dIII and *Bam*HI sites in pUC19; pQP1003H, *Hin*dIII (polylinker)-*Hin*dIII(bp 999) fragment from pQP1003 reinserted into the *Hin*dIII site in pUC19. *DRT101* derivatives: pQP 1011AC, *Hin*dIII(pUC19 polylinker)-*Acc*I(bp 509) fragment from pQP1011 inserted between *Hin*dIII and *Hinc*II sites of pUC19; pQP1011MS, *Hin*dIII(polylinker)- *Msc*I(bp 626) fragment from pQP1011 inserted into the *Hin*dIII and *Hinc*II sites of pUC19; pQP1011XB, *Hin*dIII-*Xba*I(bp 733)

fragment inserted into *Hin*dIII-*Xba*I sites of pUC19; pQP1011AV, *Hin*dIII-*Ava*II(bp 861, filled in at *Ava*II 5′ overhang using Klenow fragment and dNTPs) fragment of pQP1011 reinserted between the *Hin*dIII and *Hinc*II sites of pUC19; pQP1011HD, *Hin*dIII(polylinker)-*Hin*dIII(bp 1110) fragment reinserted into the *Hin*dIII site of pUC19.

7. Determination and Analysis of DNA Sequences

Manual DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) with base-denatured double-stranded plasmid DNA templates and the Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, Ohio). The ^{35}S-labeled fragments were subsequently separated by electrophoresis at a constant power of 50 watts through a 43 by 40 by 0.06 cm 8% acrylamide gel (acrylamide:bisacrylamide = 19:1) in TBE bufer containing 8.5 M Urea. Following electrophoresis, the gel was dried and exposed to Kodak X-Omat AR film at room temperature for about 18 h prior to film development. The *DRT* cDNA sequences were also determined by the Oregon State University Central Services Laboratory, using an Applied Biosystems Model 373A DNA sequencer and a *Taq* Dye Primer Cycle Sequencing kit in accordance with instructions of the manufacturer (bulletin No. 237605). For manual sequencing of the extreme ends of the originally selected *DRT* cDNA inserts I used as primers 5′-ACTTTAACGTCAAGGAG-3′, for sequencing from the *GAL1* promoter of pSE936(Fig.4) into the inserts and 5′-TGTGGAATTGTGAGCGG-3′,

for sequencing from the *lac* promoter of pSE936 into the inserts. For automated sequencing, the *Eco*RI cDNA fragments were used to produce various restriction subfragments. These were subcloned into pUC19 for sequence determination by using M13 mp*lac* Universal and Reverse primers (United States Biochemical Corp.). For *DRT102, 112* and the smaller *DRT111* fragment, and where no appropriate restriction sites were available, the entire *Eco*RI cDNA fragments were subcloned into pUC19 and sequences at the termini determined using the Universal and Reverse primers. As these nucleotide sequence data were obtained, internal primers were prepared as synthetic oligonucleotides on a model 380A automated DNA synthesizer (Applied Biosystems Inc.), and used for further sequencing. All nucleotide sequences were determined on both DNA strands to ensure accuracy.

The *DRT* cDNA nucleotide sequences and their predicted amino-acid sequences were used to search GENBANK release 69 using the Intelligenetics FASTDB search program, and their structures were analyzed by using the Intelligenetics Suite release 5.4 programs SEQ, PEP, QUEST, and GENALIGN.

8. Southern Hybridization Analysis

Genomic DNA from *Arabidopsis*, broccoli and Chinese cabbage (*Brassica pekinensis*) tissues was prepared by the CTAB procedure(Rogers and Bendich, 1985). Maize (*Zea mays*) DNA was provided by Dr. Carol Rivin of Botany Department, Oregon State

University. DNA from *E. coli* and frog (*Xenopus laevis*) liver was isolated by standard procedures (Ausubel *et al.*, 1987). A known amount of total DNA was digested to completion with restriction endonuclease *Eco*RI, and applied to a 0.9% agarose gel. After electrophoresis, the fragments were transferred to a nylon membrane (0.45-μm pore size; Nytran; Schleicher & Schuell) by capillary action for 18 h. The membrane was then dried at 85°C for 2 h. Probes were ^{32}P-labeled by the random-primer method (Feinberg and Vogelstein, 1983), using a commercial kit (United States Biochemical Corp.) with 0.1 μg of *Eco*RI-*DRT*-cDNA fragments. After removal of unincorporated nucleotides on a Sephadex G-50 column (Pharmacia, Uppsala, Sweden), the activity of the probes was measured. The procedure of Maniatis *et al.* (1982) for Southern hybridization, was employed with a minor modification. Prehybridization solutions contained 50% formamide, 3X SSC (1X SSC is 150 mM NaCl, 15 mM sodium citrate), 3X Denhardt's solution, 0.2% SDS, 10 mM EDTA, 0.06 M phosphate, and 0.1 mg of denatured, sheared herring sperm DNA per ml. Hybridization solution was the same as prehybridization solution except with the addition of ^{32}P-labeled probes. After a 4-h prehybridization at 38°C, a volume of radiolabeled probes was added such that hybridization solutions contained approximately 5 X 10^6 cpm/ml. The hybridization reaction mixtures were incubated with gentle shaking at 38°C for 18 h. The filter was then removed, and washed at 38°C in 0.1X SSC-0.2% SDS four times. Autoradiography was performed for 48 h at

-80°C, with Kodak X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) and with an intensifying screen.

9. RNA Preparation and Northern Analysis

Isolation of plant total RNA was by a modification of the phenol/SDS method for plant RNA preparation (Ausubel *et al.*, 1987). Fresh plant materials(5 g) were ground to a fine powder with a prechilled mortar(70-mm o.d.) and pestle under liquid nitrogen, suspended in 50 ml of grinding buffer(0.18 M Tris, 0.09 M LiCl, 4.5 mM EDTA, 1% SDS, pH 8.2), ground at room temperature for an additional 5 min, and transferred to a 250-ml centrifuge bottle. Then 16 ml of phenol equilibrated with TLE buffer(0.2 M Tris, 0.1 M LiCl, 5 mM EDTA, pH 8.2) and 16 ml of chloroform were added and mixed well. The slurry was heated at 50°C for 20 min, and centrifuged at 10000 rpm for 20 min at 4°C. The aqueous layer was extracted sequentially with equal volumes of TLE-equilibrated phenol, twice with phenol:chloroform (1:1), and once with chloroform. RNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold ethanol at -20°C overnight. Total RNA was recovered by centrifugation for 15 min at 10000 rpm, 4°C, in a microfuge. Poly(A)$^+$ RNA was bound to oligo(dT)-cellulose columns, using a mRNA separator kit (Clontech Labaratories, Inc.) in accordance with instructions of the manufacturer (bulletin No. PR81112) as follows. About 2 mg total RNA was disolved in 1 ml of elution buffer (10 mM Tris-HCl[pH 7.4], 1 mM EDTA), and loaded to the oligo (dT)-cellulose spun columns.

The columns were centrifuged at 350 X g for 2 min, washed twice with 0.25 ml of high salt buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl), then twice with 0.25 ml of low salt buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1 M NaCl), and eluted with elution buffer. The RNA concentration was determined spectrophotometrically.

For RNA gel blot analysis, samples of poly(A)$^+$ RNA were denatured in 50% formamide, 6% formaldehyde at 60°C for 15 min, separated electrophoretically on a 1.2% agarose gel containing 1.5% formaldehyde, and subsequently transferred to a nylon membrane(Ausubel *et al.*, 1987). Probes used for radioactive labeling were the *Eco*RI fragments from plasmids pQP1003(*DRT100*; Fig.16), pQP1011(*DRT101*; Fig.18) and pQP1021(*DRT102*; Fig.19), a 1.2-kb *Sst*I-*Eco*RI fragment from *Arabidopsis thaliana* actin gene *AAc1*(Nairn *et al.*, 1988) (provided by Dr. R. J. Ferl, University of Florida), and a 1.2-kb *Hind*III fragment from *Arabidopsis thaliana* chalcone synthase(CHS) gene(Feinbaum and Ausubel, 1988)(gift of Dr. R. L. Feinbaum, Harvard University). Prehybridization incubation was for 6 h at 38°C in 5X SSC(1X SSC is 0.12 M NaCl, 0.015 M sodium citrate), 5X Denhardt's solution(1X Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 50% formamide, 25 mM KPO_4(pH7.4), and 50 μg/ml sheared herring sperm DNA. Hybridization was carried out overnoght at 38°C in the same buffer except with 10% dextran sulfate and ^{32}P-labeled probes added. Filters were washed twice

in 1X SSC, 0.1% SDS at room temperature for 15 min, then twice in 0.25X SSC, 0.1% SDS for 15 min, and exposed to Kodak X-Omat AR film with intensifying screen for various periods.

The relative amounts of *DRT* and CHS mRNAs by densitometric scanning were normalized to actin mRNAs present in the same samples, detected by hybridization with *AAc1* DNA probe. Film densitometry was performed with a GS 300 densitometer (Hoefer Scientific Instruments), and the maximum value obtained was taken as 100%. Several exposures of each blot were analyzed to ensure linearity of response.

IV. RESULTS

A. Photoreactivation and Dark Repair in Arabidopsis

As stated in Section IIA1, the ozone layer prevents all UV-C(100-280 nm) light from reaching the earth's surface. However, laboratory sources of UV-C radiation induce CBPDs and other DNA photoproducts of biological interest, with very high efficiency. Since repair of CBPDs is of interest, 254-nm irradiation was used as a convenient source of CBPDs. To determine whether UV had any biological effects on *Arabidopsis*, I irradiated, with various doses of 254-nm UV light, young (six-leaf) plants growing on petri plates. The plants were subsequently incubated under photoperiodic light, or in the dark. After one week of growth in the light, plants irradiated to 500 and 1000 J/m^2 showed some leaf yellowing but were about the same size as unirradiated plants (Fig.5), and those irradiated to 2000 J/m^2 were slightly reduced in size. In the dark, unirradiated plants grew poorly and all irradiated plants showed in addition severe yellowing and marked growth inhibition; 2000-J/m^2-irradiated plants appeared virtually dead.

Did these morphological effects reflect DNA damage by UV irradiation and, in the case of irradiated plants incubated in the light, repair by photoreactivation? UV repair

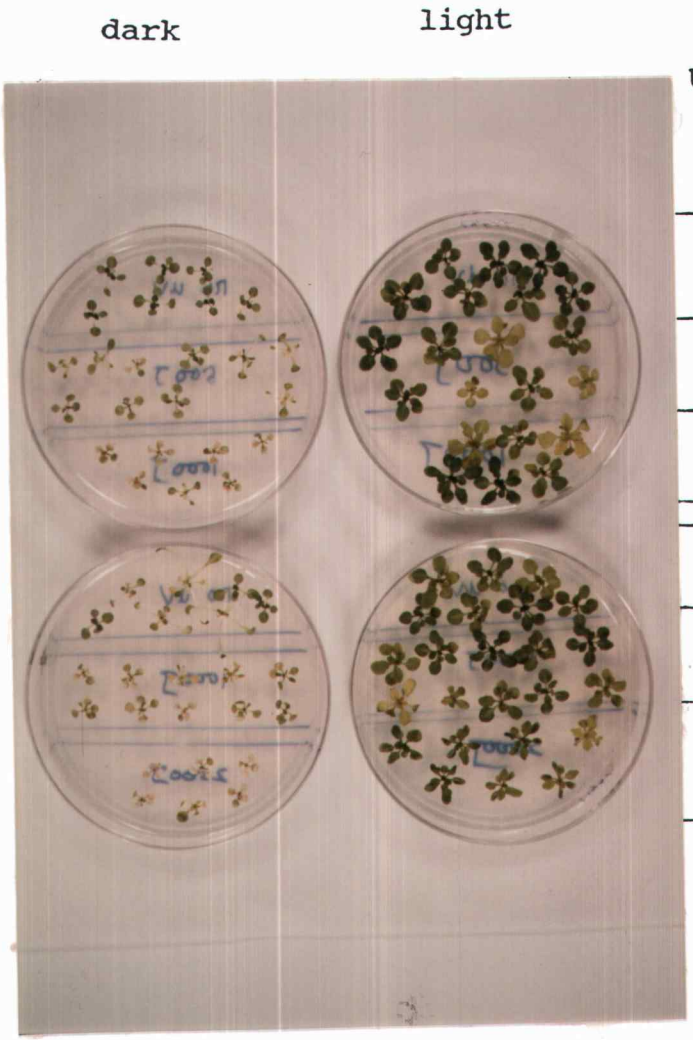

Fig. 5. Effects of UV irradiation on *Arabidopsis*. Six-leaf young plants, growing on MSS-agar plates, were irradiated with 254-nm UV light, and subsequently incubated in the dark, or under photoperiodic light, at 22°C in a Conviron 8601 growth chamber for 7 days.

processes were assayed *in vivo*. Plants were grown on MSS-agar containing ^{3}H-thymidine, which efficiently radiolabeled the plant DNA, irradiated young plants with 254-nm UV light (1000 J/m^{2}), and incubated the irradiated plants in the light or in the dark. The extracted plant DNA was treated with CBPD-specific UV endonuclease, and measured the frequency of UV-endonuclease sensitive sites (ESS) by alkaline-sucrose sedimentation. The results (Fig.6) showed that light-dependent removal of CBPDs was relatively rapid - 50% removed in 1 h, and 90% in 6 h. On the other hand, removal of CBPDs in the dark was much slower - 50% removal in 8 to 17 h, and only 85% after 96 h; this might be overestimated because ^{3}H-dT in DNA was diluted by replication and thus looked like repair. These experiments suggested that photoreactivation appears to be the predominant repair pathway through which CBPDs are removed in *Arabidopsis*.

B. Thermosensitivity and UV-B inducibility of Arabidopsis photolyase

Photolyase activity was assayed by transformation of UV-repair-deficient *E. coli* mutants (*recA uvrB phr*), which cannot be transformed by plasmids containing even a single UV lesion. In the presence of 365-nm photoreactivating light, *Arabidopsis* extracts showed good repair activity (Fig.7). Photoreactivation for 40 min at room temperature removed about 65% of lethal lesions on the UV-irradiated plasmids. The rate of photoreactivation was

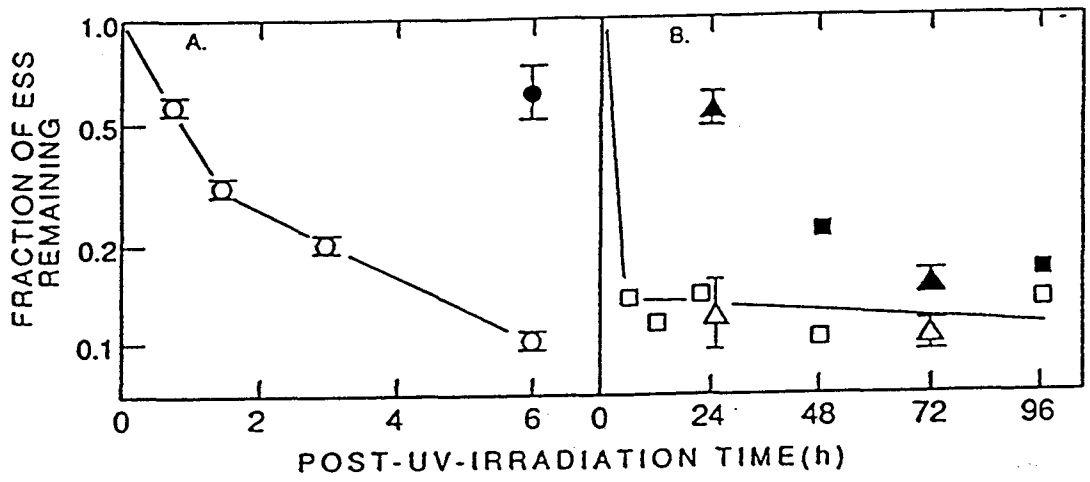

Fig.6. Repair of cyclobutane pyrimidine dimers *in vivo*. Plants were grown at 22°C on MSS-agar containing [^{3}H]-thymidine (2 uCi/ml), irradiated at 254 nm to 1000 J/m^2, and incubated under growth-chamber illumination (open symbols) or wrapped in foil (filled symbols). A, Short-term experiment, with continuous illumination. Data (O,●) correspond to averages from two or three ESS determinations. B, Long-term experiments, with photoperiodic illumination. Data are a composite from two experiments and correspond to one ESS determination (□,■) or average of two determinations (Δ,▲).

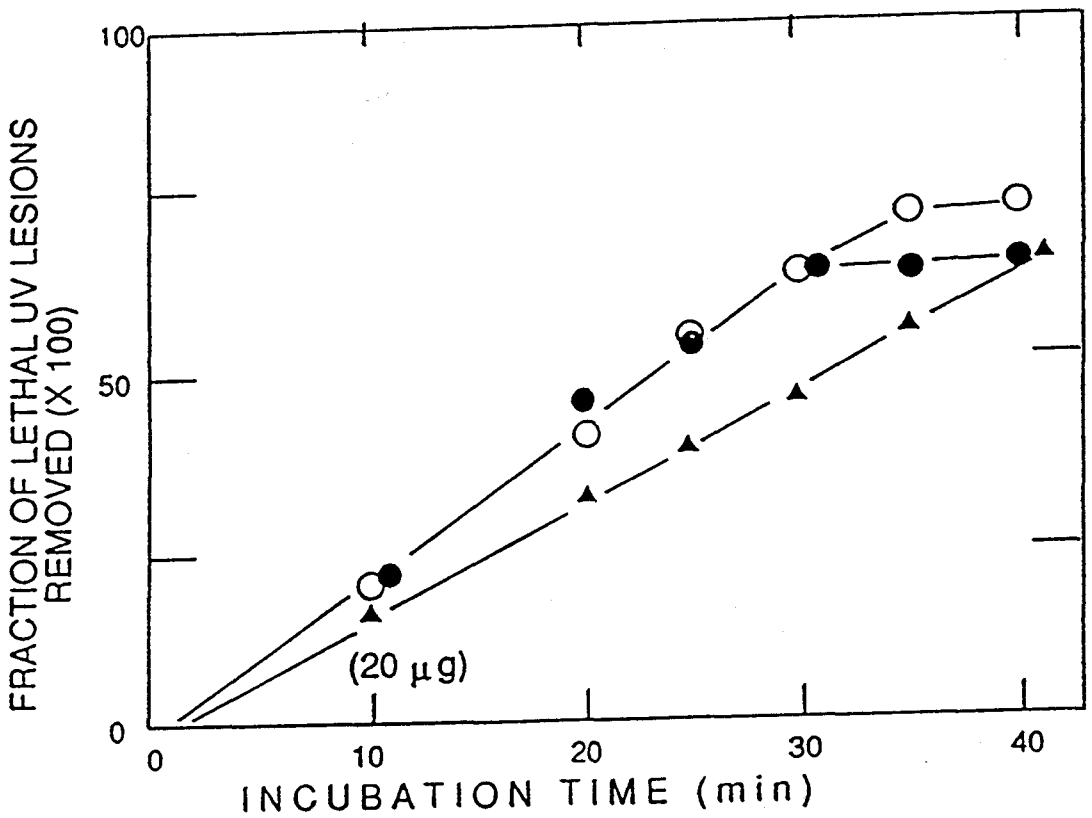

Fig.7. Time course of photoreactivation *in vitro*. Photolyase extracts were prepared from stems and leaves of 8-leaf-stage plants, and assayed at 23°C for 40 µg (●) or 20 µg (▲) of the extract using 100 ng of UV-irradiated plasmid DNA. Purified *E. coli* photolyase (0.4 ng) was assayed in parallel (○).

linear with less than 20 μg of protein added, but did not increase with increased amounts of protein from 80 to as much as 200 μg (data not shown). This might suggest that a certain amount of the lethal lesions were not photoreactivatable. No repair activity was detected in boiled extracts, or when reactions mixtures were incubated in the dark, indicating the repair observed was due to photoreactivation rather than excision repair. The *Arabidopsis* photolyase activity was highest at 4- to 12-leaf growth stages (7 and 12 days old, respectively). It was not detectable in *Arabidopsis* seeds. Activity was about 50% of the maximum in 2-leaf(4-day old) and 20-day-old plants (Pang and Hays, 1991).

The *Arabidopsis* photolyase was quite temperature-sensitive in extracts (Fig.8): half-lives were about 12 min at 30°C and 1.3 min at 50°C. The enzyme activity was partially protected from heat inactivation by the substrate, UV-irradiated DNA. The *in vivo* experiments (Fig.9) showed that after 45-min postirradiation growth at 30°C, photolyase activity was decreased relative to control plants (postirradiation incubation at 22°C), and was rapidly inactivated at 37°C. Photoreactivation of plants at 34°C (incubated in a greenhouse) was intermediate between the activities of the 30 and 37°C plants.

To determine whether UV-B induces photolyase expression in *Arabidopsis*, I first grew plants in a growth chamber, with four different protocols for shielding by Mylar film

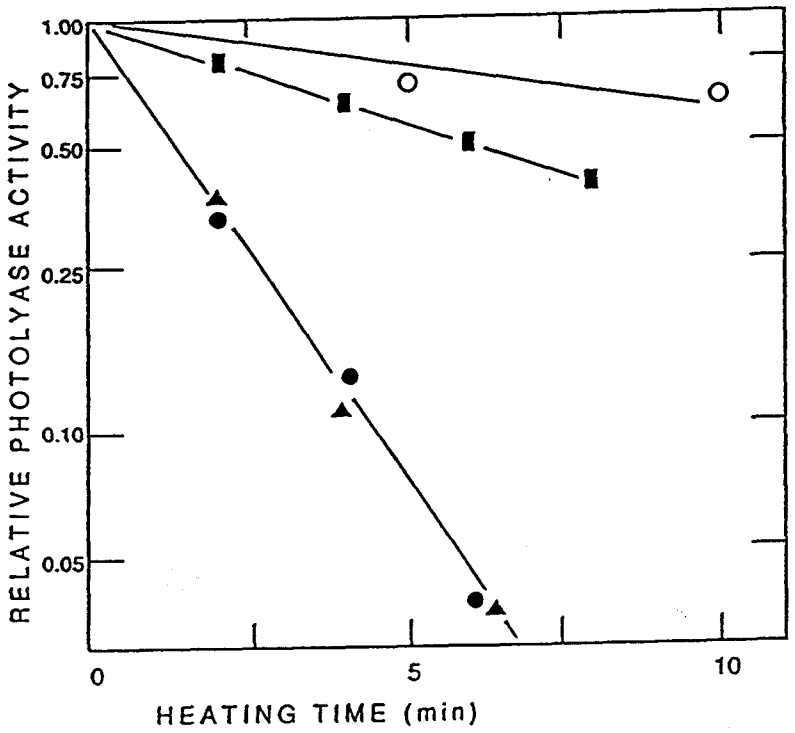

Fig.8. Temperature sensitivity of *Arabidopsis* photolyase *in vitro*. Extracts of 8-leaf-stage plants in PHR buffer (200 µg in 500 µl) were heated in the absence of added DNA at 30°C (O) or 50°C (●) or at 50°C in the presence of 200 ng of unirradiated calf thymus DNA (▲) or of calf thymus DNA irradiated to 400 J/m^2 (■), for the times indicated, and cooled immediately to 0°C. Aliquots (40 µg) were assayed for photolyase activity by incubation with 100 ng UV-irradiated plasmid DNA for 30 min at 23°C.

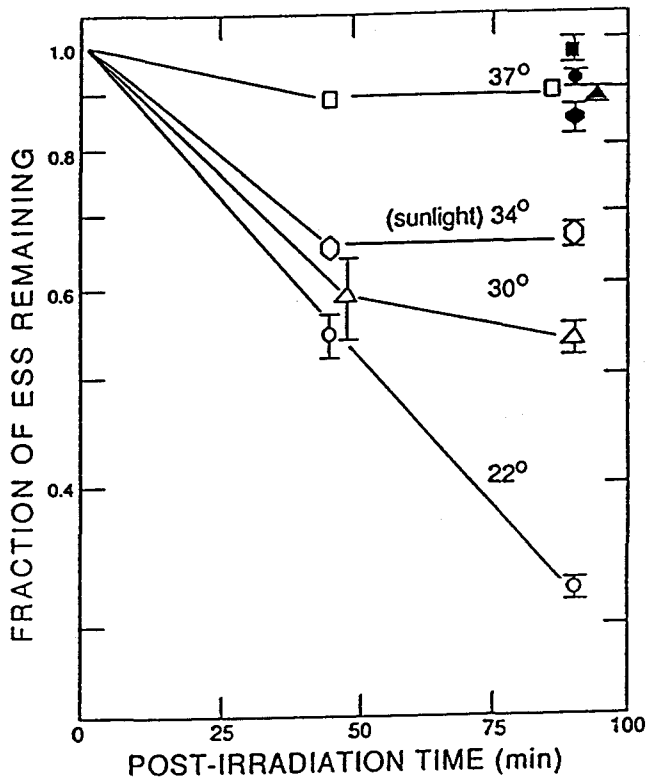

Fig.9. Temperature sensitivity of *in vivo* photoreactivation in *Arabidopsis*. Plants were grown on MSS-agar plates containing ^{3}H-thymidine (2 μCi/ml) to 8-leaf stage and shifted to a growth-chamber warmed to 30°C (Δ,▲) or 37°C (□,■) or to a greenhouse under direct sunlight (○,●) for 1 h. Control plants (○,●) remained at 22°C. Plants were then irradiated at 254 nm to 1000 J/m^2, and incubated at the same temperature for the indicated times, in the light (open symbols) or dark (filled symbols). Extraction of DNA and analysis for UV-ESS was as described under "Experimental Materials and Approaches".

(completely removes all UV wavelengths), for 9 days (8-leaf stage), then treated plants for 7 h with UV-B light. The UV-B intensity was nearly equal to the highest currently expected on the earth (solar noon, equator, high altitudes). No difference in photolyase activities was detected between plants grown entirely or partially without Mylar shielding (Fig.10A, open symbols) and those incubated with shielding (filled symbols). Subsequent treatment of these plants with UV-B light, however, caused a doubling of the rate of photoreactivation of lethal lesions by extracts, corresponding to a four-fold increase in the transformation efficiency of photoreactivated plasmids (Fig.10B, open symbols). Mylar-shielded plants showed no increase (filled symbols) in photoreactivation.

C. Selection and Isolation of Arabidopsis DRT cDNAs

For selection of cDNAs by complementation of *recA⁻uvrC⁻phr⁻ E. coli* mutations, I infected 10^9 QP2898 bacteria, which had been lysogenized with a *cI(ind⁻)* derivative of λKC, with 5 X 10^9 phage from a λYES *Arabidopsis* cDNA library. After 30-min growth in non-selective broth, about 25% of the cells, when plated out, were Ap^R, and presumably harbored excised plasmids. As soon as these transformants (about $2X10^8$ total) were just visible on Ap-LB plates, I washed them off the plates and amplified one-third of these plasmid-containing colonies to about 2 X 10^{11} cells. I irradiated the entire culture with 254-nm UV light at a dose of 0.5 J/m^2 and treated

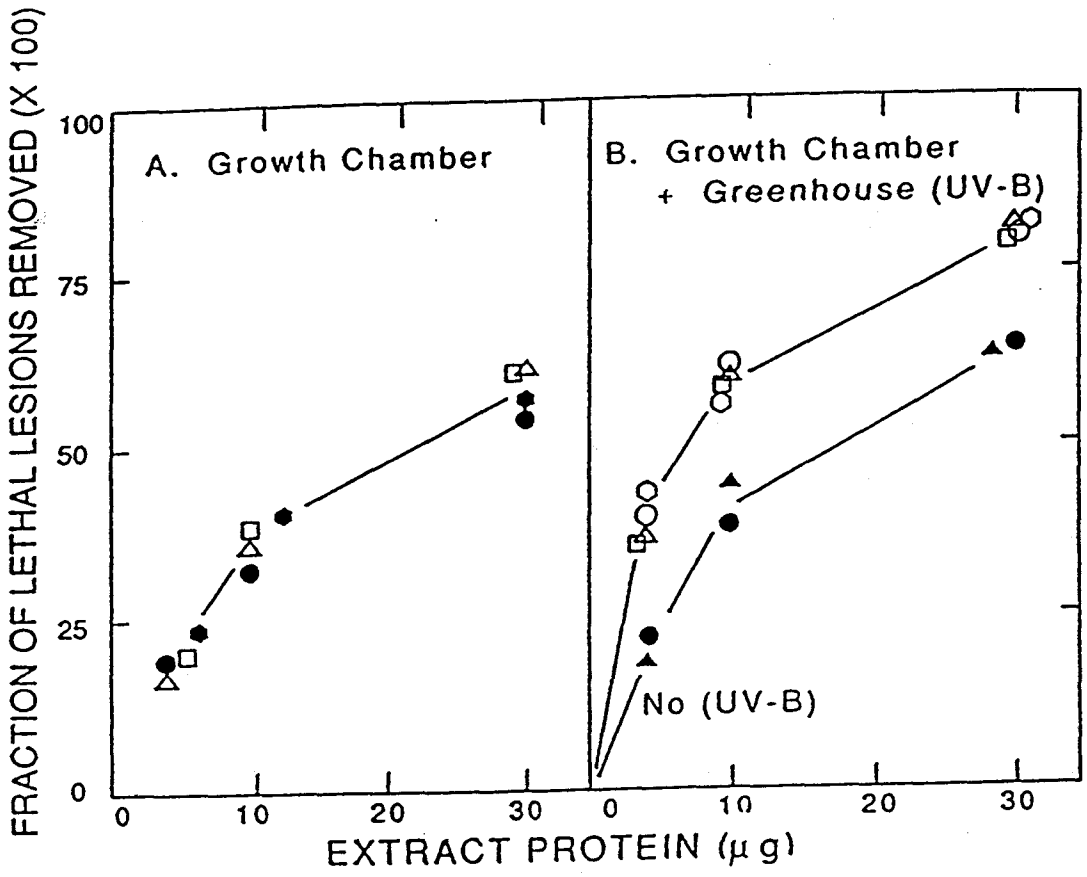

Fig.10. UV-B induction of *Arabidopsis* photolyase. A, Effect of shielding against UV-B during growth-chamber incubation. (●), Container shielded with Mylar filter during days 1-10; (Δ), no shielding days 1-10; (□), Mylar during days 1-7, no shielding days 8-10; (⬢), no shielding days 1-7, Mylar during days 8-10. B, Effect of UV-B irradiation. Open symbols, 7 h unfiltered UV-B (approximately 280 J/m^2); filled symbols, 7 h Mylar-filtered light.

the irradiated cells with photoreactivating light. After incubation at 38°C for overnight, Ap^R survivors arose at a frequency of about 10^{-7}. I screened 840 of these survivors for resistance to a second UV challenge, and found that six colonies were heritably UV resistant. Transformants of naive QP2898 bacteria with plasmids from these six clones again conferred UV resistance. Restriction analysis and the DNA sequences of about 200 bp at each of the ends of the six cDNAs showed that they belonged to four unique cDNAs. These were designated *DRT100, DRT101, DRT102* and *DRT103* (here *DRT* refers to DNA-*D*amage-*R*epair/*T*oleration); the cDNA inserts were 1.35 kb, 1.25 kb, 1.0 kb and 1.6 kb, respectively.

E. coli ruvC recG bacteria lack activities that resolve recombination intermediates, and therefore are deficient in homologous recombination and are DNA-damage-sensitive (Lloyd, 1991). For selection of cDNAs that complemented *ruvC recG* mutations, I used the same procedures as those for $RecA^-UvrC^-$ Phr^- bacteria except that the cDNA-plasmid-containing cells (total about 10^{11} cells) were spread on LB-Ap plates containing 0.06% methyl methanesulfonate (MMS), a concentration at which non-cDNA-containing bacteria could not grow. The 25 survivors were further tested for resistance to UV irradiation. Four isolates conferred UV resistance, and corresponding plasmids contained cDNA inserts: one of 1.5 kb, and three of 0.8 kb. Sequence information obtained from the ends of the three 0.8-kb cDNAs demonstrated that they were

identical. The 1.5- and 0.8-kb cDNAs were designated *DRT111* and *DRT112*, respectively.

D. Characterization of Arabidopsis DRT cDNAs

1. Complementation of E. coli Repair-deficient Mutations

I first compared the ability of the *DRT* cDNAs to confer UV resistance in *recA uvrC phr* (strain QP2898; Fig.11A) and *recA uvrB phr* (strain FD2566; Fig.11B) bacteria. *DRT100, 101, 102* and *103* did not show activity specific for $UvrB^-$ or $UvrC^-$ phenotypes. When irradiated with 0.3 J/m^2, the highest UV dose employed, *DRT100* (triangles) and *DRT101* (diamonds) increased UV survival 50- to 70-fold, and *DRT102* (squares) and *DRT103* (hexagons) increased resistance 10- to 20-fold (Figs 11A, 11B). Treatment of the irradiated bacteria under photoreactivating light (open symbols) did not increase the resistance conferred by *DRT100, 101* or *102*, but *DRT103* increased survival only in the light.

Only *DRT100* increased resistance of *recA uvrB phr* bacteria to a DNA-crosslinking chemical, mitomycin C, and to a DNA alkylating agent, methyl methanesulfonate (MMS) (Fig.12). At the highest doses applied, *DRT100* increased resistance to mitomycin C and MMS by factors of 7.4 or 9.4, respectively. Since Drt101, 102 and 103 were apparently inactive with respect to DNA damage caused by chemical agents, they might be specific for UV damage.

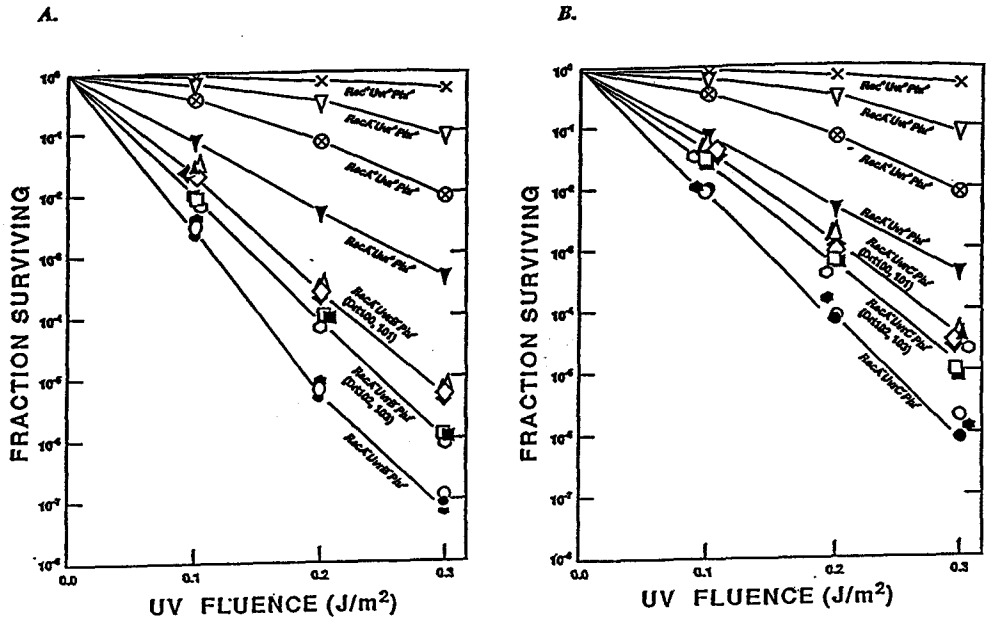

Fig.11. Resistance to UV of *E.coli* mutants expressing *Arabidopsis DRT* cDNAs. Bacterial strains with indicated chromosomal (plasmid) phenotypes were grown to log phase, irradiated at 254 nm to indicated fluences, treated with 365-nm light or not, and surviving fractions were determined, as described under "Experimental Materials and Approaches". A and B. RecA$^+$Uvr$^+$Phr$^+$ strain C600 (pSE936) in light (X) or dark (⊗). RecA$^-$Uvr$^+$Phr$^+$ strain DJ1 (pSE936) in light (▽) or dark (▼). Open, filled, symbols correspond to light, dark in all subsequent designations. A. Plasmid-bearing derivatives of RecA$^-$UvrB$^-$Phr$^-$ strain FD2566. B. Plasmid-bearing derivatives of RecA$^-$UvrC$^-$Phr$^-$ strain QP2898. Drt phenotypes (plasmids): None (pSE936), (○,●); Drt100 (pQP1000). (△,▲); Drt101 (pQP1010), (◇,◆); Drt102 (pQP1020), (□,■); Drt103 (pQP1030), (○,●).

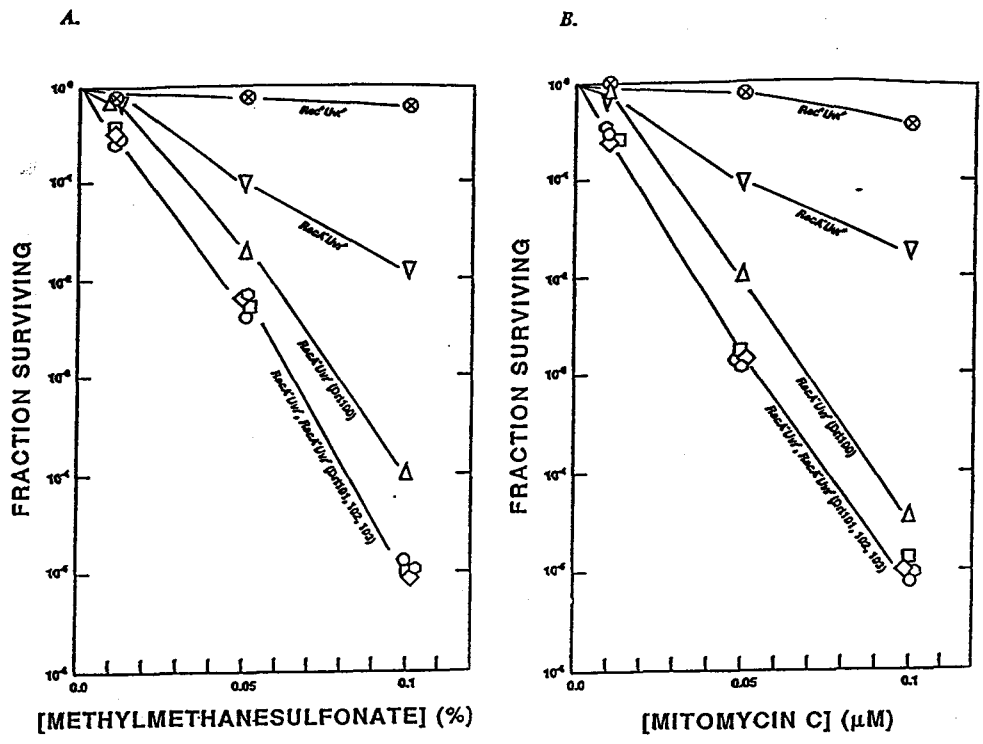

Fig.12. Resistance to chemical DNA-damaging agents. Bacterial strains with indicated chromosomal (plasmid) phenotypes were grown to log phase, treated with indicated concentrations of (A.) methylmethanesulfonate or (B.) mitomycin C, and surviving fractions were determined, as described under :Experimental Materials and Approaches". $RecA^{+}Uvr^{+}$ strain C600 (pSE936), (⊗). $RecA^{-}Uvr^{+}$ strain DJ1 (pSE936), (▽). Derivatives of $RecA^{-}UvrB^{-}Phr^{-}$ strain FD2566, with following plasmid phenotypes (plasmid indiicated): None (pSE936), (○); Drt100 (pQP1000), (△); Drt101 (pQP1010) (◇); Drt102 (pQP1020), (□); Drt103 (pQP1030), (○).

DRT100, *101*, *102* and *103* cDNAs were subcloned into a high-copy-number bacterial expression vector pUC19. To maximize bacterial expression of the *DRT* cDNAs, they were inserted downstream of and in alignment with the *lac* promoter. When fully derepressed by the presence of IPTG, Drt100 increased UV resistance as much as 160-fold (Fig.13A, filled triangles), compared with a maximum of 67-fold in the low-copy-number plasmid pSE936 (Fig.11). Similar results were obtained with resistance to mitomycin C and MMS: fully derepressed *DRT100* in pUC19 further increased survival of RecA$^-$ UvrB$^-$Phr$^-$ bacteria up to 16-fold (Fig. 13B,C, filled triangles). Although the activity of *DRT100* in pUC19 was not as high in the absence of inducer as when fully derepressed, it did increase resistance to these DNA-damaging agents (Fig.13A,B,C, open triangles) in the absence of IPTG, presumably because multiple copies of the *lac* operator titrated out the endogenous *lac* repressor (Zagursky and Hays, 1983). *DRT101* cDNA proved to be aligned with the yeast *GAL1* promoter in the vector pSE936; I do not know if the Drt101 activity in bacteria resulted from bacterial transcription initiated at the yeast promoter or at a minor plasmid promoter. When subcloned into pUC19 and aligned with the *lac* promoter, *DRT101* increased UV resistance 111-fold, as compared to 64-fold in the original pSE936 construct. Neither *DRT102* nor *DRT103* conferred a further increase in UV survival of RecA$^-$UvrB$^-$Phr$^-$ bacteria when transferred from pSE936 to pUC19. No derivatives of pUC19 with cDNAs in inverted

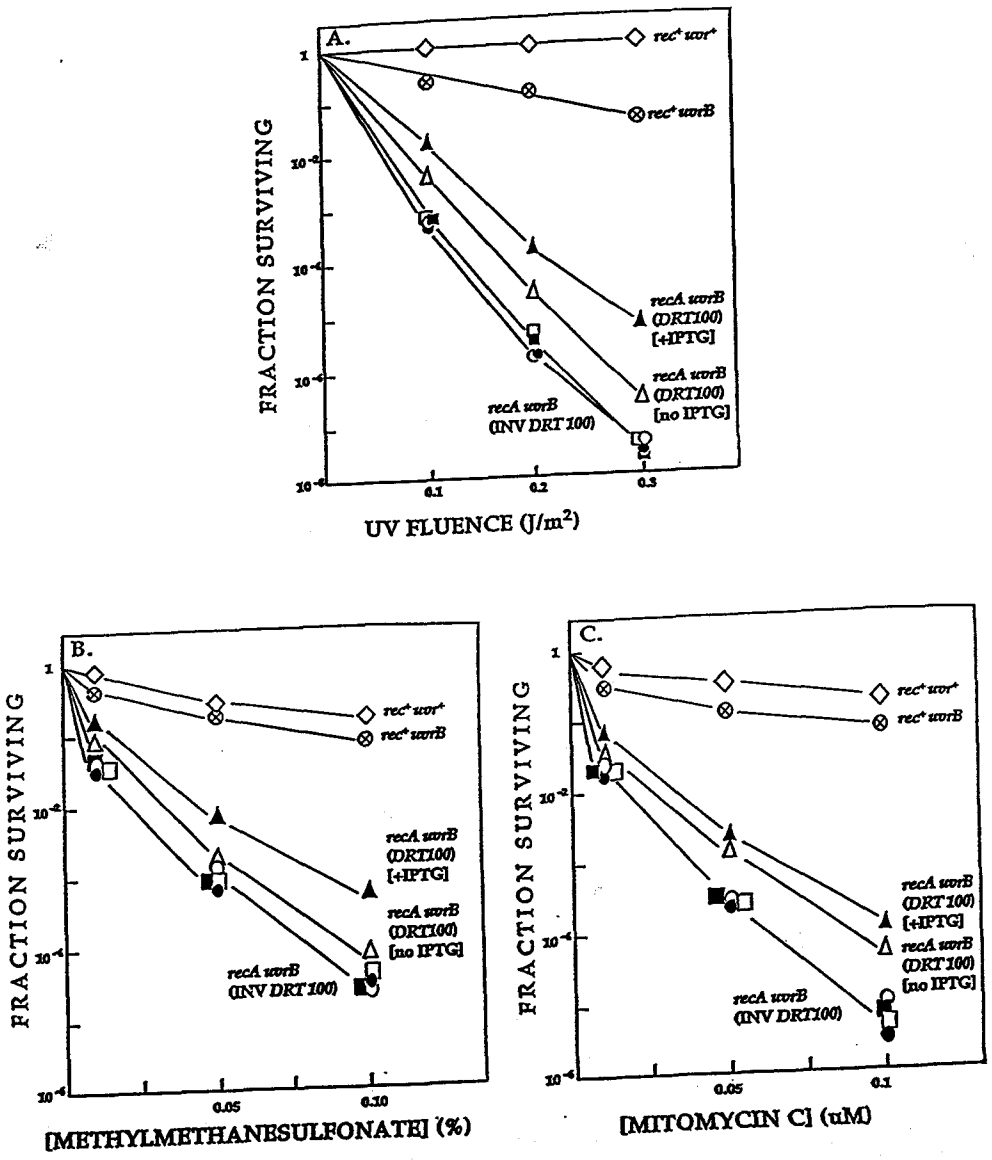

Fig.13. Complementation of *E.coli* DNA-damage-sensitivity mutations by *DRT100* in high-copy-number expression vector. *E.coli* strains C600 [*rec*$^+$*uvr*$^+$ (◇)] and FD2565 [*rec*$^+$*uvrB* (⊗)] containing pUC19, and strain FD2566 (*recA uvrB*) containing respectively plasmids pQP1001 [*DRT100* (△,▲)], pQP1002 [INV *DRT100* (□,■)], or pUC19 (○,●), were grown in the presence (filled symbols) or absence (open symbols) of IPTG.

orientation with respect to the *lac* promoter exhibited any complementation activity(Fig.13A,B,C, square symbols; data not shown); therefore, resistance was not due to the DNA sequence itself, but to the expressed product of the cDNA.

Bacteria deficient in DNA-damage repair and toleration functions typically show reduced plating efficiency for UV-irradiated phages (Hays *et al.*, 1985). Plating efficiencies on RecA$^-$Uvr$^-$Phr$^-$ bacteria were increased by *DRT* cDNAs in pSE936: up to 4-fold by Drt100, 3.9-fold by Drt101 and 2.2-fold by Drt103, corresponding to relative survivals of 41%, 40% and 22% compared to survival with RecA$^+$Uvr$^+$Phr$^+$ bacteria at the lowest dose(2 J/m^2) of phage irradiation (Table 2).

Resistance conferred by *DRT111* and *DRT112* to UV light (Fig.14A) and to the DNA-crosslinking agent mitomycin C (Fig.14B), was measured in RuvC$^-$ bacteria as well as in the RuvC$^-$RecG$^-$ double mutants in which the two *DRT* cDNAs had been originally selected and isolated. Survival of UV-irradiated RuvC$^-$ single mutants was increased only slightly - 5-fold for Drt111 and 3.3-fold for Drt112 (Fig.14A, open symbols), corresponding to complementation efficiencies (relative to Ruv$^+$ bacteria) of 0.5% and 0.3%, respectively. However, Drt111 and 112 increased resistance of RuvC$^-$RecG$^-$ double mutants to UV irradiation by up to 44- and 13-fold (closed symbols), corresponding to complementation efficiencies of 0.45% and 0.13%. Similarly, the two cDNAs increased resistance of RuvC$^-$ single mutants to mitomycin C by only two-fold or less

Table 2. Plating of UV-irradiated phages

UV	Relative plating-efficiency factor [Apparent complementation efficiency]*				
	$RecA^+Uvr^+Phr^+$ (pSE936)	$RecA^-UvrC^-Phr^-$ (pSE936)	(pQP1000)	(pQP1010)	pQP1030)
2 J/m^2	9.8 [100%]	(1)	4.0 [41%]	3.9 [40%]	2.2 [22%]
4 J/m^2	12.9 [100%]	(1)	2.9 [22%]	1.7 [13%]	1.9 [15%]
6 J/m^2	23.2 [100%]	(1)	2.6 [11%]	1.7 [7.3%]	1.5 [6.5%]
8 J/m^2	43.2 [100%]	(1)	2.5 [5.8%]	1.8 [4.2%]	1.5 [3.5%]

* Efficiencies of plating of λ*plac5 lacZ118 red3 cI857* phages, irradiated at 254 nm to indicated fluences at a concentration of $5X10^7$ PFU per ml in TM buffer, were determined using bacterial strains C600 ($RecA^+Uvr^+Phr^+$) and QP2898 ($RecA^-UvrC^-Phr^-$). Relative plating-efficiency factor equals plating efficincies for C600 (pSE936) or QP2898 (pQP1000, 1010, 1030) bacteria divided by those for QP2898 (pSE936) bacteria which were $8.8X10^{-2}$, $5.2X10^{-3}$, $3.8X10^{-4}$ and $3.7X10^{-5}$ for 2, 4, 6 and 8 J/m^2, respectively. Range between the two independent determinations was less than ± 14% of averages.

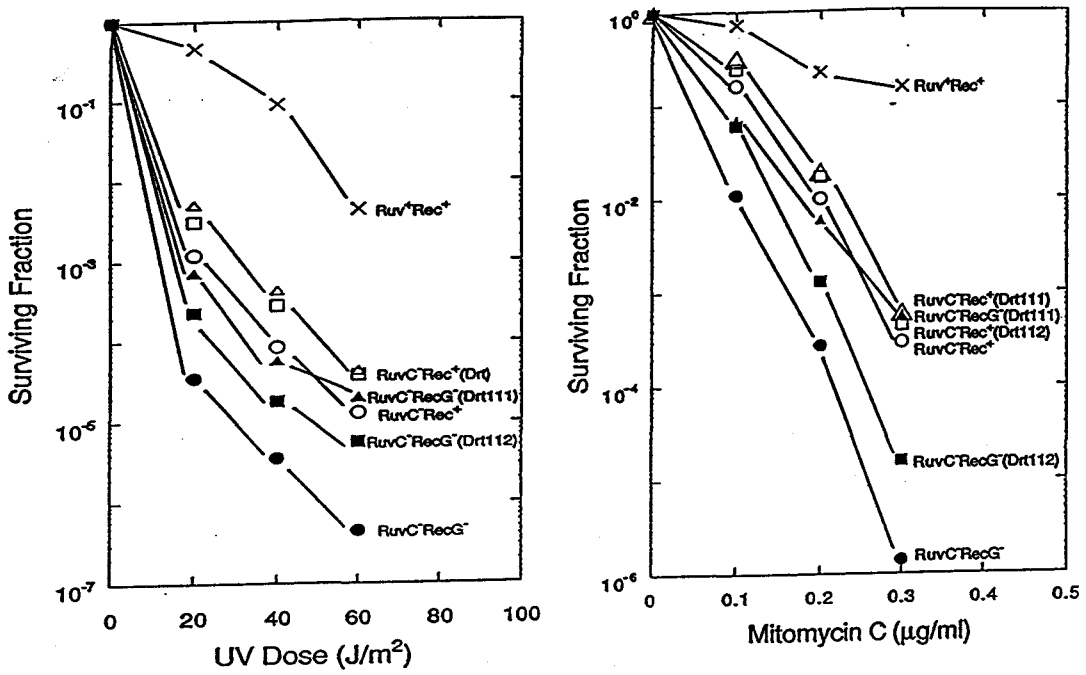

Fig.14. Resistance to DNA-damaging agents of bacteria expressing Drt11 and Drt112. Indicated bacteria were (A) irradiated with 254-nm UV light to fluences indicated, or (B) spread on plates containing indicated mitomycin C concentrations, and surviving bacteria scored, as described under "Experimental Materials and Approaches". Strains [phenotypes] employed: (X), AB1157 (pSE936) = [Ruv^+Rec^+]; (O), CS85 (pSE936) = [$RuvC^-Rec^+$]; (Δ), CS85 (pQP1110) = [$RuvC^-Rec^+$(Drt111)]; (□), CS85 (pQP1120) = [$RuvC^-Rec^+$(Drt112)]; (●), N3398 (pSE936) = [$RuvC^-RecG^-$]; (▲) N3398 (pQP1110) = [$RuvC^-RecG^-$(Drt111)]; (■), N3398 (pQP1120) = [$RuvC^-RecG^-$(Drt112)].

(Fig.14B, open symbols), but increased survival of RuvC⁻RecG⁻ bacteria by 407-fold and 11-fold respectively(filled symbols) at the highest concentration (0.3 μg/ml) of mitomycin C employed; this corresponds to complementation efficiencies of 0.38% and 0.01%, respectively. I also tested resistance, conferred by Drt111 and Drt112 to MMS, the DNA alkylating agent originally used for selection of the two *DRT* cDNAs. At a concentration of 0.015%, Drt111 and 112 increased survival of RuvC⁻RecG⁻ bacteria by 20- and 4-fold, respectively. Resistance of these double mutants to 10 μM 4-nitroquinoline-N-oxide(NQO) was increased 7-fold by Drt111 and 3-fold by Drt112.

Since the *DRT101* cDNA in vector pSE936 was aligned with the yeast *GAL1* promoter, it was possible to test Drt101 for complementation of yeast excision repair mutations. I therefore transformed the *S. cerevisae rad2* mutant with pQP1010, and compared effects on UV survival of non-induced (2% glucose), partially induced (1% galactose, 1% glucose) and fully (2% galactose) induced levels of *DRT101* expression. At 8 J/m^2, *DRT101* increased resistance 3.3, 8.1, and 12.6-fold, respectively. Fig 15 shows the survival curves for cells grown in YPD broth containing 1% galactose plus 1% glucose. Plasmids pQP1000 and pQP1020, in which the *DRT100* and *DRT102* cDNAs are aligned with the *lac* promoter, did not complement the *rad2* mutation(data not shown).

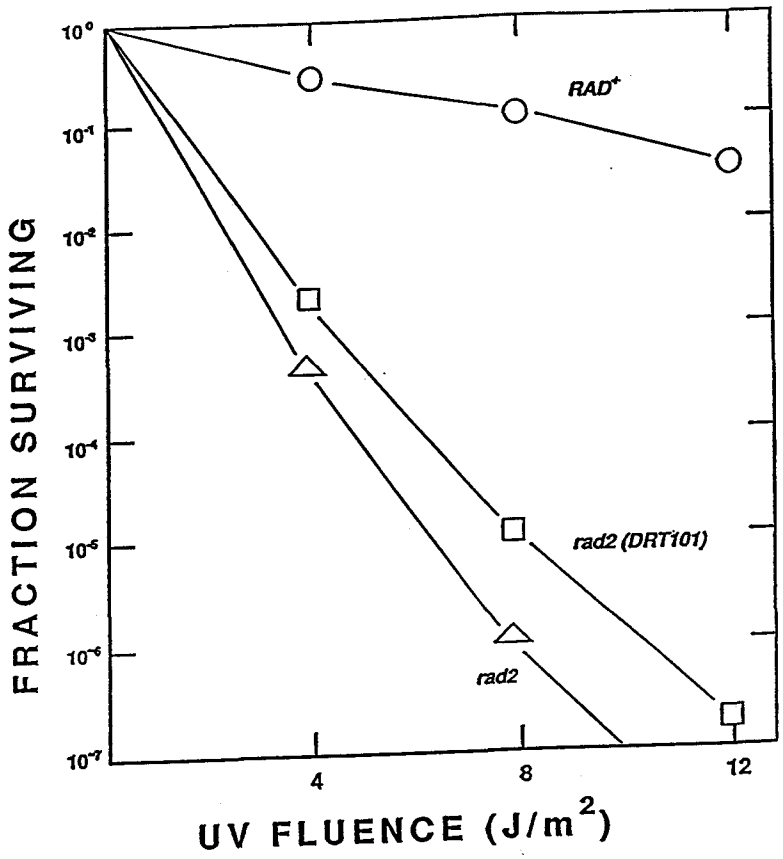

Fig.15. Complementation of yeast *rad2* mutation by Drt101. Yeast strain LN2-1I1 containing plasmids pQP1010 or pSE936, and strain BY747 containing pSE936, were grown at 30°C in YPD-broth containing 1% glucose plus 1% galactose, irradiated at 254 nm to the indicated fluences, spread on YPD-plates, and incubated in the dark at 30°C. Surviving fraction equals viable cell count before UV treatment divided by cell count after treatment. Phenotypes of cell [strains (plasmids)]: *RAD*$^+$ [BY747(pSE936)], (O); *rad2* [LN2-1I1(pSE936)], (Δ); *rad2*(Drt101) [LN2-1I1(pQP1010)], (□).

2. Complementation of RecA⁻ Phenotypes by Drt100 Activity

Since Drt100 increased resistance of *recAuvrphr* mutants to a variety of DNA-damaging agents, it might have been complementing RecA⁻ rather than Uvr⁻ phenotypes. I thus tested the effect of Drt100 on plating of λ*red*⁻ *gam*⁻ phages. These phages can grow in *E. coli* cells only if homologous recombination between monomer circles, promoted by the host RecA function, produces sufficient levels of dimers which are substrates for encapsidation (Enquist and Skalka, 1973). This test therefore provides a sensitive, but not quantitative measurement for RecA recombination activity. Drt100 increased by 18-fold the plating efficiency of *red*⁻ *gam*⁻ phages on RecA⁻ UvrC⁻Phr⁻ bacteria (Table 3, column 2). The homologous recombination system in *E. coli* also increases the efficiency of phage P1 lytic growth (Cohen, 1983). As shown in column 3 of Table 3, the plating efficiency of P1(vir) phages on RecA⁻UvrC⁻Phr⁻ bacteria was increased up to 65-fold by Drt100. The efficiencies with which Drt100 complemented RecA⁻ deficiencies in propagation of λ*red*⁻ *gam*⁻ and P1 phages were very low, as indicated in the brackets of Table 3, and might not be strong evidence that Drt100 complemented RecA⁻ phenotypes. I further examined, therefore, the effect of Drt100 activity on the ability of RecA⁻ recipients to form stable transconjugants during Hfr X F⁻ conjugal crosses, a well-studied homologous recombination process. From the

Table 3. Partial correction of Rec⁻ phenotypes by *DRT100*

RecA phenotype of Uvr⁻Phr⁻ bacteria [plasmid]*	Relative λ*red*⁻*gam*⁻ plating efficiency ($X10^8$)	Relative phage P1 plating efficiency ($X10^4$)	Recombinants per 10^6 recipients, Hfr X F⁻cross
	[RecA⁻ complementation efficiency (%)]		
RecA⁺ [None]	10^8 [100%]	10^4 [100%]	2690 [100%]
RecA⁻ [None]	0.8±0.06 [0%]	0.4±0.3 [0%]	0.3 [0%]
RecA⁻ [Drt100]	14±4 [0.000013%]	26±8 [0.0026%]	16 [0.6%]
RecA⁻ [INV Drt100]	0.9±0.2 [0%]	0.3±0.1 [0%]	ND ND

* Bacterial strains: RecA⁺Uvr⁻Phr⁻, FD2565 (pUC19) (phage plating) or QP2895 (pUC19) (conjugation); RecA⁻Uvr⁻Phr⁻, QP2898 (pUC19) (phage plating) or QP2897 (pUC19) (conjugation); RecA⁻ Uvr⁻Phr⁻ (Drt100), QP2898 (pQP1001 or pQP1003) (phage plating) or QP2897 (pQP1003) (conjugation); RecA⁻Uvr⁻Phr⁻ (INV Drt100), QP2898 (pQP1002 or pQP1004).

ND: not determined.

results obtained (Table 3, column 4), it was clear that Drt100 increased conjugal recombination - a 50-fold increase with an apparent complementation efficiency of 0.6%. Drt100 did not promote DNA transfer: the efficiency of mating was the same in $RecA^+$, $RecA^-$ and RecA(Drt100) bacteria (data not shown).

An alternative explanation for the complementation of $RecA^-$ phenotypes by Drt100 would be stimulation of autoproteolysis of LexA(and λ) repressor(s), thus mediating induction of *E. coli* SOS responses to DNA damage. This was tested in three ways, with negative results (data not shown). First, Drt100 did not increase the efficiency of spontaneous or UV-stimulated induction of wild-type λ prophages in $RecA^-$ bacteria, under conditions where UV-irradiated $RecA^+$ bacteria released 3 X 10^{-6} PFU/cell. Second, in $RecA^-$ bacteria irradiated with 5 J/m^2 UV light Drt100 did not increase the frequency of rifampicin-resistant mutants; the mutation frequency was increased by 6-fold in wild-type cells irradiated with the same UV fluence. Finally, Drt100 did not increase induction in $RecA^-$ bacteria of *sfiA*, a known SOS gene, above background levels, under conditions where *sfiA::lacZ* expression was induced 25-fold in $RecA^+$ cells.

3. Complementation of Recombination Deficiency by Drt111 and Drt112 Activities

The assays here tested the ability of the Drt111 and Drt112 activities to complement the recombination deficiencies

of resolvase-deficient recipients in an Hfr conjugation. In these experiments, matings were done in liquid between the ampicillin-sensitive Hfr Cavalli strain EG333 and the resolvase-deficient recipients harborinng Ap^R-conferring plasmids, and scored quantitatively. The recipient strains (CS85 and N3398) carried *thr-1 leuB6* mutations; recombinant transconjugants were thus selectable as Ap^R Thr^+ Leu^+ colonies on minimal plates. The results were shown in Table 4. In the *ruvC* mutant background Drt111 and Drt112 increased recombination efficiency by 2.5- and 1.7-fold, corresponding to apparent complementation efficiencies of 75% and 44% of wild-type levels, respectively. In *ruvC recG* double mutants Drt111 and Drt112 increased recombination frequencies by 35- and 21-fold, corresponding to complementation efficiencies of 11% and 5.8%, respectively.

4. DNA and Protein Sequence Analyses

DRT100 The nucleotide sequence of the 1.35 kb *DRT100* cDNA was determined as described in Experimental Materials and Approaches. The nucleotide sequence and the predicted amino acid sequence of *DRT100* are presented in Fig 16. The 1187-bp open reading frame (ORF), starting from the 5′ end of the cDNA, encodes a 395-amino-acid polypeptide, in which the N-terminal portion is rich in Ser/Thr and small side-chain residues and has a net positive charge; the Gly-Arg-Val-Thr sequence, encoded by nucleotides 208-219, is identical to the

Table 4. Increased conjugal recombination by Drt111 and Drt112

Recipient (plasmid) phenotype[a]	Number of recipients ($\times 10^{-7}$)[b]	$Thr^+Leu^+Ap^R$ recombinant ($\times 10^{-7}$)[b]	Recombinant frequency[c]
Rec^+Ruv^+ (pSE936)	35 ± 4	5.6 ± 0.6	(1.0)
Rec^+RuvC^- (pSE936)	32 ± 4	1.0 ± 0.4	0.21
Rec^+RuvC^- (Drt111)	21 ± 2	2.5 ± 0.2	0.75
Rec^+RuvC^- (Drt112)	24 ± 2	1.7 ± 0.2	0.44
$RecG^-RuvC^-$ (pSE936)	37 ± 2	0.02 ± 0.004	0.003
$RecG^-RuvC^-$ (Drt111)	38 ± 6	0.67 ± 0.02	0.110
$RecG^-RuvC^-$ (Drt112)	41 ± 8	0.41 ± 0.7	0.058

[a] Bacterial strains (line) were: (line 1), AB1157 (pSE936); (2), CS85 (pSE936); (3), CS85 (pQP1110); (4),CS85 (pQP1120); (5), N3398 (pSE936); (6), N3398 (pQP1110); (7), N3398 (pQP1120).

[b] Data are averages and standard deviations for two trials.

[c] Relative recombinant frequency equals ratio of recombinant frequency for indicated bacteria, divided by ratio for $RecA^+Ruv^+$ bacteria.

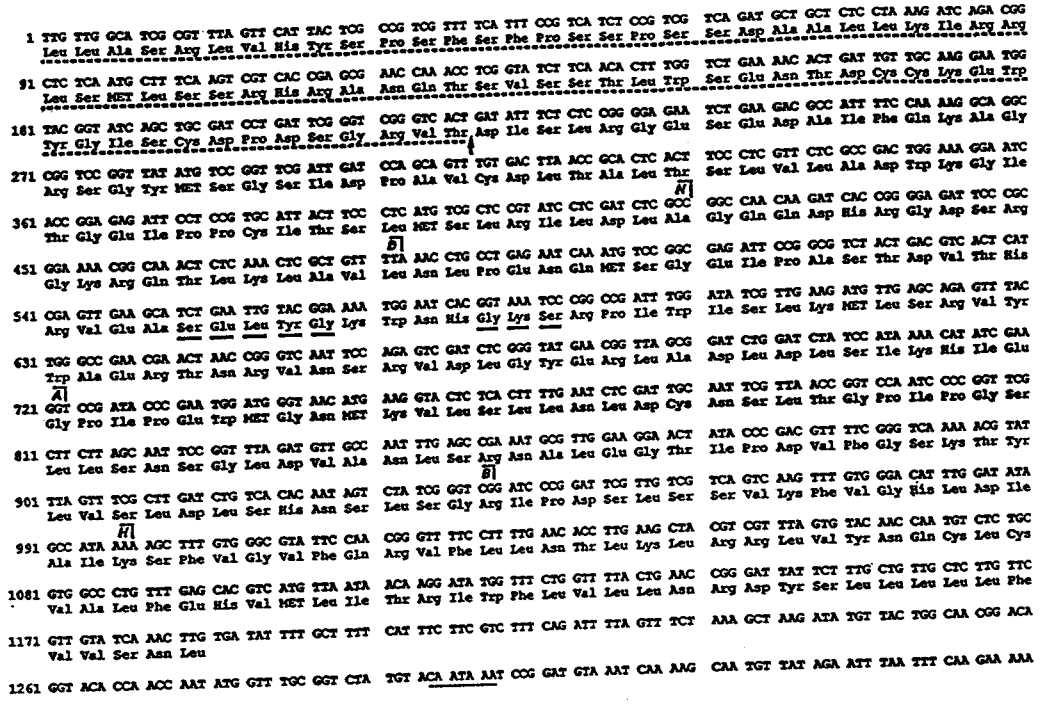

Fig.16. Sequences of *Arabidopsis DRT100* cDNA and of longest open reading frame. DNA sequences were determined in both directions. Features indicated: ---, putative chloroplast transit peptide sequence; i, putative site for protease cleavage during processing in chloroplast; -, amino acids in putative nucleotide binding motif; ..., putative polyadenylation signal; *N,D,A,B,H,* endpoints of *DRT100* coding sequences retained in plasmids pQP1002N, 1003D, 1003A, 1003B, and 1003H (see "Experimental Materials and Approaches"). (The cDNA isolated begins with GG at bp -2.)

proposed consensus site for processing chlorophyll a/b binding proteins. This structure feature suggests that Drt100 may be a nuclear-encoded chloroplast protein containing a transit peptide (Keegstra *et al.*, 1989). The putative mature Drt100 would contain 322 residues, slightly smaller than bacterial RecA proteins (Murphy *et al.*, 1990).

From bp 553 to 588, *DRT100* encodes eight amino acids identical or highly conserved with respect to putative nucleotide-binding motifs (Walker boxes) in RecA-like yeast Dmc1 (Bishop *et al.*, 1992) and Rad51 (Shinohara and Ogawa, 1992) proteins, as well as in a bacterial RecA consensus sequence (Walker *et al.*, 1982) (Fig.17A). However, no significant global homology was shown between Drt100 and any of these proteins. The C-terminal part of *DRT100* contains four repeats of leucine-rich motifs (LRMs), which are thought to be involved in specific protein-protein interactions. In general, LRM repeats contain 22-25 amino acids, including several conserved leucine positions; two of yeast repair proteins, Rad1 and Rad7, are the examples of the superfamily with such LRM repeats (Schneider and Schweiger, 1991). Drt100 contains four tandem LRM repeats with 4 or 5 conserved leucines and 11-12 other positions of conservation in each, encoded by bp 685-972 (Fig.17B).

The first ATG of the *DRT100* at nucleotide 97 is preceded by a stretch of purine-rich residues that might function as ribosome-binding site (Shine and Dalgarno, 1975) in bacteria.

A. Putative nucleotide binding motif.

RecA	v	E	I	Y	G	p	e	s	s	G	K T
Dmc1	T	E	V	F	G	e	f	r	c	G	K T
Rad51	T	E	L	F	G	e	f	r	t	G	K S
Drt100	S	E	L	Y	G	k	w	n	h	G	K S

B. Leucine-rich motifs

A

L a d L *D* L S i k h I E G P I P E w m G n m K v

L s l L N L d c N S L *T* G P I P g S L l S n *S* g

L d v a N L S r N a L E G P I P D v f G S k *T* Y

L V s L *D* L S h N S L *S* G r I P D S L s S l K F

B

Fig.17. Protein motifs in Drt100. (A) Comparison of predicted Drt100 protein sequence with those of a bacterial RecA consensus and two yeast RecA-like proteins, in the putative nucleotide-binding region (see text). (B) Folding of predicted Drt100 sequence on itself to reveal repeated 24-residue leucine-rich motifs (LRMs) (see text). Upper case letters, positions of identity or high conservation; low case, positions of nonconservation; A and B, C-terminal endpoints of deletion proteins corresponding to plasmids pQP1003A and pQP1003B (see legend to Fig.16), respectively.

Whether the complementing activity expressed by plasmids pQP1001 and pQP1003 comes from a putative 363-amino-acid product initiated at this ATG, or from the 426-residue LacZ-Drt100 fusion protein, remains to be determined in further *in vitro* studies. Inspection of the 3′ region revealed the common polyadenylation signal AATAAA (Wickens and Stephenson, 1984) upstream of the poly(A) tail. However, the pentanucleotide sequence CAYTG which is found adjacent to the polyadenylation site in many eukaryotic mRNA (Berget, 1984), is not found in the 3′ part of the *DRT100* sequence.

DRT101 The open reading frame (ORF) of *DRT101* sequence (Fig.18) predicts a 276-amino-acid polypeptide. Its N-terminus (about 60-90 residues) also possesses the structure features of chloroplast transit peptides: rich in Ser/Thr and small side-chain amino acids, and a net positive charge; but it does not have a consensus processing site as described above. Comparing Drt101 with the sequences in the SWISSPROT data bank, I found no similarity with any known protein. Drt101 shows no significant homology to the known DNA repair proteins; however, some short amino-acid-sequence blocks conserved between Drt101 and either the yeast excision repair protein Rad10(Reynolds *et al.*, 1985) or the human repair protein ERCC-1(van Duin *et al.*, 1986) may be significant. These are (Drt101 vs Rad10): S_{151}nlIN vs S_{118}tgIN, L_{185}ykDlpSR vs L_{145}tvDyiSR, L_{219}qDaiNSE vs L_{168}vDdnNSE; and (Drt101 vs ERCC-1): A_{38}EfdI vs A_{61}EyaI, L_{113}QhLG vs L_{158}QsLG.

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CGTCTTCAAGGCAACTGCAG AATGATTACTTTGAGAATCT ATCTACTTCGTTTAGGTGTT   60

TGATTGATTTGATTCCAAGC AGTGGAAATGATAAGTTAGA GATTAGCAAATTGCTTGTAG  120

ATGGTGCTAATGGTGTAGGT GGACAGAAGATTGAGAAGCT AAGAGGGTCTTTGAGTAATT  180

TAGATGTTGAGATTCGTAAC ACAGGGAGAGATGGTGGTGT GCTTAATGAAGGTGTAGGTG  240

CTGATTTTGTGCAGAAAGAA AAGGTTTTGCCTGTAGGATT TGGGTTTAAGGAAAGTTGGG  300

ATG AGG TGT GCG AGT TGG ATG GTG ATG AGA    TCG ATT GGT TAC TTT TAC AGT CCT TCA GAT  360
MET Arg Cys Ala Ser Trp MET Val MET Arg    Ser Ile Gly Tyr Phe Tyr Ser Pro Ser Asp   20

CCT CTG AAA AGG TTG AGC TAC TTG GCG GTG    ATA AGA TTC TGT CTT TGT TTG CTC TCT TCA  420
Pro Leu Lys Arg Leu Ser Tyr Leu Ala Val    Ile Arg Phe Cys Leu Cys Leu Leu Ser Ser   40

TCA AAG AGC AAC AAA ATG CTC TGG AGC ATG    ATG AAG AAA GGA AGC AGT CTC GTC TTG CTG  480
Ser Lys Ser Asn Lys MET Leu Trp Ser MET    MET Lys Lys Gly Ser Ser Leu Val Leu Leu   60

ATG TGC AGA CAC TTA CGC GAA TGG TGC GTC    TAC AGA TTA CCT AAA GCA TTT GGG TTT ACG  540
MET Cys Arg His Leu Arg Glu Trp Cys Val    Tyr Arg Leu Pro Lys Ala Phe Gly Phe Thr   80

ATG CTG TTT CTG CTG AAA CTG GAG TTC AAG    CAT TTA CAC GAG AAA GCA GCA GAG TTT GAT  600
MET Leu Phe Leu Leu Lys Leu Glu Phe Lys    His Leu His Glu Lys Ala Ala Glu Phe Asp  100

ATT GGA ATC TAC TTT GAA GCT AAT GGC CAC    GGG ACT ATT CTC TTC TCG GAA TCT TTC CTA  660
Ile Gly Ile Tyr Phe Glu Ala Asn Gly His    Gly Thr Ile Leu Phe Ser Glu Ser Phe Leu  120

TCT TGG TTA GTT TCC AAA CAA AAG GAT CTT    ACG GCT AAA GGT CAG GGT GGT TCT GAA GAG  720
Ser Trp Leu Val Ser Lys Gln Lys Asp Leu    Thr Ala Lys Gly Gln Gly Gly Ser Glu Glu  140

CAC AAA GCT GTT TCT AGA CTA ATG GCG GTG    AGT AAT CTG ATT AAC CAA GCG GTA GGT GAT  780
His Lys Ala Val Ser Arg Leu MET Ala Val    Ser Asn Leu Ile Asn Gln Ala Val Gly Asp  160

GCT CTA AGT GGA GTG CTC TTG GTT GAA GTG    ATT CTA CAA CAC CTG GGA TGG TCG ACA GAG  840
Ala Leu Ser Gly Val Leu Leu Val Glu Val    Ile Leu Gln His Leu Gly Trp Ser Thr Glu  180

AAG TGG AAT GAG CTA TAC AAG GAC CTT CCT    AGC AGG CAG ATC AAG GTC GAA GTT CCA GAT  900
Lys Trp Asn Glu Leu Tyr Lys Asp Leu Pro    Ser Arg Gln Ile Lys Val Glu Val Pro Asp  200

AGA ACA GCG GTT GTG ACC ACA AGC GAA GAA    ACC GAG GCT CTG AGA CCT ATG GGG ATT CAA  960
Arg Thr Ala Val Val Thr Thr Ser Glu Glu    Thr Glu Ala Leu Arg Pro MET Gly Ile Gln  220

GAT GCT ATT AAT TCT GAA ATC AAG AAG TAC    TCG CGT GGC AGA GCT TTT ATA AGG CCA TCG 1020
Asp Ala Ile Asn Ser Glu Ile Lys Lys Tyr    Ser Arg Gly Arg Ala Phe Ile Arg Pro Ser  240

GGT ACA GAA GAT GTG GTG AGA GTA TAT GCA    GAG GCT TCC ACT CAA GAA GAT GCT GAT TCT 1080
Gly Thr Glu Asp Val Val Arg Val Tyr Ala    Glu Ala Ser Thr Gln Glu Asp Ala Asp Ser  260

TTG GCT AAT TCT GTG GCT CAG CTC GTC AAA    AGC TTC CTT GGT TCA AGC TAA AGAACCATC   1140
Leu Ala Asn Ser Val Ala Gln Leu Val Lys    Ser Phe Leu Gly Ser Ser  .               276

TGAATTTCTGCAAGAGTATG TTAATGGGAGATTTTTTTAA TTTTCGTGTTCACCCTCTTA 1200

GCTTTTACATCTTTTGTGTA ACCCGAATGACAAGCTTTCG TCTCAGCTTCTTTT       1254
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Fig.18. Sequences of *DRT101* cDNA and predicted Drt101 polypeptide. The various restriction fragments of plasmid pQP1010 were purified and replacement-inserted into appropriately cleaved plasmid pUC19. DNA sequences of the fragments in pUC19 derivatives were determined for roughly 450 nucleotides in each direction, as described under "Experimental Materials and Approaches".

DRT102 The 990-bp *DRT102* cDNA sequence is presented in Fig 19. The first ATG of the sequence is followed by the longest ORF of 690 bases. Given the fact that the other reading frames encode much smaller polypeptides (maximum of 76 amino acids), I conclude that the 230-residue polypeptide translated in Fig 19 specifies the Drt102 protein. The ORF is preceded by an untranslated region of 95 bp, in which each of the three reading frames contains at least one termination codon; there is an apparent polyadenylation signal followed by a long poly(A) tail in the 3′ region. No known proteins identical to Drt102 were found in the SWISSPROT databank. Again, no strong homology was found between Drt102 and any known repair protein except that three small blocks of homology between Drt102 and the human apurinic/apyrimidinic (AP) endonuclease 1(HAP1) (Robson and Hickson, 1991) may be significant: K_{38}TSP vs K_{52}TSP, A_{50}WIK vs A_{74}WIK, and D_{158}LvviKGlKs vs D_{219}LrnpKGnKk.

DRT103 I made use of the restriction sites in the *DRT103* cDNA to produce various restriction fragments which were subcloned into plasmid pUC19, and subjected to double-strand nucleotide sequence analysis (Fig.20). The largest open reading frame of *DRT103* goes to the end of the cDNA isolated, suggesting that the present cDNA clone may not be complete. No homology to sequences in GenBank could be found. Despite its light-dependent activity, the Drt103 amino acid

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CCCTGACGTAGCCTGAAGTC GGTCGCCGCGTCTCCGCTTC ATCGTCTTCCGAAGTTCGCG GCTTAGTCTGCTGCGGCACC   80

GGCGTCGGAGTTGCG      ATG TTC GCC AAC AAG  TTC CCG GGC GTC TAC GCA GCC ACT TGT CTC   140
                     MET Phe Ala Asn Lys   Phe Pro Gly Val Tyr Ala Ala Thr Cys Leu    15

TCC GTC GAA GAC GCC GTC AAC GCT CGA TCA  ATA AGC AAT TGC AAT GTC CTC GCA TTC TCC   200
Ser Val Glu Asp Ala Val Asn Ala Arg Ser  Ile Ser Asn Cys Asn Val Leu Ala Phe Ser    35

GGC ATC AAA ACA TCC CCG GAA ACC GCC TTG  GAA ATC TTC GAC GCT TGG ATC AAA ACT CCT   260
Gly Ile Lys Thr Ser Pro Glu Thr Ala Leu  Glu Ile Phe Asp Ala Trp Ile Lys Thr Pro    55

TTC AAA TCT CCT TGT CCT GCG TCC GGA TCC  GAA CCA TGG AGC TCA GTT ATC TCT TCC TTC   320
Phe Lys Ser Pro Cys Pro Ala Ser Gly Ser  Glu Pro Trp Ser Ser Val Ile Ser Ser Phe    75

CTC GAC AAT TCT CTC TCC GAG ATG TCT CAG  ATT GGA AAG TCA ACC GCC GGC GAT TCA ACA   380
Leu Asp Asn Ser Leu Ser Glu MET Ser Gln  Ile Gly Lys Ser Thr Ala Gly Asp Ser Thr    95

ACC AAG AAG ATC GAT GAA ACA ACC GCG TCT  TGC GTA ATT TGC TGC TTG GCG AAG AAC AGA   440
Thr Lys Lys Ile Asp Glu Thr Thr Ala Ser  Cys Val Ile Cys Cys Leu Ala Lys Asn Arg   115

GAG TTC ACT CCA GTG GAC ATC ATG CCG GGA  GGC TCG ATG AAG ATC GTT AGA GAG ACG CCG   500
Glu Phe Thr Pro Val Asp Ile MET Pro Gly  Gly Ser MET Lys Ile Val Arg Glu Thr Pro   135

ACG TCG GCG ATT GTA AGA TTC AAA GCG GGA  AGT GTG GAA CCG GCG CAT CAC CAC ACA TTC   560
Thr Ser Ala Ile Val Arg Phe Lys Ala Gly  Ser Val Glu Pro Ala His His His Thr Phe   155

GGC CAT GAC CTT GTA GTC ATA AAG GGA AAG  AAA AGT GTG TGG AAT CTG AGC AAG AAG GAG   620
Gly His Asp Leu Val Val Ile Lys Gly Lys  Lys Ser Val Trp Asn Leu Ser Lys Lys Glu   175

AGA GCT GAT CTC GTT GAC GGC GAT TAC CTA  TTC ACT CCC GCC GGT GAT GTT CAC CGA GTC   680
Arg Ala Asp Leu Val Asp Gly Asp Tyr Leu  Phe Thr Pro Ala Gly Asp Val His Arg Val   195

AAA TAT CAC GAA GAC ACT GAG TTC TTC ATC  ACT TGG GAT GGC CAT TGG GAC ATA TTC CTT   740
Lys Tyr His Glu Asp Thr Glu Phe Phe Ile  Thr Trp Asp Gly His Trp Asp Ile Phe Leu   215

GAC GAA GAC CTC GAA ACT GCA AAG AAA GCC  ATC GAA GAA GAA GCT TGA    AGGTGTAAACTT   800
Asp Glu Asp Leu Glu Thr Ala Lys Lys Ala  Ile Glu Glu Glu Ala  .                    230

TGTGTAGTACTCTCTTCTTT TGAATGTTGTAAGTTCTGAG AGTAAACTTTCTTAAATTGA TGTACTTTGTAAGAATCTTT 880

GATTTCAAAAGATGGTGTAA TATTACAAATACTTTCTCTT ATGAACACTATATAAAGGGT TTCTAC(A)44          990
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Fig.19. Sequences of *DRT102* cDNA and predicted Drt102 polypeptide. The 1-kb *Eco*RI fragment from plasmid pQP1020 was electrophoretically purified and ligated into plasmid pUC19. The DNA sequence of about 450 bp at each end was determined as described under "Experimental Materials and Approaches". Based on these sequences, the following internal primers were chemically synthesized and used to complete the sequence determinations (oligonucleotide sequences are given 5′ to 3′, with endpoints indicated): sense strand, C_{290}GAACCATGGAGCTCAG$_{306}$ and C_{632}GTTGACGGCGATTACCTA$_{650}$; anti-sense strand, complements of above sense-strand oligonucleotides.

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GTTCATCCTCTCCCACTTGTTTTCTCCTCTTTGGGTTTCTCTGTGATTCCTGATTATCATTTA
TCTTGATTTTTAAACTCTTAGGAGATCGAATTTATGTTTTTTTTAACCTGAAAGATCCAATTT
TCCGTTTCTGATTAAGCCCGCCAAGTGTCTAAGAGGAATCCATAATTGTTGAATTGTAAAGGA
TTGTTTCCGATTATAGCACTGAGAGACTTTATAGAAAGAGTGTGACTTTC ATGTATGATGAT
                                                    M  Y  D  D

TTGGGATTGTTTGGTTTCATATTGTTGAAGGCCCCTTGTTTATTGTTGATTAACCTAATGATA
L  G  L  F  G  F  I  L  L  K  A  P  C  L  L  L  I  N  L  M  I
TTGTTTTATGTCATGAGCGTTCCAAATAATCTATGTTTTGGATTAGTTTTGCAAGATGATGTA
L  F  Y  V  M  S  V  P  N  N  L  C  F  G  L  V  L  Q  D  D  V
CAAAAATCTGTAATTCACTTTGCATTGGTTCATTGGGTGATAGATTTAGCTGAATGGTGGTGG
Q  K  S  V  I  H  F  A  L  V  H  W  V  I  D  L  A  E  W  W  W
TGGCTACATTTTAGATTGTATTTCTTTCTTTGGTTAATCTTTTTATGTCCTTACTTGTTAGTT
W  L  H  F  R  L  Y  F  F  L  W  L  I  F  L  C  P  Y  L  L  V
GGTTTCAGAGATTCTATACTATGCTATGCCTGTCATGAAGCGTACCTAAATTTTCTTCTGAGT
G  F  R  D  S  I  L  C  Y  A  C  H  E  A  Y  L  N  F  L  L  S
GGTCAACTTTGTAGGTCAGATTTAAAGGAAGTAAAAATGAGAAGACAGGGAAACTTTGCGGAC
G  Q  L  C  R  S  D  L  K  E  V  K  M  R  R  Q  G  N  F  A  D
TCATCACCTGCCAGTGCATATGGAGCTGGACAGATACAGGATCCCCATTCTGATTTTCAAGGT
S  S  P  A  S  A  Y  G  A  G  Q  I  Q  D  P  H  S  D  F  Q  G
CAATTAGAGGCATTCACGCCTGAGAGGGATCAGCCATACTCAGATTCACAAGCTGAGGGTCAA
Q  L  E  A  F  T  P  E  R  D  Q  P  Y  S  D  S  Q  A  E  G  Q
TGGAGATGGGAAAGAGATGGCCCTAATATGTCAAGGCCAATGGCTACTGCTGTTTACAATGAA
W  R  W  E  R  D  G  P  N  M  S  R  P  M  A  T  A  V  Y  N  E
GGACAACAAGGTGTTGATTCATCGAGGACGTATTACCGTGGCCAAATAGATCCAAAATCAGGA
G  Q  Q  G  V  D  S  S  R  T  Y  Y  R  G  Q  I  D  P  K  S  G
ATGGAGAAGCAAGGCAGTGATCCAAGAGCTCAACCACAACACCAAGAAAATCCAAAAACCGGT
M  E  K  Q  G  S  D  P  R  A  Q  P  Q  H  Q  E  N  P  K  T  G
TATGATAATAACCGTGGGGTGCAGACATTTGAAGGTCTTGAACAGAAGTTCATGGATGATATA
Y  D  N  N  R  G  V  Q  T  F  E  G  L  E  Q  K  F  M  D  D  I
ACAAGACTAGCAAAAGATCAAATCGAAGCAGAGGATGCAGAGATCGCCAGACATCGAGAGAAA
T  R  L  A  K  D  Q  I  E  A  E  D  A  E  I  A  R  H  R  E  K
ATAAACACTATAAACGCTAGATACGAAGAACAGTTAGCTACACTTAGAGCACGGCACACAGGT
I  N  T  I  N  A  R  Y  E  E  Q  L  A  T  L  R  A  R  H  T  G
AAGAGAGAAGAGATCATGAGGAAAGAATCACTAGCTAGGCAGCAACAATTCAAGCAGCAAACC
K  R  E  E  I  M  R  K  E  S  L  A  R  Q  Q  Q  F  K  Q  Q  T
ATGGGGATGATGGACCAATACCATCCAAACGTGGTTGGTCAGGCCAATCTAATGCCATCTGGT
M  G  M  M  D  Q  Y  H  P  N  V  V  G  Q  A  N  L  M  P  S  G
CATCCCCAAGGCTACATTGGCAGTGTTCAAGATCCAGCGGCTGTGGCGGATGCACCACCAAGA
H  P  Q  G  Y  I  G  S  V  Q  D  P  A  A  V  A  D  A  P  P  R
TCATATGGCTCAGATCGGTTTGAAGCATACGGAGAGAGAGCTAGGTTTCAGGGAGGAAACAGA
S  Y  G  S  D  R  F  E  A  Y  G  E  R  A  R  F  Q  G  G  N  R
GATCACGGGTTCGAGCCTAGGGGTCCATACCCTGGTGGGAATGTCTATGACACCAGCTCACGT
D  H  G  F  E  P  R  G  P  Y  P  G  G  N  V  Y  D  T  S  S  R
TCTACTGATGTGATATTACTTGAACATATTGTGTTTTTAGGGGATTCACTCACTCCCACTTTT
S  T  D  V  I  L  L  E  H  I  V  F  L  G  D  S  L  T  P  T  F
TTTGTGGCTTTATA
F  V  A  L
```

Fig.20. Sequences of *DRT103* cDNA and predicted Drt103 polypeptide. The various restriction fragments of pQP1030 were purified and re-inserted into vector pUC19. DNA sequences of these fragments were determined in both direction by using M13 Universal and Reverse primers, as described under "Experimental Materials and Approaches".

sequence showed only marginal similarity to those of microbial photolyases. Interestingly, nucleotides 432-443 within the *DRT103* ORF encodes a Trp-Trp-Trp-Trp moiety, which might play a role in photosensitizing CBPDs to cleavage via tryptophan absorbance and energy transfer, as do certain Trp-containing peptides and proteins (Sutherland and Griffin, 1980).

DRT111 The 1400-bp *DRT111* sequence obtained is shown in Fig 21. A search of the GenBank data base (release 69) found no significant homology using the Intelligenetics FASTDB program. From bp 716 to 757, *DRT111* encodes a conserved nucleotide binding domain that is highly homologous to the motif I of *E. coli* RecG protein, which is involved in resolution of RecA-mediated recombination intermediates (Lloyd and Sharples, 1991). The first 90 residues of Drt111 show features of chloroplast transit peptides as described above: high frequency of serine and threonine residues, large numbers of small hydrophobic amino acids, and net positive charge.

DRT112 The *DRT112* cDNA is the smallest cDNA isolated, only 738 bp in length (Fig.22). It predicts a 167-amino-acid polypeptide with a MW of 17,260 Dalton. Its size is nearly the same as the three known prokaryotic resolvases: phage T4 endonuclease VII (Kosak and Kemper, 1990), 18 kilodalton (kDa); phage T7 endonuclease I (Parsons and West, 1990) 17 kDa; and *E. coli* RuvC resolvase (Sharples and Lloyd, 1991), 19 kDa. No obvious homology could be found among these proteins, nor with Drt112. Instead, the Drt112 amino acid sequence is

```
                                                30                                                  60
CAG ATC TTT CCA ATT GAA AGC GAA CCG TGA   ATT TTG AGT CTC TTT ACC GGA GGA GAA AAA

                                                90                                                 120
ATG CTT GGT GGA TTA TAC GGA GAT CTT CCT   CCA CCG ACC GAT GAT GAG AAA CCC AGT GGA
Met Leu Gly Gly Leu Tyr Gly Asp Leu Pro   Pro Pro Thr Asp Asp Glu Lys Pro Ser Gly  20

               150                                                 180
AAC TCC TCT TCC GTC TGG TCA CGC AGT ACC   AAA ATG GCT CCA CCT ACA CTT CGC AAA CCA
Asn Ser Ser Ser Val Trp Ser Arg Ser Thr   Lys Met Ala Pro Pro Thr Leu Arg Lys Pro  40

               210                                                 240
CCA GCT TTT GCT CCC CCG CAA ACA ATC TTA   AGA CCT CTC AAC AAA CCT AAA CCT ATC GTT
Pro Ala Phe Ala Pro Pro Gln Thr Ile Leu   Arg Pro Leu Asn Lys Pro Lys Pro Ile Val  60

               270                                                 300
TCG GCT CCG TAC AAG CCT CCT CCG AAT TCA   TCG CAA TCG GTG CTT ATT CCG GCG AAC GAA
Ser Ala Pro Tyr Lys Pro Pro Pro Asn Ser   Ser Gln Ser Val Leu Ile Pro Ala Asn Glu  80

               330                                                 360
TCA GCA CCT TCG CAT CAG CCT GCA TTG GTT   GGT GTG ACT TCA TCG GTG ATT GAA GAG TAC
Ser Ala Pro Ser His Gln Pro Ala Leu Val   Gly Val Thr Ser Ser Val Ile Glu Glu Tyr 100

               390                                                 420
GAT CCA GCG AGA CCT AAC GAT TAC GAG GAG   TAT AAG AGG GAG AAG AAG AGG AAA GCT ACG
Asp Pro Ala Arg Pro Asn Asp Tyr Glu Glu   Tyr Lys Arg Glu Lys Lys Arg Lys Ala Thr 120

               450                                                 480
GAA GCT GAG ATG AAA CGA GAG ATG GAT AAG   AGA AGG CAA GTG TAT CCG GAA AGA GAT ATG
Glu Ala Glu Met Lys Arg Glu Met Asp Lys   Arg Arg Gln Val Tyr Pro Glu Arg Asp Met 140

               510                                                 540
AGA GAA AGA GAA GAG AGG GAG AGG AGA GAG   AGA GAG ATA ACA GTG ATC CTC TCG GTT GAT
Arg Glu Arg Glu Glu Arg Glu Arg Arg Glu   Arg Glu Ile Thr Val Ile Leu Ser Val Asp 160

               570                                                 600
ATC TCC GGT GAG GAA CGT GGA AGA GAC CCT   GCT AGA GTA GTG GTG GAA GTG TTG GGA AGG
Ile Ser Gly Glu Glu Arg Gly Arg Asp Pro   Ala Arg Val Val Val Glu Val Leu Gly Arg 180

               630                                                 660
GAA GAT CCT CGT CTC CTC CCT GGG AAT GTT   GAT GGG TTT AGT ATT GGG AAA TCG AAG CCG
Glu Asp Pro Arg Leu Leu Pro Gly Asn Val   Asp Gly Phe Ser Ile Gly Lys Ser Lys Pro 200

               690                                                 720
AGT GGG TTA GGA GTA GGA GCA GGT GGA CAG   ATG ACA CCT GCT CAG AGG ATG ATG CCG AAG
Ser Gly Leu Gly Val Gly Ala Gly Gly Gln   Met Thr Pro Ala Gln Arg Met Met Pro Lys 220

               750                                                 780
ATG GGA TGG AAA CAA GGA CAA GGG CTT GGG   AAA TCA GAG CAA GGG ATT CCT ACG CCA TTG
Met Gly Trp Lys Gln Gly Gln Gly Leu Gly   Lys Ser Glu Gln Gly Ile Pro Thr Pro Leu 240

               810                                                 840
ATG GCT AAG AAG ACT GAT CGT AGA GCT GGT   GTC ATT GTC AAT GCT AGT GAG AAT AAA TCT
Met Ala Lys Lys Thr Asp Arg Arg Ala Gly   Val Ile Val Asn Ala Ser Glu Asn Lys Ser 260

               870                                                 900
TCT TCG GCG GAG AAG AAG GTT GTT AAG AGT   GTT AAT ATC AAT GGT GAA CCA ACC AGG GTT
Ser Ser Ala Glu Lys Lys Val Val Lys Ser   Val Asn Ile Asn Gly Glu Pro Thr Arg Val 280

               930                                                 960
TTG CTT CTT AGA AAC ATG GTT GGG CCA GGA   CAA GTA GAT GAT GAG CTA GAA GAT GAG GTA
Leu Leu Leu Arg Asn Met Val Gly Pro Gly   Gln Val Asp Asp Glu Leu Glu Asp Glu Val 300

               990                                                1020
GGA GGC GAG TGT GCC AAA TAC GGT ACA GTC   ACT CGT GTA CTG ATA TTC GAG ATC ACT GAA
Gly Gly Glu Cys Ala Lys Tyr Gly Thr Val   Thr Arg Val Leu Ile Phe Glu Ile Thr Glu 320

              1050                                                1080
CCG AAC TTC CCT GTA CAC GAA GCA GTA AGA   ATC TTT GTT CAG TTT TCA AGA CCC GAG GAA
Pro Asn Phe Pro Val His Glu Ala Val Arg   Ile Phe Val Gln Phe Ser Arg Pro Glu Glu 340

              1110                                                1140
ACA ACT AAA GCC CTT GTA GAC CTC GAT GGG   AGA TAC TTT GGA GGA AGA ACC GTA CGT GCA
Thr Thr Lys Ala Leu Val Asp Leu Asp Gly   Arg Tyr Phe Gly Gly Arg Thr Val Arg Ala 360

              1170                                                1200
ACG TTC TAT GAT GAA GAG AAA TTC AGT AAG   AAC GAG TTG GCT CCA GTT CCA GGT GAA ATC
Thr Phe Tyr Asp Glu Glu Lys Phe Ser Lys   Asn Glu Leu Ala Pro Val Pro Gly Glu Ile 380

              1230                                                1260
CCT GGC TAT TAA TAA CTC CTA AGA CAA AGA   AGA AAC TTC ATC TTC TAG GAG ATT CTT GTT
Pro Gly Tyr  .                                                                  384

              1290                                                1320
AGA GAA AGA AAT CAT AGA CTG AGG TTT TAA   AAA CAT TTG TAA AGT ACT TAG AAA TCT TGG

              1350                                                1380
GTT ATC TCT TTA TTG TTG TTC AAT AAA GCT   TCA CAA GGA TCA AGA AAA AAA AAA AAA AAA

                    1399
AAA AAA AAA AAA AAA AAA A
```

Fig.21. DNA and predicted protein sequences for *DRT111*. DNA sequences were determined as described under "Experimental Materials and Approaches".

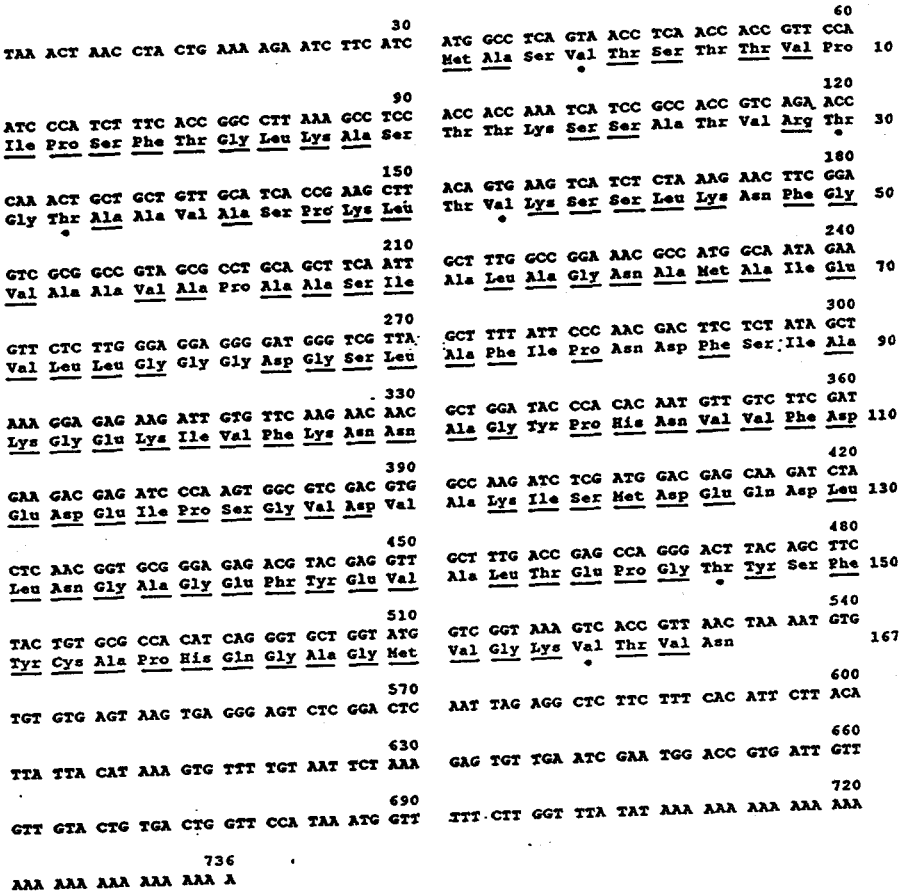

Fig.22. DNA and predicted protein sequences for *DRT112*. DNA sequences were determined as described under "Experimental Materials and Approaches". Amino acids identical with plastocyanin (see text) are underlined; conservative substitutions are dotted underneath.

about 75% identical with *Arabidopsis* plastocyanin, a nuclear-encoded chloroplast protein involved in electron transfer between photosystem I and the cytochrome b/f complex (Vorst *et al.*, 1988). It has been shown that the *Arabidopsis* genome contains only a single plastocyanin gene (Vorst *et al.*, 1988). Our Southern analysis showed that the *DRT112* probe yielded at high stringency only a single strong signal which presumably corresponds to *DRT112* itself; while at low stringency there were two additional low-MW bands which might correspond to the plastocyanin gene (data not shown).

5. Deletion Analyses of Drt100 and Drt101

DRT100 cDNAs with various deletions were constructed and transformed into RecA$^-$UvrB$^-$Phr$^-$ mutants to test for functionality. The results of these experiments are summarized in Table 5. Construct pQP1003B, which encodes only 240 residues after the putative chloroplast protease site, was only slightly less active than the intact cDNA in pQP1003; while pQP1003H, in which the last(3′)186 bp of the ORF were deleted, was completely active. This suggests that the C-terminal 62 amino acids of Drt100 are dispensable, at least for complementation of the UV-sensitive phenotype of the *E. coli* mutants. It has been shown that at least 35 C-terminal residues of the *E. coli* RecA protein are dispensable (Benedict and Kowalczykowski, 1988). Interestingly, the active construct pQP1003B encodes all but the last leucine-rich-motif(LRM) repeat (see Fig.17B), whereas pQP1003A, in which none of the

Table 5. UV-resistance activity of partial *DRT100* deletions

Plasmid	*DRT100* coding sequence retained	Survival (X 10^6) at 0.3 J/m^2 UV*
pUC19	none	0.06
pQP1003N	bp 1-420	0.08
pQP1003D	bp 1-482	0.09
pQP1003A	bp 1-721	0.07
pQP1003B	bp 1-941	4.4
pQP1003H	bp 1-999	6.1
pQP1003	bp 1-1185	5.0

* Deletion derivatives of plasmid pQP1003, retaining the indicated *DRT100* coding sequence (Fig.16), were transformed into RecA$^-$UvrB$^-$Phr$^-$ bacteria (FD2566), and the resistance of transformants to 0.3 J/m^2 254-nm irradiation measured. Data correspond to averages for duplicated plates (range less than 25% of value shown).

four LRM repeats is intact, did not display detectable complementing activity. This suggests that the domain structure of Drt100 may be critical to its function.

The activities of deletion derivatives of *DRT101* are compared in Table 6. Construct pQP1011AV, which encodes a truncated protein of 187 amino acids, showed no complementation activity. Removal of six amino acids from Drt101 C-terminus (pQP1011HD), however, had no effect.

6. Southern Hybridization Analysis

I hybridized a *DRT100* cDNA probe at high stringency to genomic DNAs from various plants, and also from frog (*Xenopus laevis*) liver and wild-type *E. coli* cells (Fig.23). Homology to *DRT100* was detected in genomic DNA from *Arabidopsis* and two other *Brassicaceae*, Chinese cabbage and broccoli, but not in DNA from maize, frog, and *E. coli*.

7. Northern Hybridization Analysis

To determine the effect of UV-B irradiation on *Arabidopsis DRT* gene expression, poly(A)$^+$RNA was isolated from plants exposed to various amounts of UV-B light, and steady transcript levels examined by Northern blot hybridizations (Fig.24A-D). The level of *AAc1*(actin) mRNA did not change in UV-B irradiated plants; therefore, *DRT* mRNA levels were normalized with the *AAc1* mRNA to correct for variation in loading. In plants irradiated with UV-B light for 1, 2, 3 and

Table 6. UV-resistance activity of partial *DRT101* deletions

Plasmid	Drt101 amino acids retained[a]	Survival (X 10^6) at 0.3 J/m^2 UV[b]
pUC19	none	0.088
pQP1011	all (1-276)	8.6
pQP1011HD	1-270	8.8
pQP1011AV	1-187	0.10
pQP1011XB	1-144	0.11
pQP1011MS	1-109	0.12
pQP1011AC	1-69	0.17

[a] See Fig.18.

[b] RecA$^-$UvrB$^-$Phr$^-$ bacteria (strain FD2566) were transformed to ampicillin resistance with the indicated Drt101-deletion plasmids, and cultures were grown in broth and treated with UV light.

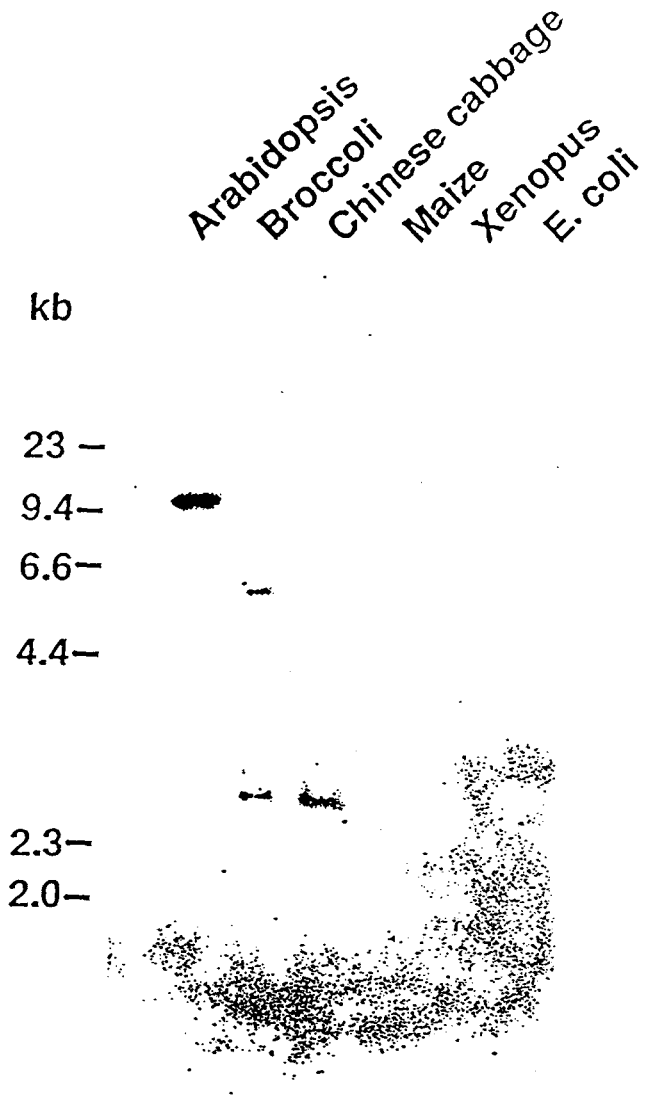

Fig.23. Hybridization of *DRT100* cDNA probe to plant DNA. Lanes contain genomic DNA from *Arabidopsis* leaves and stems (15 μg), broccoli florets (20 μg), Chinese cabbage (*Brassica pekinensis*) leaves (20 μg). Extraction of DNA and hybridization analysis of products of digestion with *Eco*RI endonuclease were as described under "Experimental Materials and Approaches".

Table 7. Quantitation of *DRT* mRNA levels by densitometric scanning*

Treatment	*DRT100*	*DRT101*	*DRT102*	CHS
UV-B				
0 h	1.16	1.23	1.29	0.56
0.5 h	1.93	1.98	1.38	0.77
1 h	2.31	2.71	1.35	0.77
2 h	3.31	3.27	1.48	2.89
3 h	4.08	4.53	1.60	2.91
4 h	2.60	2.63	1.41	3.34
6 h	1.15	1.46	1.44	0.67
Dark	1.29	1.12	1.45	0.54
mitomicin C				
0 ug/ml	0.70	0.81	ND	0.17
5 ug/ml	3.26	5.60	ND	0.21
10 ug/ml	7.07	11.86	ND	0.18
15 ug/ml	3.86	6.59	ND	0.23
methylmethanesulfonate				
0 %	0.86	0.85	ND	0.16
0.005%	4.10	0.69	ND	0.18
0.01%	8.03	0.67	ND	0.25
0.05%	3.74	0.52	ND	0.22

* Data are the ratio of *DRT* or CHS to actin.

ND: not determined.

4 h, *DRT100*(Fig.24A) and *DRT101*(B) mRNA levels increased, with maximal accumulation occurring at 3-h irradiation. Densitometric measurements determined that the levels of *DRT100* and *DRT101* mRNA were 3.3- and 3.8-fold, respectively, higher in plants treated with 3-h UV-B light than in control plants (lanes 0 and Dark). *DRT102* transcript level did not increase in response to UV-B irradiation (C). About 6-fold increase in *Arabidopsis* chalcone synthase (CHS) mRNA levels was detectable after exposure of the plants to UV-B for 2-4 h(D, lanes 2-4). (Overexposure of the autoradiogram depicted in Fig.24D revealed the presence of a low level of CHS mRNA.) The increase in the levels of *DRT100, 101* and CHS mRNAs was not due to a general accumulation of mRNA under UV-B light conditions, because neither the level of mRNA from the gene coding for actin (random variations in actin signal in Fig. 24 most likely reflects differences in loading) nor the level of *DRT102* mRNA was increased by UV-B irradiation.

Since *DRT100* increased resistance of *E.coli* DNA-repair-deficient mutants to various DNA-damaging agents, whereas *DRT101* and *102* showed specificity for UV resistance, I examined the specificity of the response of the *DRT* genes to the DNA-damaging agents, mitomycin C and methyl methanesulfonate (MMS). Here I show that the *DRT100* mRNA level increases in plants treated with mitomycin C and MMS (Fig.25A). Induction by both mitomycin C and MMS was maximal (10.1-fold for mitomycin C and 9.3-fold for MMS) at the

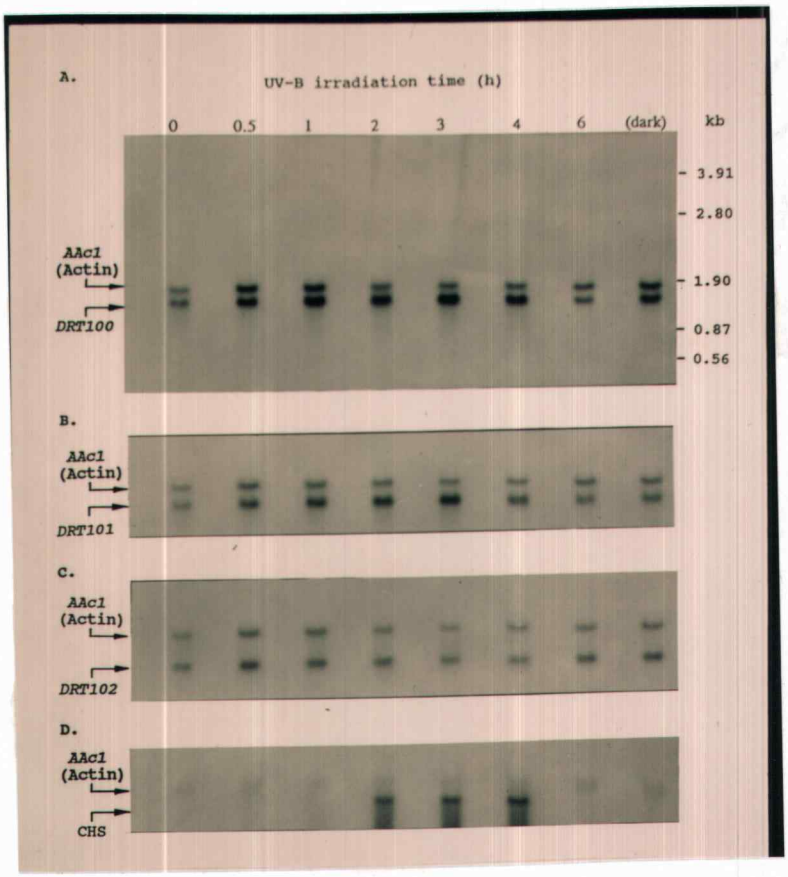

Fig.24. Northern blot analysis of *DRT* mRNA levels in plants exposed to UV-B irradiation. Blots were probed with random-primed (A) *Arabidopsis AAc1* (actin) and *DRT100* specific probes, (B) *AAc1* and *DRT101* probes, (C) *AAc1* and *DRT102*, (D) *AAc1* and *Arabidopsis* chalcone synthase (CHS) gene probes. The positions of RNA size standards (Promega Co.) are indicated to the right in (A).

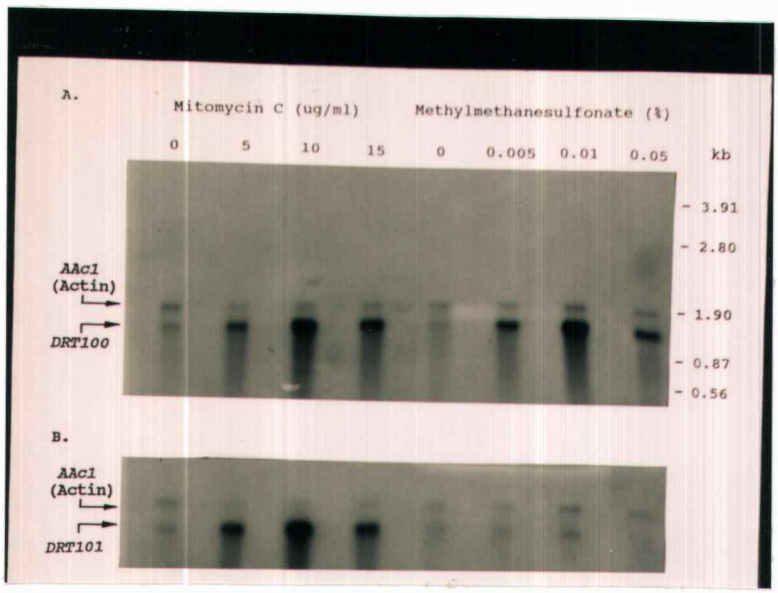

Fig.25. *DRT* mRNA levels in plants treated with DNA-damaging chemicals. Poly(A)$^+$ RNA (3.5 μg in each lane) isolated was electrophoresed, and Northern blots hybridized with the following specific DNA probes: (A) *Arabidopsis AAc1* (actin) and *DRT100*, (B) *AAc1* and *DRT101*. RNA markers are indicated to the right in (A).

intermediate doses employed, 10 ug/ml of mitomycin C and 0.01% of MMS (A, lanes 10 and 0.01). When plants were exposed to higher doses (15 µg/ml mitomycin C and 0.05% MMS), at which the treated plants showed marked yellowing and withering, the induction of *DRT100* transcript decreased to 5.5-fold for mitomycin C and 4.3-fold for MMS. Surprisingly, the *DRT101* transcript was not exclusively induced by UV irradiation, but also by mitomycin C (Fig.25B). At doses of 5, 10 and 15 µg/ml, the *DRT101* mRNA accumulation increased by 6.9-, 14.6-, and 8.1-fold, respectively (lanes 5, 10 and 15). However, treatment of the plants with MMS did not increase the level of *DRT101* mRNA. Again, *DRT102* was not inducible by either mitomycin C or MMS (data not shown). In contrast to UV-B induction, the CHS mRNA level was not increased by both chemical DNA-damaging agents (data not shown). (Overexposure of the autoradiogram of both *DRT102* and CHS blots revealed the presence of a low levels of *DRT102* and CHS mRNAs in all samples.) I infer that these chemical agents are not involved in the regulation of CHS gene expression in *Arabidopsis*.

V. SUMMARY AND DISCUSSION

A. Summary of the Project Achievements

Arabidopsis thaliana was used as a model green plant for studies of DNA damage and repair following UV irradiation. My observations indicate the following: (1) DNA damage might be an important component of UV effects on *Arabidopsis* because when the intact plants were subjected to UV (254 nm) fluences which resulted in measurable CBPD levels (typically about one CBPD per 2 X 10^4 nucleotides at 1000 J/m^2), the *Arabidopsis* seedlings exhibited significant physiological effects; (2) photoreactivation is the predominant pathway of UV-induced damage repair in *Arabidopsis* - CBPDs were rapidly removed from the DNA of UV-irradiated plants grown in the light, but were repaired an order of magnitude more slowly in the dark; (3) *Arabidopsis* photolyase levels are increased significantly by UV-B irradiation - treatment of plants with 280 J/m^2 UV-B light (approximately equivalent to the daily UV-B fluence expected for 40% ozone depletion at temperate latitudes) increased photolyase activity by 2-fold, and extracts from UV-B-treated plants were able to repair as many as 80% of total lethal lesions compared to 65% by control plants; (4) *Arabidopsis* photolyase is markedly temperature-sensitive - *in vitro* half-lives were about 12 min at 30°C and 1.3 min at 50°C, and the *in vivo* photoreactivation decreased considerably when irradiated plants were transferred from 22 to 37°C.

Partial complementation of *E. coli* DNA-repair-deficient mutations was used to isolate *Arabidopsis* cDNAs encoding putative plant DNA-damage-repair/toleration (DRT) activities. The initial work yielded six unique cDNAs, encoding probably four distinct classes of activities (summarized in Table 8): (1) *DRT100* increased resistance of mutant bacteria to UV irradiation, and to mitomycin C and methyl methanesulfonate (MMS) treatments, supported the growth of λ *red*$^-$ *gam*$^-$ and P1 phages, and promoted conjugational recombination in RecA$^-$ bacteria; (2) the activities encoded by *DRT101* and *DRT102* appear to be UV-specific, since they fail to provide resistance to DNA damage by mitomycin C and MMS; (3) Drt103 conferred UV resistance in a light-dependent manner, but its amino acid sequence shows only marginal similarity to those of microbial photolyases; and (4) *DRT111* and *DRT112* increased resistance to UV light, mitomycin C, MMS, and nitroquinoline oxide, of *E. coli* resolvase-deficient bacteria (RuvC$^-$ and RuvC$^-$ RecG$^-$ mutants), and partially restored the ability of these mutants to carry out conjugal recombination.

It should be noted that the putative Drt activities summarized in Table 8 are highly speculative. Since expression of the Drt proteins has not yet been confirmed in the complementing *E. coli* mutant cells, alternative interpretations for the DNA-damaging-resistance activities of the *DRT* genes should be addressed. Under control of the bacterial *lac* promoter, the transcripts of the *DRT* genes were

Table 8. Putative activities of *Arabidopsis DRT* cDNAs

cDNA	Function in *E. coli* mutants	Speculated activity
DRT100	Increased resistance to UV,MC, MMS, and reconbination frequency	Strand-exchange protein ?
DRT101	Increased resistance to UV	UV-specific endonuclease ?
DRT102	Increased resistance to UV	UV-specific endonuclease ?
DRT103	Light-dependent increased resistance to UV	CBPD-photosensitizing protein ?
DRT111	Increased resistance to UV,MC, MMS, NQO, and recombination frequency	Recombination-intermediate resolvase ?
DRT112	Increased resistance to UV,MC, MMS, NQO, and recombination frequency	Recombination-intermediate resolvase ?

made in multiple copies. These *DRT* mRNAs could be catalytic; they could act as RNA emzymes (ribozymes) to remove DNA damage and thus contribute to the increased survival of the repair mutants. It has been known for some time that some RNAs, such as *Tetrahymena* pre-rRNA (Bass and Cech, 1984) and a group of fungal mitochondrial mRNAs (Garriga *et al.*, 1986), use proton exchange reactions to catalyze hydrolysis of nucleotide substrates. Another possibility could be that the *DRT* mRNAs might complex with certain exsisting protein subunits in the repair mutant cells. The mRNA-protein complex might then interact with the damage-containing DNA, resulting in cleavage of the damaged nucleotide. RNA-protein complexes are known to be involved in mediating biochemical reactions. These include RNase P and small nuclear ribonucleoproteins (snRNPs). RNase P, the enzyme responsible for the cleavage that produces the mature 5′ terminus of tRNA molecules, requires an RNA as well as a protein subunit for its *in vivo* activity (Cech and Bass, 1986). snRNP particles are thought to be involved in a variety of RNA processing reactions (Gerke and Steitz, 1986). Even if the Drt proteins are synthesized in the mutant cells, apparent complementation could occur in several ways. For instance, a Drt protein could suppress the deficiency of a mutant, but not be the precise analog of the protein inactivated in the mutant. Such suppression could arise because the Drt protein increases the activity of an alternative repair pathway, or increases the survival of cells with DNA damage for some other reason.

B. Temperature-sensitive and UV-B-inducible Photoreactivation in Arabidopsis

CBPDs are removed predominantly by photoreactivation in *Arabidopsis*: in plants irradiated with 1000 J/m^2 UV light, 50% of total CBPDs were photoreactivated within 1 h, whereas excision repair took 8 to 17 h to remove the same amount of the photoproducts (Fig.6). This is probably an underestimate of the true removal time because of dilution by replication. This is similar to the situations in the bacterium *E. coli* and the water plant *Wolffia microscopica*. In *E. coli*, photoreactivation can remove up to 250 CBPDs per minute (Sancar and Sancar, 1988), whereas excision repair removes only 5-20 CBPDs/min (Hays *et al.*, 1985). In intact *W. microscopica* subjected to 250 J/m^2 of UV irradiation, about 10% of all thymine moieties in the DNA were dimerized; photoreactivation of all of the induced CBPDs was completed within 3 h, but dark repair removed only about 40% of the dimers in the same period, and had removed less than 60% after 50 h (Degani *et al.*, 1980). Light appeared not to be the rate-limiting factor for photoreactivation in *Arabidopsis*, because the *in vivo* rates of light-dependent removal of CBPDs were nearly the same in the growth chamber as in the greenhouse, where the light intensities at 300-500 nm were an order of magnitude higher. It seems possible, therefore, to increase the intrinsic resistance of plants to UV-induced DNA damage by increasing levels of plant photolyase.

Plant DNA repair enzymes require appropriate physiological conditions, such as water and temperature, for maximal activity. If these conditions are not met, the greater part of the initial damage will remain uncorrected in plant DNAs, leading to chromosomal aberrations. For example, transfer of barley seeds from room temperature to 10°C, after treatment of the seeds with the alkylating agent *N'*-methyl-*N*-nitrosoguanidine, led to further decreases in the molecular weights of single-strand DNA fragments, as determined by alkaline sucrose gradient centrifugation, and to further increases in the number of aberrations (Soyfer, 1987). *Arabidopsis* photolyase is also very sensitive to temperature changes. The enzyme activity was partially or completely inactivated when plants were incubated at 30, 34 or 37°C. This may have important implications with respect to predicted changes in the terrestrial atmosphere. Depletion of stratospheric ozone will increase the intensity of solar UV-B (280-320 nm) radiation reaching the biosphere, and new satellite measurements of atmospheric ozone suggest that plants will be among those organisms most severely affected. There are also reports that global climate change due to increases in CO_2 and other greenhouse gases may elevate surface air temperatures by 1.5-5.5°C (Schneider, 1989). If CBPD induction is an important component of UV-B damage to plants, and if temperature-sensitive photoreactivation is an important defense against DNA damage in plants, then the combination of increased UV-B radiation and elevated

temperature may be highly deleterious to plants. It might be worthwhile to improve plant photolyase gene(s), that is, make their products less temperature sensitive, so that photoreactivation of UV-induced CBPDs in plant DNAs can tolerate continuously increasing temperatures.

The increase in *Arabidopsis* photolyase level achieved by treatment with a terrestrially attainable UV-B intensity suggests that plants might adapt in part to ozone depletion, by increasing levels of DNA repair activities. Similar UV induction of the genes encoding enzymes responsible for biosynthesis of flavonoids, which are plant UV-absorbing compounds, is well-known (Chapell and Hahlbrock, 1984). Expression of the yeast photolyase gene, *PHR1*, was increased up to 10-fold by UV irradiation or by DNA-damaging chemical treatments (Sebastian *et al.*, 1990). At least 20 other yeast genes, including some of the genes involved in DNA repair, are known to be UV-inducible (Roby and Szostak, 1985). The UV-B-inducibility of *Arabidopsis* photolyase suggests that plants have evolved mechanisms, at the levels of DNA metabolism, to protect themselves from genetic damage by environmental stresses.

C. The Technical Feasibility of Isolating Plant DRT Genes by Bacterial Complementation

A genetic selection technique was developed for isolation of *Arabidopsis* DNA-damage-repair/toleration(*DRT*) genes, by

complementation of repair-deficient mutations in *E.coli*. This proved to be a powerful method for cloning genes which were at least partially functional in bacteria. A number of parameters may be important to the success of this selection: (1) the expression vectors(λYES) in which the plant cDNAs are inserted must provide for high-level regulated expression of the cDNAs, and must be easily manipulated in *E.coli* cells; (2) the λKC phages must not be inducible by UV or other DNA-damaging agents; (3) the mutations used for selection must be genetically stable to prevent selection for revertants; (4) the selection pressure applied should be powerful enough that genes whose products are only partially functional in bacteria will survive the first round of selection.

The *Arabidopsis* cDNA library employed in this study has been constructed in the multifunctional λYES expression vectors, which have several attractive features that facilitate isolation of *DRT* genes by complementation: (1) the cDNA library can be replicated as phage for standard hybridization and antibody screening; (2) the phages may be converted *in vivo* to plasmids that can be selected for and maintained in *E.coli*, thus facilitating complementation studies; (3) the vectors provide regulated expression in *E.coli* for cDNA inserts aligned with the *lac* promoter. The successful application here of these vectors for isolation of the six *DRT* genes, and for isolation of *Arabidopsis* analogs of the *trpD* and *leuB* genes by complementation of *E.coli*

mutations (Elledge *et al.*, 1991), has demonstrated that these vectors, combined with creative selection and screening procedures, will facilitate the isolation of many plant genes which express functions of bacterial homologs or analogs.

UV light and other DNA-damaging agents might cause induction of phage λKC, leading to the death of the complementing hosts. This problem was overcome by introducing an *cI(ind⁻)* into the phage λKC, by homologous recombination between phages λKC and λ*xis1 red3 cI(ind⁻)*. In the presence of KC(*ind⁻*), infecting λYES phages can not grow lytically even in the presence of DNA-damaging agents because (1) the phage part is lost when the plasmid is excised and (2) *ind⁻* is dominant to *ind⁺*. Strain FD256 (Δ*recA*Δ*uvrB*Δ*phr*) appeared to be an ideal host for complementing selection because of its multi-repair-pathway deficiency and genetic stability. Unfortunately, this triple-deletion mutant grows poorly; for unknown reasons, infection of FD2566(λW1721) lysogen with YES phages gave very low efficiency of plasmid excision(11% relative to plaque-forming units). Strain QP2898(*recA::cat uvrC34*Δ*phr*), which is also deficient in recombinational repair, excision repair, and photoreactivation, grows better than FD2566; up to 26% of the YES-plasmid-excision rate could be obtained in QP2898 (W1721). I did not find *recA* or *uvrC* revertants among the survivors of drastic treatments with DNA-damaging agents.

Plants and bacteria are evolutionarily very remote. One can expect that plant genes may not be expressed well in

E.coli cells. Even properly expressed, the bacterium-translated plant proteins may not be as active as in plant cells, since the two cellular environments are quite different. It is important, therefore, to determine an appropriate selection pressure so that the plant *DRT* genes with only moderate or even lower activity can be selected out of the vast number of cDNA clones in the library. Although the selection was ultimately successful, the secondary screening step was very time-consuming. When the cells in solution are subjected to UV irradiation, some inevitably receive more UV light than others, since photoproducts are essentially distributed at random. As a result, some cells whose plasmids contained no cDNA inserts or non-*DRT* inserts escaped killing by UV light. These "background" survivors increased the time spent in screening for *DRT* clones. The fact that screening of 840 survivors yielded only six heritably UV-resistant clones(0.7%) reflects the shortcoming of this approach. Increasing UV fluences may eliminate the "background" to a certain extent; on the other hand, it may risk the loss of those *DRT* genes which confer only slight UV resistance due to their poor expression and/or function in *E.coli* cells. These problems, however, do not apply to the selection with DNA-damaging chemicals. When growing on agar containing an appropriate concentration of the agent, the bacteria are likely subjected to nearly the same exposure. Although DNA lesions will still be distributed randomly and some parent cells may be undamaged, the daughters will be exposed to the

agent and may be damaged later, thus minimizing "background" survivors. The results obtained with MMS selection described above indicated this advantage: with only 25 survivors from 10^{11} plasmid-containing transformants, I obtained four *DRT* clones, corresponding to 16% of the survivors.

D. Some Implications for Complementation Efficiencies of the Isolated DRT Genes

Apparent efficiencies for complementation of various *E.coli* repair-deficient phenotypes by *DRT100, 101, 102* and *103* were high (up to 36%) for low levels of bacterial or phage DNA damage caused by UV or chemical agents, but decreased markedly with increasing doses of treatment (see Figs. 11A; 12A, B). Similar results were also obtained with *DRT111* and *DRT112* (Fig.14A,B). This suggests that limiting amounts of these Drt activities were being saturated by high levels of damage.

The actual biochemical amounts of Drt activity in *E.coli* that result from these genes must depend on the levels of gene expression, the intrinsic biochemical efficiencies of the gene products, and on other factors that modulate biochemical efficiencies. Although the *lacZ* signals for initiation of transcription were provided by the vectors, the presence or absence of sequences that can be used as translation signals may affect the expression of the plant genes in bacteria. Expression of *DRT100* may have benefited both from fusion of its cDNA in-frame to the first 35 *lacZ* codons in the vectors,

and from the presence of an internal ATG, preceded by a purine-rich sequence AGACGG (Fig.16) characteristic of Shine-Delgarno sequence (Shine and Delgarno, 1975). It may be significant that *DRT101, 102, 103, 111* and *112* also encode rudimentary ribosome-binding sequences five to seven bp 5′ to their first internal ATG start codons - AGGAAA (Fig.18), TCGGAG (Fig.19), AGTGTG (Fig.20), GAGGAG (Fig.21), and AAGAAT (Fig.22), respectively. However, translation of the plant genes in *E.coli* may not be efficiently initiated at these internal starts. Moreover, elongation efficiency may be reduced by the presence of codons poorly used by *E.coli*. Therefore, limiting amounts of the Drt molecules due to poor expression of the plant *DRT* genes by the bacterial machinery may not be able to keep pace with the increasing damage. This might be one of the reasons that complementation efficiencies decreased as the bacterial damage increased.

The structural features of the *Arabidopsis DRT* genes may also cause low complementation efficiencies in *E.coli*. When *DRT100* is expressed as a fusion protein, the presence of extraneous β-galactosidase residues might inhibit its activity. Thus complementation activity could only result from initiation of translation at the internal ATG, which might not be efficient in *E.coli*. Since *E.coli* presumably does not remove chloroplast transit peptides, the apparent chloroplast transit sequences in Drt100, 101, 111 and 112 may mask their intrinsic biochemical activities. These putative plant Drt

proteins may be unstable, or less active in *E.coli* than in plants for other reasons. For example, the extensive domain of leucine-rich-motif(LRM) repeats(Fig.17) in Drt100 suggests that it may require interaction with other plant (Drt) proteins, or self-association in order to stabilize itself or efficiently function. Recent findings revealed that two yeast repair proteins, RAD1 and RAD7, contain tandem LRM repeats (Schneider and Schweiger, 1991). They are believed to belong to the LRM superfamily, which includes such members as yeast adenylate cyclase (Suzuki *et al.*, 1990), cell surface receptors (Hickey *et al.*,1989; McFarland *et al.*, 1989; Smiley *et al.*, 1990) and ribonuclease/angiogenin inhibitor (Schneider *et al.*, 1988), all exerting their function by specific protein-protein interactions involving LRM repeats. If we assume that Drt100 functions as a strand-exchange protein, its complementation efficiency in RecA$^-$ bacteria would be limited by the extent to which homologous recombination processes contribute to DNA-damage toleration, and the extent to which Drt100 matches the efficiency of RecA protein in mediating these processes. If Drt101 and Drt102 prove to be single-polypeptide UV endonucleases, their efficiencies for complementing *E.coli* Uvr$^-$ phenotypes must depend on their ability to recognize the UV photoproducts and incise the damaged DNA without the assistance of other proteins, a complex process which is accomplished coordinatively by UvrABC proteins in *E.coli*. Similarly, if Drt111 and Drt112 serve to resolve recombination intermediates, their abilities to

increase toleration of DNA damage by UV or other chemical agents in resolvase-deficient bacteria will be determined by the capacity of Drt111 and Drt112 to recognize Holliday junctions formed by RecA-mediated recombination, and to resolve the large number of such intermediates formed during DNA-damage-induced sister-strand exchange (Roca and Cox, 1990).

E. DRT100 and DRT101 mRNA Levels Increase in Response to a Variety of DNA-damaging Agents

The expression of the *DRT100* and *DRT101* genes of *Arabidopsis* was increased in response to UV-B irradiation and chemical treatments. At the UV-B doses examined, increases in both *DRT100* and *DRT101* mRNA levels were maximal at 3-h UV-B irradiation (Fig.24A,B); the amounts of *DRT100* and *DRT101* mRNAs were increased by 3.3- and 3.8-fold, respectively, relative to the levels in unirradiated plants. The maximal *DRT100* mRNA accumulation occurred with 3 h after UV-B irradiation, while the rise in the level of *DRT101* transcript was rapid, reaching a peak immediately after irradiation. Exposure of plants to mitomycin C and methyl methanesulfonate (MMS) led to much greater increase in the level of *DRT100* transcript than that observed with UV-B irradiation. *DRT100* mRNA levels were elevated 10.1-fold by mitomycin C, and 9.3-fold by MMS treatment, compared to 3.3-fold by UV-B light. Similar result was obtained with *DRT101* mRNA levels, which was increased up to 14.6-fold by mitomycin C.

Compared to various sets of *E.coli* damage-inducible genes which respond to specific kinds of DNA-damaging agents (Walker, 1985), *DRT100* appeared to be induced by three different kinds of DNA-damaging agents. Surprisingly, *DRT101*, whose complementing activity was specific for UV irradiation in *E.coli* repair-deficient mutants, was not only induced by UV-B irradiation but also by mitomycin C treatment. It thus seems that the induction signal for expression of both *DRT100* and *DRT101* genes could be produced by very different kinds of DNA-damaging agents. It would be premature to conclude that there is more than one mechanism for the induction of the *DRT* genes. However, at least one mechanism must involve a common signal produced as a consequence of the DNA damage caused by UV-B, mitomycin C and MMS (in the case of *DRT100*), or by UV-B and mitomycin C (in the case of *DRT101*).

Increased cellular levels of a particular mRNA can be achieved via enhanced transcription of the gene or by stabilization of the mRNA. Here, I examined increases in *DRT100* and *DRT101* mRNA levels, and thus have not yet differentiated between these two induction mechanisms. I am interested in determining whether the accumulation of these two *DRT* mRNAs in response to DNA damage is due to an increased rate of transcription of the *DRT* genes, by nuclear runoff experiments. Meanwhile, it is currently unclear whether the induction of the *DRT100* and *DRT101* transcripts enhances the ability of the plant cells to repair or tolerate DNA damage *in*

vivo. Direct demonstration of relationship between increased survival and DNA-damage induction of the *DRT* genes will likely require comparison with noninducible mutants. *DRT101* does not increase resistance of *E.coli* repair-deficient mutants to mitomycin C, but its transcript level is increased in plants treated with this DNA-damaging agent. It is not clear whether the induction of *DRT101* by mitomycin C in plants reflects a general DNA-damage-repair role there, in contrast to its UV specificity in *E. coli*. It has been reported that transcription of the yeast *PHR1* gene coding for photolyase, which is specific for UV-induced *cis,syn*-cyclobutane pyrimidine dimers(CBPDs), is induced by several alkylating agents as well as UV light (Sebastian *et al.*, 1991). Therefore, the signal for *DRT101* induction may not be the appearance of its specific substrate or substrates but rather a more general metabolic response to DNA damage.

It has been shown that UV light and pathogen stress increase transcription of the chalcone synthase(CHS) gene, which encodes the first enzyme unique to flavonoid biosynthesis (Thompson and White, 1991; see "Rationale and Significance" section). The results here indicate that the transcript level of the CHS gene in intact *Arabidopsis* plants increases in response to UV-B irradiation. This is consistent with the previous observations that, in parsley tissue culture cells (Chappell and Hahlbrock, 1984) and in tobacco teratomas carrying a chimeric CHS gene (Kaulen *et al.*, 1986),

transcription of the CHS gene is induced by UV-B light. However, treatment of plants with DNA-damaging chemicals, mitomycin C and MMS, did not increase accumulation of the CHS mRNA. This suggests that the signal for CHS induction is produced by metabolic stress in general rather than by DNA damage, and that transcription of the *DRT* and CHS genes may be controlled by different regulatory mechanisms. Transcription of some yeast DNA-damage inducible genes such as *UB14, DDRA2,* and *DDR48,* is activated in response to heat shock (Finley *et al.,* 1987; McClanahan and McEntee, 1986). It will be interesting to determine whether *DRT* transcription responds specifically to DNA damage, or is also involved in providing resistance to a variety of stress conditions.

The genome of every organism is exposed to numerous natural and synthetic agents that damage DNA, and the expression of certain sets of prokaryotic and eukaryotic genes can be induced in response to that DNA damage (see "Introduction" section above). Some of these DNA-damage-inducible gene products are involved in DNA repair and so protect cells against mutation and cell death. To date, two DNA-damage-inducible responses have been characterized in *E.coli,* namely the SOS response and the adaptive response (Walker, 1985). In eukaryotes, several yeast DNA-damage-inducible genes have been identified, including those involved in DNA repair (see "Introduction" section) and genes involved in nucleotide and DNA metabolism, such as *POL1*(Johnston *et*

al., 1987), *CDC9*(Barker *et al.*, 1985) and *RNR2*(Elledge and Davis, 1989). In human cells, genes encoding c-Fos(Angel *et al.*, 1985), collagenase(Angel *et al.*, 1987), and metallothionein(Angel *et al.*, 1986), have been proved inducible by DNA damage. In higher plants, the synthesis of a RecA-like protein in pea chloroplast was increased by treatments with UV, mitomycin C and nalidixic acid (Cerutti and Jagendorf, 1991). However, whether this increase reflects transcriptional or translational regulation has been unclear. The observations that *DRT100* and *DRT101* transcript levels were increased by UV-B irradiation and chemical treatments suggest that mechanisms through which expression of certain repair/toleration genes is induced in response to DNA damage also exist in higher plants. Further elucidation of the mechanisms involved in regulating expression of the *DRT* genes will benefit from strong evidence for transcriptional control, mediated by one or a few damage-responsive regulatory elements.

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