Isolation and Characterization of UV-sensitive Mutants from *Arabidopsis thaliana.*

By

Ishita Chatterjee

A thesis
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requirements for the degree of
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ISOLATION AND CHARACTERIZATION OF UV-SENSITIVE MUTANTS FROM *Arabidopsis thaliana*

BY

ISHITA CHATTERJEE

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Our understanding of DNA repair and mutagenesis in higher plants is very limited, in comparison to other eukaryotes. This is primarily due to the fact that the genes which control DNA repair in plants have not yet been isolated and to the relative lack of repair-defective mutants. This study involves isolation and characterization of UV-sensitive mutants of the angiosperm *Arabidopsis thaliana*. UV-sensitive mutants were isolated by screening approximately 49,000 seeds which were T_4 progeny of 4,900 independent *Agrobacterium tumefaciens* T-DNA transformed lines. A total of 55 UV-sensitive mutants were isolated on the basis of the sensitivity of their root tissue to UV-C. The mutants were named *uvs*1-55. Out of those 55 *uvs* mutants, in 11 cases the roots were UV-sensitive in the light (*uvs*1-11) and in 44 cases, the roots were sensitive in dark (*uvs*12-55). Based on 100% inhibition of root growth, *uvs*12-55 were further classified into: 1. mutants where the roots stopped growing at ~600 J/m^2; 2. those which stopped growing at ~800 J/m^2; and 3. those which stopped growing at ~1,200 J/m^2. In all *uvs* mutants (*uvs*1-55), the mutation was found to be recessive. Results of segregation analysis helped in determining the pattern in which these UV-sensitive mutations segregate in successive generations. Complementation analysis, where UV-sensitivity of the roots was observed in the F_1 generation, placed the mutants in 48 separate complementation groups. Mutants were further characterized by exposing the leaves of 15 day old seedlings to a series of UV-B and UV-C doses and by assessing the sensitivity of the seeds to ionizing radiation. In contrast to UV, ionizing radiation is highly

penetrating and causes DNA damage by predominantly single- and double-strand breaks. Thus, mutants sensitive to ionizing radiation are most likely DNA repair defective, potentially in recombinational repair. The *uvs* mutants showed characteristic patterns of UV and ionizing radiation sensitivity, quite similar to the yeast RAD mutants. Based on their sensitivity to UV and ionizing radiation the *uvs* mutants were grouped into six different classes sharing similar phenotypic characteristics with the three yeast epistasis groups (*RAD3*, *RAD6* and *RAD52*). In the final part of this study, cosegregation analysis was used to determine whether the UV-sensitive mutation and the T-DNA were linked. In mutants, where the UV-sensitivity and the T-DNA was found to be linked, the copy number of T-DNA inserts was determined. This was done by Southern hybridization using probes made from the right and left border ends and the pBR322 origin of replication component of the T-DNA insert sequence. With the exception of one mutant (where the copy number of T-DNA could not be discerned), the copy number of T-DNA was proven in all cases to be more than one.

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List of Abbreviations

amp	ampicillin
ATP	adenosine-5'-triphosphate
bp	base-pair
BSA	bovine serum albumin
C	cytosine
Ci	curie
Col	Columbia ecotype
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxyribonucleoside-5'-triphosphate
DNA	deoxyribonucleic acid
ds DNA	double stranded DNA
dTTP	2'-deoxythymidine-5'-triphosphate
F_1	first filial generation
F_2	second filial generation
FAD	1,5- dihydroflavin adenine dinucleotide
G	guanine
g, mg, ng, µg	gram, milligram, nanogram, microgram
HDF	8-hydroxy-5-deazariboflavin

h(rs)	hour(s)
J	joule
kan	kanamycin
kb	kilobase-pair
krad	kilorad
l, ml, μl	litre, milliliter, microliter
M, mM, μM	molar, millimolar, micromolar
m, cm, nm	meter, centimeter, nanometer
M_1	primary mutagenized population
M_2	secondary mutagenized population
M_3	tertiary mutagenized population
min	minute
MTFH	5,10- methylene tetrahydrofolate
n	number of observation(s)
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
Na_2EDTA	disodium ethylenediaminetetraacetic acid
no.	number
PABA	*para* - amino benzoic acid
PAR	photosynthetically active radiation
pol	polymerase
PR	photoreactivation

rad	radiation-absorbed dose
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
ss DNA	single stranded DNA
T_1	first generation of progeny derived after transformation
T_2	second generation of progeny derived after transformation
T_3	third generation of progeny derived after transformation
T_4	fourth generation of progeny derived after transformation
T-DNA	transfer DNA
TE	Tris-EDTA
tRNA	transfer RNA
UV	ultraviolet
UV-A	ultraviolet-A (320 - 400 nm)
UV-B	ultraviolet-B (290 - 320 nm)
UV-C	ultraviolet-C (100 - 290 nm)
v/v	volume/volume
Ws	Wassilewskija ecotype
WT	wild type
w/v	weight/volume

w/w	weight/weight

1 Introduction

Environmental agents such as solar ultraviolet (UV) light, ionizing radiation, and certain chemicals, such as alkylating agents are a continual threat to DNA (Setlow 1966; Smith and Hanawalt 1969; Cerutti 1976; Singer and Grunberger 1982; Goodhead 1989; Frankenberg-Schwager 1990). DNA is also susceptible to spontaneous damage, that includes loss and chemical modification of bases (Lindahl and Nyberg 1972; Loeb and Preston 1986; Lindahl 1993) and breakage of DNA strands (Friedberg et al. 1995). These structural alterations can change DNA bases into miscoding (mutagenic) or noncoding (lethal) lesions that can eventually mutate or kill cells (Friedberg et al. 1995). DNA lesions can also block the progress of DNA and RNA polymerases (Chan et al. 1985; Protic-Sabljic and Kraemer 1986; Mitchell et al. 1989; Donahue et al. 1994) thereby affecting replication, recombination and transcription. Therefore, alterations in the structure of DNA must be dealt with in a manner that neither interferes with transcription nor causes an alteration in the nucleotide sequence. This task is accomplished by DNA repair (Friedberg et al. 1995).

Plants are photosynthetic autotrophs, using sunlight to prepare their food. They are thus, inevitably exposed to UV and oxygen, which are important genotoxic agents. Plants, like other organisms, must have developed mechanisms to tolerate and/or repair DNA damage. There is considerable evidence from various sources regarding the existence of DNA repair system in plants (Nuffer 1957; Ikenaga and Mabuchi 1966; Fuji 1969; Howland 1975; Soyfer 1979; McLennan 1987; Small 1987; Mayerhofer et al. 1991; Pang and Hays 1991; Britt et al. 1993; Chen et al. 1994, 1996). However, in comparison to other eukaryotes, our understanding of the mechanisms involved in DNA damage and repair systems in higher

plants is still in its infancy (Britt 1996). Even though plants occupy the position of "primary producers" in the ecological food chain, it is surprising, that, until recently, very little attention has been directed towards understanding DNA repair and mutagenesis in plants. During the last several years the concern regarding the depletion of the stratospheric ozone layer (for review, see World Meteorological Organization, 1995) and the resulting increase in UV radiation reaching the earth's surface (Madronich et al. 1995 and the references therein) has stimulated interest in characterizing the mechanisms involved in combating UV-induced stress in higher plants.

In this review, UV-induced DNA damage and its repair processes in plants will be the focus of interest. An outline of other protective mechanisms, associated with UV-induced stress responses, also will be given. In the final section of this review, a brief description of *Arabidopsis thaliana* (henceforth referred to as *A. thaliana)*, the experimental organism used for this study, will be provided.

1.1 Changing solar ultraviolet climate

Ultraviolet radiation constitutes approximately 7% of the total solar output of electromagnetic radiation. UV radiation spectrum is divided into three wavelength bands known as UV-C (100-290 nm); UV-B (290-320 nm) and UV-A (320-400 nm) (IARC 1992). Radiation below 295 nm is generally absorbed by molecular oxygen and ozone, present in the stratosphere. Thus, only UV-A and UV-B, but virtually no UV-C wavelengths, reach the earth's surface (Stapleton 1992). A significant concern of global magnitude is the depletion of the stratospheric ozone layer (Stolarski et al. 1992; Ambach

and Blumthaler 1994; Herman et al. 1995) and consequently, the increase in UV radiation entering the biosphere (Blumthaler and Ambach 1990; Crutzen 1992; Madronich 1993; Jokela et al. 1995). Atmospheric pollutants such as chlorofluorocarbons (CFCs) are the main agents held responsible for ozone depletion (NASA 1987; Frederick 1990; McFarland and Kaye 1992; Gleason et al.1993; Kerr and McElroy 1993).

Attenuation of UV radiation by the atmosphere is dependent on UV wavelength. In the event of ongoing ozone depletion, the expected spectral irradiation changes by the turn of the next century would occur within a waveband of about 13 nm (between 297-310 nm) in the UV-B region (Caldwell et al. 1989; IARC 1992). Even in the case of an unlikely 40% global ozone reduction, UV-C radiation would not penetrate the earth's atmosphere (Green et al. 1974, 1980; Caldwell et al. 1989; Frederick et al. 1989). Furthermore, since the ozone absorption coefficient is very low at longer wavelengths, UV-A radiation is basically unaffected by changes in the stratospheric ozone concentration (Caldwell 1981). Hence damage due to ozone depletion would be mostly due to UV-B and a portion of UV-A (Coohill 1983; Freeman et al. 1987; Caldwell et al. 1989; IARC 1992).

1.2 Biological effects of increased ultraviolet radiation

Increased UV-B entering the earth's atmosphere is expected to impair growth and photosynthesis of plants (Tevini et al. 1989a; Cen and Bornman 1990; Strid et al. 1994; Teramura and Sullivan 1994; Caldwell et al. 1995), affect the motility and reproductive capacity of phytoplankton (Sebastian et al. 1994), change the composition of phytoplankton

in aquatic ecosystems (Cullen and Neale 1994; Grobe and Murphy 1994 and references therein), affect plant-herbivore interactions (Bothwell et al. 1994) and may eventually lead to disruption of some food chains (Veen and Buma 1995).

UV-induced damage to plants can be broadly categorized into (i) damage to physiological processes which includes alterations in photosynthesis (Bornman et al. 1984; Vu et al. 1984; Greenberg et al. 1989; Strid et al. 1990; Tevini et al. 1991; Jordan et al. 1992; Wilson et al. 1995), reduction in biomass (Tevini et al. 1981; Lydon et al. 1986; Sullivan and Teramura 1988), decrease in pollen germination (Flint and Caldwell 1984), epidermal deformation (Tevini et al. 1981, 1989b), and activation of the flavonoid biosynthetic pathway (Tevini et al. 1981, 1991; Beggs and Wellman 1985; Stapleton 1992; Bornman and Teramura, 1993), and (ii) damage to DNA, which may lead to heritable mutations.

This review focuses on DNA damage and repair in higher plants; thus, readers interested in more information on UV-induced damage to physiological processes are directed to the publications cited in Table 1.1.

1.2.1 DNA lesions caused by ultraviolet light

DNA repair and mutagenesis studies require information on the wavelength output of the UV-lamp used in such experiments and most importantly, the spectrum of DNA damage products induced by such wavelengths.

Table 1.1 Literature survey of recent publications in the area of UV-induced damage to physiological processes in higher plants.

Topics discussed	Reference
Effect of UV-B on photosynthesis.	Teramura and Sullivan 1994; Fiscus and Booker 1995.
UV-B induced stress responses.	Stapleton 1992; Middleton and Teramura 1994.
UV-B damage and protection at the molecular level.	Strid et al. 1994.
Penetration of UV-B radiation in foliage.	Day et al. 1992.
Effect of UV-B on flavonoids and other protective pigments.	Stapleton and Walbot 1994; Liu et al. 1995.
Effect of UV-B on ecosystems.	Bothwell et al. 1994; Johanson et al. 1995.

1.2.1.1 Lesions formed by direct damage to DNA bases

The molecular lesions formed in DNA by UV-C radiation are largely the result of direct absorption of photons by bases in the DNA. Of the UV-induced DNA damage products formed, 5-5, 6-6 cyclobutane-type pyrimidine dimers (cyclobutane dimers) (Freeman et al. 1989) and pyrimidine (6-4) pyrimidone photoproducts ([6-4] photoproducts) (Mitchell and Nairn 1989) due to dimerization of adjacent pyrimidines, constitute approximately 75% and 25% respectively (Mitchell and Nairn 1989).

Cyclobutane dimers (Fig. 1.1) are formed between adjacent pyrimidines by saturation of their respective 5,6 double bonds, thereby forming a four-membered ring structure (Friedberg et al. 1995). Cyclobutane dimers exist in 12 isomeric forms. Only four forms, *cis-syn*, *cis-anti*, *trans-syn*, *trans-anti* have been observed in significant amounts (Khattak and Wang 1972), and the *cis-syn* isomer is most commonly detected in double-stranded (B-form) DNA. Formation of [6-4] photoproducts (Fig. 1.2) also involves the formation of a covalent link between two pyrimidines, but by bonding of carbon 6 of the 5'-pyrimidine and carbon 4 of the 3'-pyrimidine (Wang and Varghese 1967; Patrick and Rahn 1976; Franklin et al. 1982).

Both cyclobutane dimers and [6-4] photoproducts have demonstrable roles in UV-induced killing and mutagenesis (Kunz and Glickman 1984; Mitchell et al. 1985; Wood 1985; Franklin and Haseltine 1986; Glickman et al. 1986). The presence of cyclobutane dimers distorts the DNA helix. This occurs due to considerable rotation of the adjacent pyrimidines (from the usual B-form alignment) in their new conformation. The distorted helix produces kinks and unwinds the DNA strand in the region of the dimer (Pearlman et

Figure 1.1 Formation of cyclobutane pyrimidine dimer.

Cyclobutane pyrimidine dimers are formed by covalent bonding between two pyrimidines. Cyclobutane rings (Marked in the diagram) are formed by saturation of the respective 5, 6 double bonds (Modified from Friedberg et al. 1995).

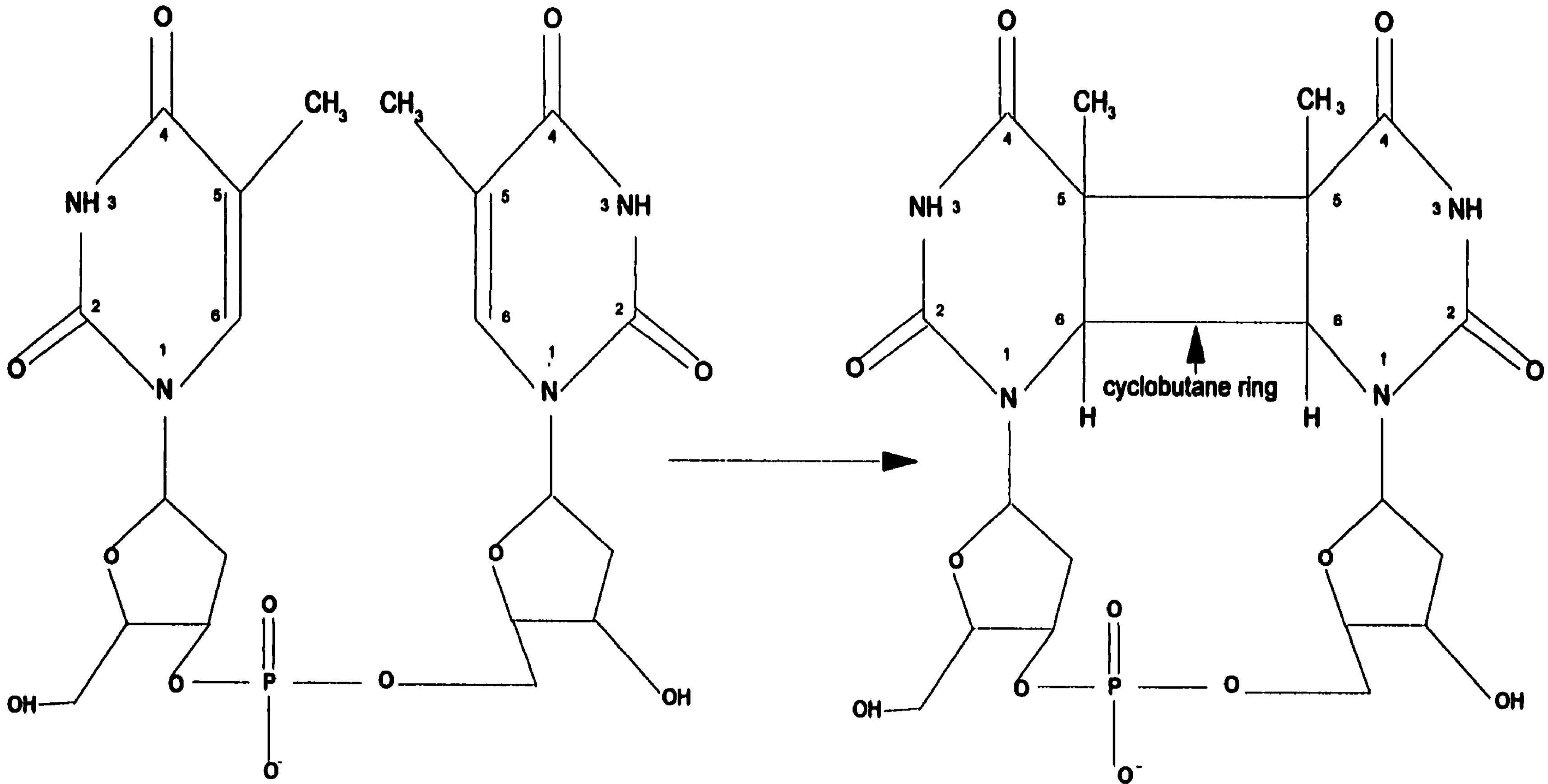
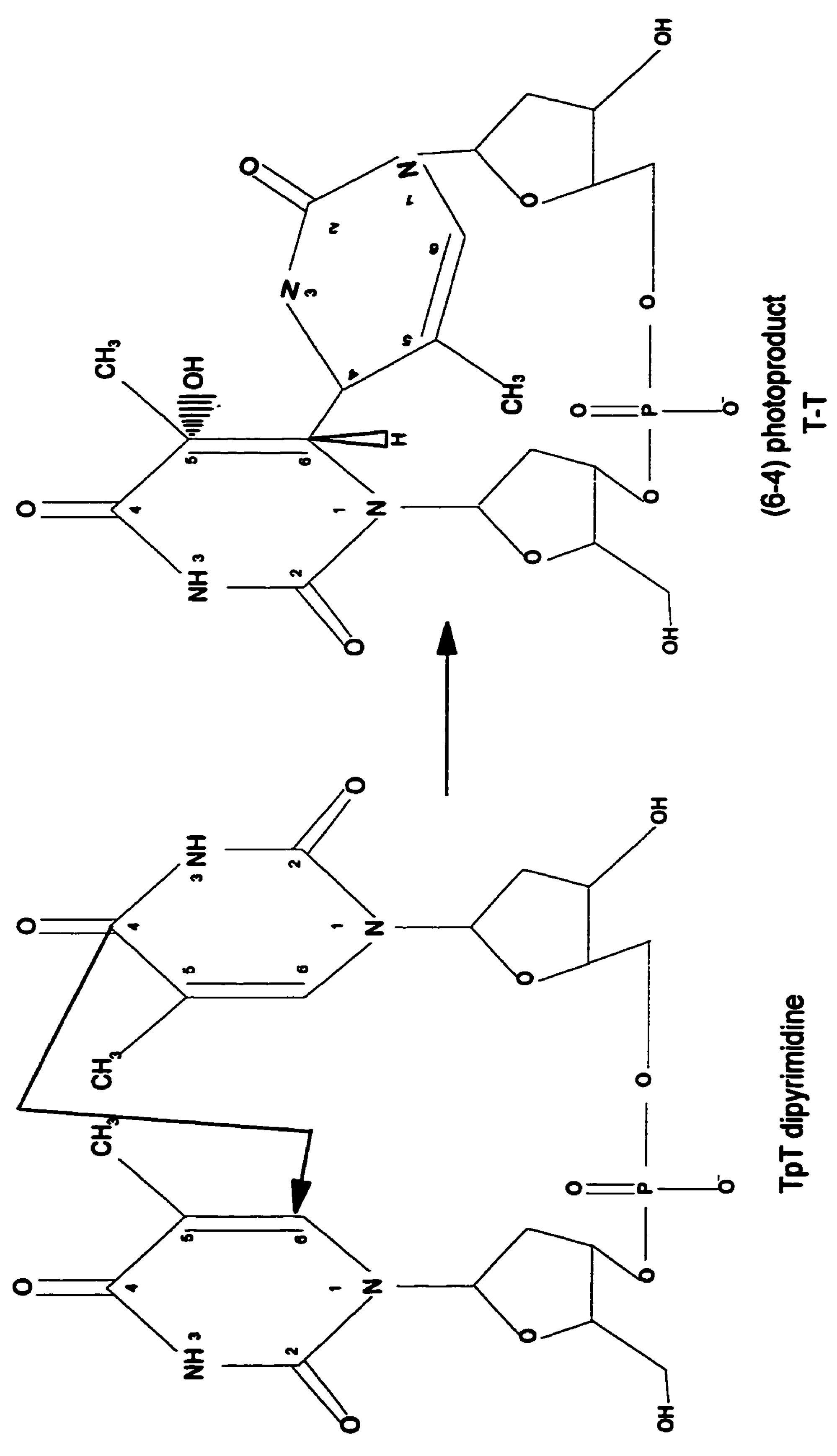

cyclobutane ring
TpT dipyrimidine
Cyclobutane dimer
cis, syn T<>T

Figure 1.2 Formation of [6-4] photoproduct.

In the diagram on page 10, formation of thymine-thymine photoproduct is shown. It is produced by linkage between C-6 position of one thymine and C-4 position of an adjacent thymine. The spontaneous breakage of the exocyclic group associated with the 6-4 bond formation transfers the amino group of the 3'-pyrimidine to the carbon 5 of the 5'-pyrimidine. This produces a shift of the 3'-pyrimidine by 90° relative to the 5'-pyrimidine causing a rotation of the 3'-pyrimidine out of the plane of the DNA helix (Modified from Taylor and Cohrs 1987).


TpT dipyrimidine
(6-4) photoproduct
T-T

al. 1985; Husain et al. 1988; Wang and Taylor 1991). In the case of [6-4] photoproducts, the spontaneous breakage of the exocyclic group associated with the 6-4 bond formation transfers the amino group of the 3'-pyrimidine to the carbon 5 of the 5'-pyrimidine. This produces a shift of the 3'-pyrimidine by 90° relative to the 5'-pyrimidine. Thus, induction of [6-4] photoproducts causes more rotation of the 3'-pyrimidine out of the plane of the DNA helix and accordingly more helix distortion in DNA structure than that caused by cyclobutane dimers (Franklin et al. 1985; Taylor et al. 1988; Kim and Choi 1995). Both photoproducts are unable to form stable hydrogen bonds and therefore, cannot pair correctly with any base (Ciarrochi and Pedrini 1982). In the event of DNA replication prior to or in the absence of DNA repair, the damaged nucleotides are used as templates. But as the photoproducts are unable to pair correctly, DNA synthesis past these lesions is error prone, producing miscoding deviants (Witkin 1976; Chan et al. 1985). In microbes and mammals, cyclobutane dimers have been found to inhibit the progress of DNA polymerases (Lawrence et al. 1990; Taylor and O'Day 1990); thus, in the absence of repair, a single dimer is capable of arresting replication. It has been demonstrated in mammals that both cyclobutane pyrimidine dimers and [6-4] photoproducts affect transcription by inhibiting the progress of RNA polymerase II, which has been found to remain stalled at the site of DNA damage (Protic-Sabljic and Kraemer 1986; Mitchell et al. 1989; Donahue et al. 1994). Thus, in addition to eliminating the expression of the transcriptional unit, the presence of pyrimidine dimers may also reduce the overall concentration of free RNA polymerase.

The inhibitory effect of a pyrimidine dimer on the progress of DNA and RNA polymerases has not yet been demonstrated in plants. But as the developmental pattern of

plants makes them inherently more resistant to the consequences of mutagenesis (Walbot 1985), it can be assumed that plant systems might be comparatively relaxed and may sacrifice accuracy of DNA replication to reduce toxicity of UV-induced damage (Britt 1995).

UV-C also produces non-dimer DNA lesions, such as DNA single-strand breaks (Tyrrell et al. 1974; Cerutti and Netrawali 1979; Rosenstein and Ducore 1983; Peak and Peak 1986), DNA-protein cross links (Rosenstein and Ducore 1983; Peak and Peak 1991), photoproducts involving purines, e.g. 8,8-adenine dehydrodimer, formed after irradiation of poly (dA) (Porschke 1973; Gasparro and Fresco 1986), pyrimidine hydrates, formed by adding a molecule of water across the 5,6 double bond of the pyrimidine base to form 5,6-dihydro-6-hydroxy derivatives (Fisher and Johns 1976; Boorstein et al. 1990; Vairapandi and Duker 1994), and thymine glycol, resulting from saturation of the 5,6-double bond of some thymine residues (Yamane et al. 1967; Demple and Linn 1980, 1982).

1.2.1.2 Lesions formed by indirect damage to DNA bases

In addition to the products formed in DNA by direct absorption of photons, UV radiation also reacts indirectly with DNA (Straight and Spikes 1985). Induction of indirect DNA damage is a property of UV in the range of 290 - 400 nm (which includes UV-B and UV-A radiation). Cells have both DNA and non-DNA photoreceptors, the later category absorbs maximally in the range of 290 - 400 nm. Thus, indirect DNA damage may result from absorption of UV wavelengths by non-DNA chromophore molecules (sensitizer molecules)

that then transfer the energy to DNA (Lamola 1974; Piette et al. 1986). This phenomenon is referred to as photosensitization.

A commonly occurring photosensitization process is enzymatic photoreactivation (described in detail in section 1.4.1). Photoreactivation is a biological reaction for reversing *cis-syn* cyclobutane pyrimidine dimer into its monomerized state. Photosensitization reactions in relation to DNA damage products include formation of thymine-thymine dimers in DNA by triplet excitation energy (Lamola 1974). Photosensitizers include both endogenous agents such as riboflavin and aromatic amino acids (Piette et al. 1986) and exogenous drugs e.g. 8-methoxypsoralen (Gasparro 1988).

1.2.1.3 Choice of UV-C lamps in DNA repair and mutagenesis studies

Experiments with UV are performed using both polychromatic and monochromatic UV sources. Polychromatic UV sources consists of varying proportions of UV-B and UV-A wavelengths. These type of sources are used in the elucidation of potential interactions among wavelengths thereby simulating the spectral output of the sun. In contrast, monochromatic UV sources emit specific wavelengths in the UV-A, UV-B or UV-C regions, and are used to ascertain the effectiveness of an individual wavelength. For studies involving DNA repair and mutagenesis, monochromatic UV-C sources are generally used (see section 1.2.1.4 for details). UV-C radiation sources are highly rated for studies involving DNA repair and mutagenesis. DNA has a high absorption maximum in the UV-C range (at 260 nm). The energetic UV-C radiation induces more DNA lesions in a shorter time than UV-B and UV-A (Mitchell et al. 1991; Bykov and Hemminki 1996). UV-C

induced damage is mainly restricted to direct damage to DNA, but UV-B induced damage includes DNA photoproducts, formed by direct interaction with DNA as well as other types of indirect DNA damages (Eisenstrak 1989). In addition, prolonged exposure to UV-B wavelengths converts the [6-4] photoproduct to its Dewar isomer (Taylor and Cohrs 1987; Taylor et al. 1990). Therefore, even though with longer exposure periods, UV-B can induce similar quantity of DNA lesions as does UV-C, however, isomerization of [6-4] photoproducts owing to prolonged UV-B exposure might interfere with the proper detection of DNA damage products. Therefore, DNA repair studies which try to elucidate the type and quantity of DNA photoproducts caused by UV and also the molecular mechanisms involved in their induction and repair (or lack of repair), require the choice of a UV source which can cause direct DNA damage in a short time. This is possible with a monochromatic UV-C lamp. Finally, powerful monochromatic UV-C sources (254 nm) are easily available and are less expensive than high-output UV-B sources.

Evidence suggests that experimental data obtained by using germicidal UV-C cannot be extrapolated to explain the effects of UV-B and UV-A. UV-C damages DNA mostly by direct interaction of DNA bases while UV-B damages DNA by both direct and indirect interactions. UV-C wavelengths are not ecologically relevant as atmospheric ozone layer completely filters out UV-C (see section 1.1), allowing only wavelengths within the UV-A and UV-B regions to enter the biosphere. In the case of *Escherichia coli*, action spectra for DNA damage in the UV-B range consist of two different phases, contributed by direct DNA damage (short wavelength UV-C type contribution) and indirect DNA damage (long wavelength contribution) (Peak and Peak 1986). In addition, an assay of different

photoproducts after UV-A, B, and C irradiation of naked DNA and DNA in human skin explants (Vladimir and Hemminki 1996) indicated that photoproducts induced by UV-C in human skin occur in much lower quantities compared to those from purified DNA (Bykov and Hemminki 1995). But the use of UV-B in the same experiment gave opposite results: the induction of mutations was higher in skin than in purified DNA. Thus, as in *E. coli*, action spectra for DNA damage in humans may also be biphasic in the UV-B range, with a short wavelength type and a long wavelength type contribution. In the short wavelength (UV-C type) contribution, photoproducts are formed by direct excitation of nucleotide bases (Hauswirth and Wang 1977). In the long wavelength contribution, DNA damage is introduced by two mechanisms; the first being the same as the pathway used by short wavelength; the second is presumed to act via photosensitized molecules (Lamola 1970; Moysan et al. 1991). UV-A has been known to induce different types of DNA damage (Freeman et al. 1987; Tyrrell and Keyse 1990; Cadet et al. 1992) of which, the lesion types induced by UV-C consists of a very small class (Vladimir and Hemminki 1996). Thus UV-B and UV-A are more ecologically relevant in the study of molecular events in UV damage to DNA. But, in order to study the molecular mechanisms involved in UV-induced damage to DNA and also its repair, it is essential to dissect out DNA damage and repair mechanisms from other cellular damages. This can be performed better with UV-C (which causes mainly direct DNA damage and that too in a short time), than with UV-B (which causes both direct and indirect DNA damage over a longer time span relative to UV-C). In addition, UV-C is a potent choice for mutagenesis studies (like this study) which involves

the isolation and ranking of UV-sensitive mutants according to their DNA damage repair capabilities.

1.3 Protective mechanisms in plants in response to UV stress

Both animals and plants are affected by increased UV radiation. Animals, especially humans can move and thus avoid sunlight if they choose, however, terrestrial plants cannot. Terrestrial plants use sunlight for photosynthesis (for recent reviews see Tevini et al. 1989a; Tevini 1993; Greenberg et al. 1995), and thus cannot avoid exposure to UV.

One of the important developmental distinctions between plants and animals is that, in animals, movement of the whole creature as well as movement of the body cells (blood cells, cells of the immune response) govern behaviour and physiology. But in higher plants, neither the whole body nor the individual cells move. Thus, the developmental pattern of plants have helped them to use physiological modifications as their principal form of defense mechanism (Walbot 1985).

The molecular mechanism by which UV is received by cells in any system and consequently how it affects gene expression is not quite clear. In plants, most of the processes involved in protection are believed to occur via UV-B photoreceptor(s) as well as phytochrome and a blue light/UV-A receptors (Ballaré et al. 1991; Ensminger and Schäfer 1992). One of the potential mechanisms in response to UV stress is a change in leaf transmittance properties, thereby minimizing the amount of UV radiation reaching the epidermis (Tevini et al. 1989b, 1991; Cen and Bornman 1993; Wilson and Greenberg 1995). Increase in the activity of the oxygen detoxification enzymes such as superoxide

dismutase and glutathione reductase, is another potential protection mechanism (Murali et al. 1988; Kramer et al. 1991; Middleton and Terramura 1993; Strid 1993). UV also induces the production of UV absorbing pigments e.g. flavonoids (Beggs and Wellman 1985) and anthocyanin (Hashimoto et al. 1991). These pigments generally accumulate in the epidermal layer and block the transmittance of 90-95% of incoming UV rays thereby preventing them from reaching the internal photosynthetic tissues (Robberecht and Caldwell 1978; Hahlbrock and Scheel 1989). As an example: mutants with defects in flavonoid biosynthesis are UV-hypersensitive (Li et al. 1993).

Thus, morphological features and pigments can afford considerable protection and reduce the dose of ultraviolet light reaching the plant cell DNA (McLennan 1987). For the more exposed cells, particularly those of the germ line, plants must also have evolved mechanisms to reverse, repair or tolerate UV induced DNA damage (Langer and Wellmann 1990; Pang and Hays 1991; Quaite et al. 1992; Britt et al. 1993), in addition to protective mechanisms. The plant germline, unlike that of animals, is not reserved (Walbot 1985). Gametes in animals are formed early in development from a zygote and they migrate to the gonads where they become functional. In contrast, gametes in plants develop and differentiate at a later stage from vegetative cells of the body. Therefore, plants must have evolved DNA repair or tolerance mechanisms to lessen the load of somatic mutations and so minimize the chances of transferring mutations to the next generation (Klekowski 1988).

1.4 Cellular responses to UV-induced DNA damage

In response to DNA damage caused by UV, the cell uses one or more of the following mechanisms to remove or tolerate the DNA lesions.

(i) Reversal of DNA damage; e.g. enzymatic photoreactivation (Rupert and Harm 1966; Setlow 1968; Cook 1970; Rupert 1975; Sancar 1990, 1994).

(ii) Excision of DNA damage (for review see Sancar 1996); e.g. base excision repair (Demple and Harrison 1994; Dodson et al. 1994; Sancar 1995), nucleotide excision repair (Sancar and Rupp 1983; Yeung et al. 1983; Huang et al. 1992, 1994 a, b; Reardon et al. 1993; Svoboda et al. 1993; Huang and Sancar 1994; Guzder et al. 1995) and mismatch correction (Au et al. 1992; Alani et al. 1994; Friedberg et al. 1995; for review see Modrich and Lahue 1996).

(iii) Tolerance of DNA damage; e.g. replicative bypass of template damage (Woodgate et al. 1989; Rajagopalan et al. 1992), translesion synthesis (Echols and Goodman 1990).

The repair and tolerance mechanisms mentioned above are well characterized in microbes and mammals. But in higher plants, so far, only photoreactivation and nucleotide excision repair mechanisms have been observed to have roles in the repair or removal of UV-induced DNA lesions; while postreplicative repair has not yet been detected (Britt 1995). Thus, in this introduction descriptions of photoreactivation and excision repair (mainly nucleotide excision repair) will be the main focus. Table 1.2 summarizes the UV-induced DNA damage and repair studies published to date in plants.

Table 1.2 A survey of UV-induced DNA damage and repair studies in plants.

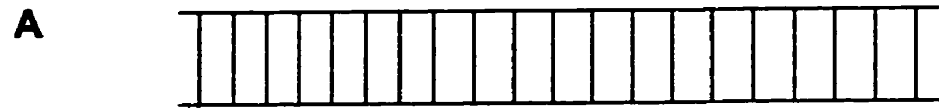
Type of study	Reference
Evidence of the genetic effects of UV	Nuffer 1957.
Photoreactivation of pyrimidine dimer	Ikenaga and Mabuchi 1966; Trosko and Mansour 1968; Pang and Hays 1991; Britt et al. 1993; Quaite et al. 1994.
Light dependent pathway for elimination of pyrimidine [6-4] pyrimidinone photoproduct	Chen et al. 1994.
Dark repair of pyrimidine dimers	Soifer and Tsieminis 1974; Howland 1975; Britt et al. 1993.
Repair in chloroplasts	Small and Greimann 1977; Small 1987.
Repair in pollen	Fuji 1969; Jackson and Linskens 1978.
Enzyme catalyzing repair of UV-induced DNA damage	McLennan and Eastwood 1986; Batschauer 1993; Murphy et al. 1993.
Arabidopsis cDNAs partially complementing repair proficiency of *E. coli* mutants	Pang et al. 1992, 1993.
Isolation of UV-sensitive mutants	Britt et al. 1993; Harlow et al. 1994; Jenkins et al. 1995.

1.4.1 Photoreactivation of cyclobutane pyrimidine dimers

As mentioned in section 1.2.1.2, cyclobutane pyrimidine dimers formed between adjacent bases in the same DNA strand are the most predominant UV-induced DNA photoproduct. Cyclobutane dimers are unable to form stable bonds with other bases, and therefore, are not directly mutagenic. They kill cells mainly by blocking replication and transcription (Setlow 1964) and on rare occasions when the DNA is replicated past the lesions (translesion synthesis), mutations are introduced at the lesion site. In addition to tolerating such damages, cells can also protect themselves against DNA damage by eliminating the photoproduct from their genome either by excision repair or by photoreactivation.

Photoreactivation involves reversible binding of the photoreactivating enzyme (photolyase) to UV-irradiated DNA, a step which can take place in the dark. Then, in the presence of visible light (300 - 600 nm) (for review see Harm 1976), the carbon-carbon bonds linking adjacent C5 and C6 atoms in th e cyclobutane ring of the *cis-syn* cyclobutane dimer are broken, thereby restoring the pyrimidines to their original nondamaged configuration (Harm 1976; Sutherland 1981). Figure 1.3 depicts the steps of enzymatic photoreactivation. The reaction directly reverses the UV-lesion; no bases are excised or inserted as in the excision repair process (Wulff and Rupert 1962; Rupert 1964). The *trans-syn* cyclobutane dimer can also be repaired by photoreactivation but the photolyase has a 10^4-fold lower affinity for the *trans-syn* isomer (Kim et al. 1993).

Figure 1.3 Schematic illustration of photoreactivation. This is a process involving the enzyme-catalyzed monomerization of pyrimidine dimers. A. Represents native DNA. B. Pyrimidine dimer present in DNA. C. A complex is formed with DNA and photoreactivating enzyme. D. Absorption of a 350-450 nm photon by the enzyme leads to monomerization of pyrimidine dimer. E. Represents DNA after reversal of damage. (Modified from Friedberg et al. 1995).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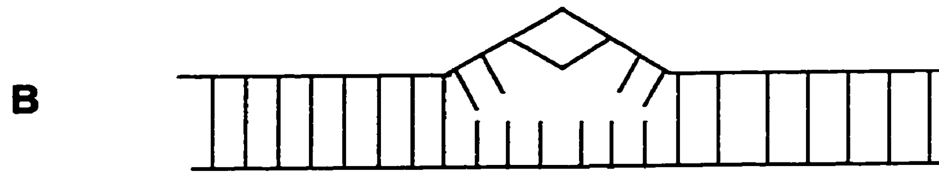
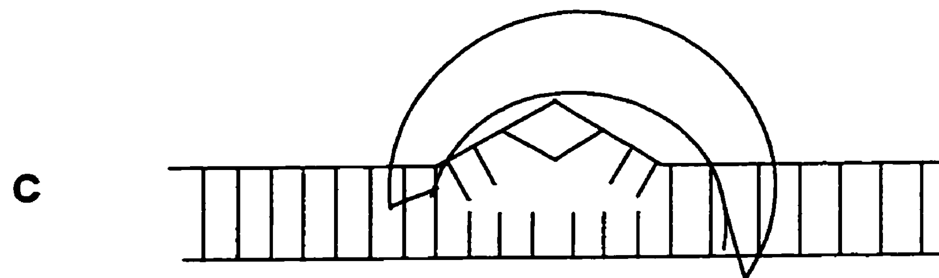
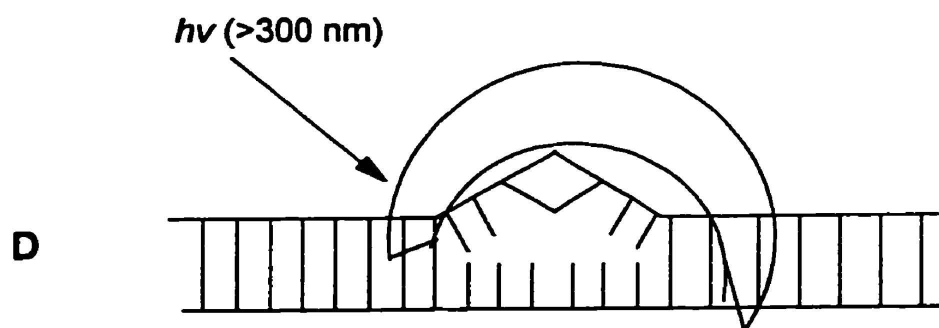
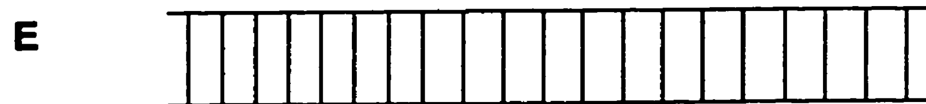

B


C


hv (>300 nm)
D


E

1.4.1.1 Properties of photolyase

Photoreactivating enzymes or photolyases have been extensively characterized in *E. coli.* (Friedberg et al. 1995). Studies in *E. coli* revealed that the enzyme photolyase contains two chromophores/cofactors (Jorns et al. 1984). One of the chromophores is 1,5-dihydroflavin adenine dinucleotide (FAD) (Iwatsuki et al. 1980; Sancar and Sancar 1984; Eker et al. 1988). FAD is always present in its reduced state. The second chromophore is either 5,10-methylene-tetrahydrofolate (MTFH) or 8-hydroxy-5-deazariboflavin (HDF) (Eker et al. 1981). Based on the different chromophores the enzymes have been divided into two groups, the folate class and the deazaflavin class (Sancar and Sancar 1988). Photolyases from both *E. coli* and *Saccharomyces cerevisiae* belong to the folate class (Li et al. 1993). The enzymes from *Stretomyces griseus* and *Aspergillus nidulans* belong to the deazaflavin class. The difference among the two classes is essentially the differences in the action spectra for the enzymes. Members of the folate class have maximum photoreactivation activity at wavelengths of ~380 nm (Johnson et al. 1988); members of the deazaflavin class have maximal activity at ~ 440 nm (Sancar 1990).

Photoreactivating activity has been observed in many organisms including most classes of vertebrates like fish, reptiles, amphibians and marsupials (Li et al. 1993; Friedberg et al. 1995). In many cases photolyases have been cloned. No photolyases have been detected in placental mammals, including humans (Kato et al. 1994).

1.4.1.2 Reaction mechanism

The fundamental photochemistry of photoreactivation in both the folate and deazaflavin class of photolyase has been found to be conserved. The photoreactivation reaction involves the binding of the enzyme to cyclobutane dimer in a light-independent step. This is followed by the absorption of a 350 - 450 nm photon by the second chromophore (MTFH or HDF). This chromophore then transfers the excitation energy to the reduced FAD cofactor which in turn initiates monomerization of the pyrimidine dimer by transferring an electron to the dimer, thereby splitting the 5-5 and 6-6 bonds of the cyclobutane ring to regenerate the two original pyrimidines. At the end of the reaction the catalytically active FAD is regenerated.

The reaction steps of photoreactivation:

Dark Step **Light Step**

E + Py<>Py → E : Py<>Py → E : Py-Py → E + Py-Py

where, Py <>Py : pyrimidine dimer,

E : photoreactivating enzyme,

Py-Py : repaired dimer.

1.4.1.3 Light dependent repair of [6-4] photoproducts in DNA

Todo et al. (1993) have demonstrated a light-dependent enzyme activity from the fruit fly, *Drosophila melanogaster* which leads to the disappearance of [6-4] photoproducts from DNA. The products of the enzyme reaction have yet to be determined and it is also not clear

whether the reaction truly represents a reversal of base damage. However, when UV-irradiated plasmid DNA of a repair defective *E. coli* was treated with the *D. melanogaster* fraction, photoreactivation of transforming activity of the irradiated DNA was restored (Todo et al. 1993). Recently, a gene encoding the *D. melanogaster* [6-4] photolyase was cloned and it has been found to have similarity with the amino acid sequence of cyclobutane pyrimidine dimer photolyase, belonging to the folate class and with the blue-light photoreceptor, an essential light detector for early plant development (Ahmad and Cashmore 1993).

1.4.2 Photoreactivation in plants

Ikenaga and Mabuchi demonstrated the presence of photoreactivation in plants as early as 1966. They showed that the frequency of UV-induced endosperm mutations generated in maize pollen dropped extensively when the pollen was exposed to visible light after UV irradiation. There is evidence for both the reversal of the damaging effect of UV light on various plant tissues (Bawden and Klechowski 1952) and for an increased rate of loss of thymine dimers from the DNA of UV-irradiated cells upon exposure to visible light in *Nicotiana tabacum* (Mansour 1968; Trosko and Mansour 1969), *Ginkgo biloba* (Trosko and Mansour 1969) and *Daucus carota* (Howland 1975; Howland and Hart 1977), in the seedlings of *Lathyrus sativus* (Soifer and Tsieminis 1974, 1977), and in whole plants of *Wolffia microscopia* and *Spirodela polyriza* (Degani et al. 1980). Photoreactivation of UV induced killing of *A. thaliana* seedlings was observed by Fuji (1965). In higher plants, photolyase activity has been detected in maize pollen and bean (McClennan 1987). The

photolyase activity of 5 day old shoots of the lima bean *Phaseolus lunatus* and the pinto bean *Phaseolus vulgaris* showed an uneven tissue distribution. There was a decline in enzyme activity with the development of shoots. Pang and Hays (1991) demonstrated UV-inducible photolyase activity in protein extracts of *A. thaliana*. The photolyase activity appeared early, but not immediately, during development and persisted for 30 days. The photolyase was found to be temperature sensitive, half-life at 30°C was 12 min, at 40°C it was 1.8 min, and at 50°C it was 1.3 min (Pang and Hays 1991). The action spectra for the *in vitro* activity of the partially purified *A. thaliana* photolyase closely resembled the action spectrum of the purified methylenetetrahydrofolate type photolyases of *E. coli* and yeast (Pang and Hays 1991). A potential plant photolyase cDNA was isolated from mustard (*Sinapis alba*) (Batschauer 1993). The polypeptide encoded by the gene has strong sequence similarity to *E. coli* and yeast photolyases. The cDNA hybridized to a light-regulated mRNA but when expressed in *E. coli* failed to display any photolyase activity. Recently, Chen et al. (1994) have demonstrated the presence of a light-dependent pathway for the removal of the [6-4] photoproduct in *A. thaliana*. It is comparable to the light-dependent [6-4] photoproduct removal pathway demonstrated in the fruit fly *D. melanogaster* (Todo et al. 1993). Unlike the cyclobutane pyrimidine dimer photolyase activity which requires prior induction by visible light, [6-4] photoproduct specific photolyase is constitutively expressed and does not need induction by visible light. Britt et al. (1993) have isolated an *A. thaliana* mutant sensitive to UV known as *UV resistance* (*uvr*)-1. *uvr*1 is defective in dark repair of the [6-4] photoproduct but can remove it in

presence of light thereby proving that the light-dependent pathway for removal of (6-4) photoproduct is independent of the *UVR1* gene product (Britt et al. 1993).

1.4.3 Excision repair

Excision repair is a major repair pathway for removing a modified base from DNA, in which case it is known as base excision repair, or, removing an oligonucleotide in which case the excision repair is known as nucleotide excision repair (Friedberg et al. 1995; Sancar 1996).

The key reaction in base excision repair is the use of lesion specific glycosylases which cleave the glycosylic bond joining the base to the sugar (Demple and Harrison 1994; Dodson et al. 1994; Friedberg et al. 1995; Sancar 1995). Following removal of the single damaged base, a combination of exonucleases, repair polymerase and DNA ligase act on the nicked DNA to restore it to its original sequence. High specificity is essential to the reaction mechanism because an enzyme that hydrolyzes the N-glycosyl bond must at one point come in close contact with the modified nucleotide, recognizing the chemical group unique to the modified base (Sakumi and Sekiguchi 1990). In contrast, nucleotide excision repair uses the same proteins to remove a variety of DNA lesions (Selby and Sancar 1990; Van Houten and Snowden 1993). Thus, instead of synthesizing an enzyme specific for each of the infinite number of possible lesions, the cell is able to make use of the nucleotide excision repair to remove most of the lesions.

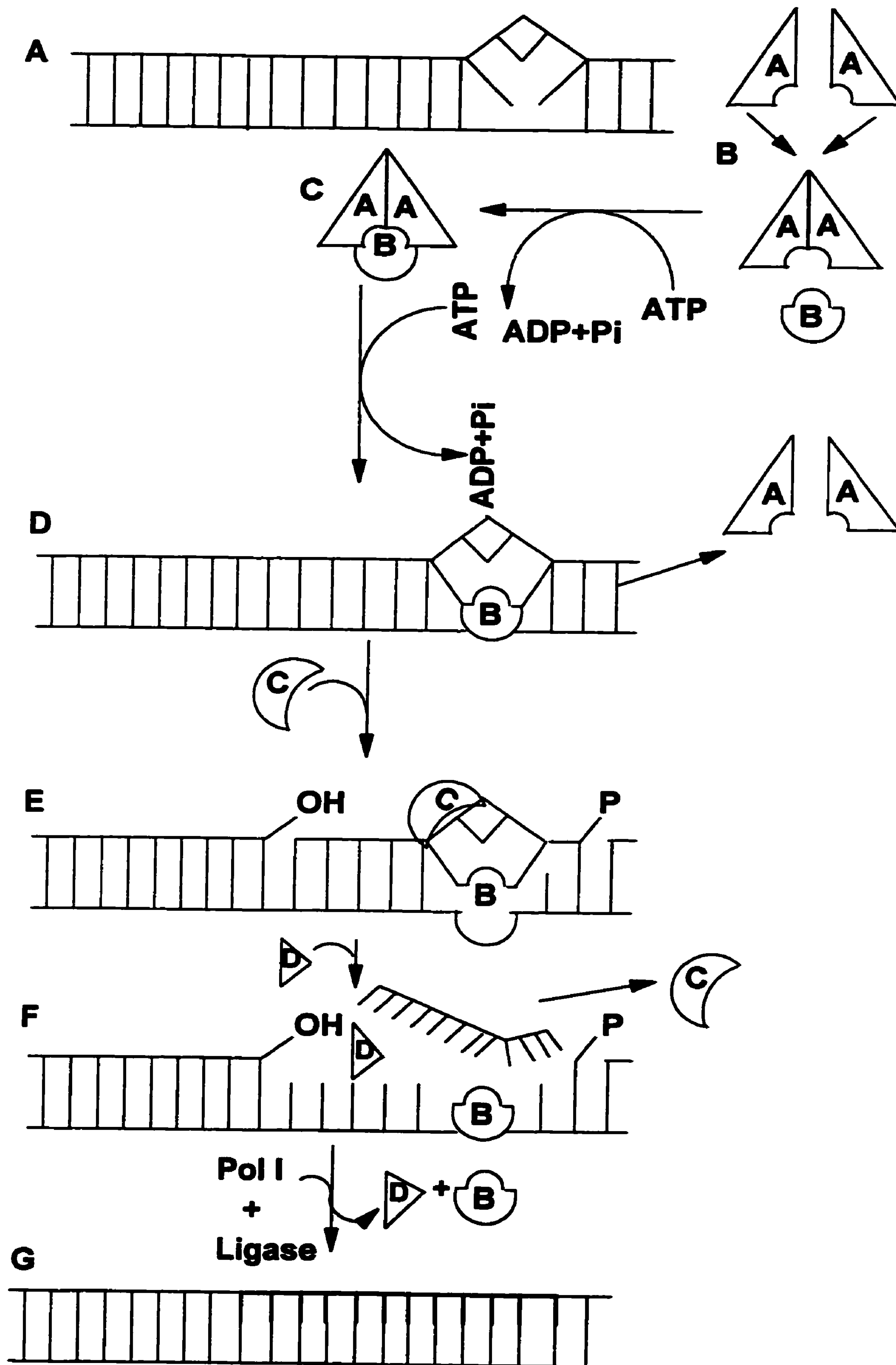
Nucleotide excision repair consists of five basic steps: damage recognition; incision on both sides of the DNA lesion; excision or displacement of the oligomer containing the DNA

lesion; repair synthesis to fill the single-strand gap left by displacement of the lesion containing oligomer; and ligation to seal the nick between the newly synthesized strand and rest of the DNA helix (Friedberg et al. 1995; Sancar 1996).

Nucleotide excision repair has been extensively characterized in *E. coli*. A model is presented in Figure 1.4. The first three steps of this process are carried out by three proteins encoded by the *uvrA*, *uvrB* and *uvrC* genes (Sancar and Sancar 1988). Prior to recognizing the damage, the UvrA and UvrB subunits form an ATP-dependent $UvrA_2B$ complex. The $UvrA_2B$ complex recognizes damaged DNA (in a processive fashion) and interacts with the damaged site which leads to the dissociation of the UvrA dimer and the formation of a stable UvrB-DNA pre-incision complex (Orren and Sancar 1989, 1990). The released $UvrA_2$ complex is now free to bind to another molecule of UvrB, which can then be directed to a new site of DNA damage. Following the release of UvrA, UvrC recognizes the UvrB-DNA complex, binds to it, and a conformational change in the complex enables UvrB to make the 3' incision, which in turn triggers a second conformational change enabling UvrC to make the 5' incision. UvrD is required to release UvrC and the excised oligomer from the post-incision complex. The remaining-UvrB-gapped DNA complex is almost as stable as the pre-incision UvrB-DNA complex. UvrB is released from the post-incision complex and DNA polymerase I, using the four deoxyribonucleoside triphosphate (dNTP) DNA precursors, fills the gap leaving a nick at the 3' incision site, which is subsequently ligated.

In comparison to prokaryotes, nucleotide excision repair is not well defined among eukaryotes. This paucity of information is probably due to larger and more complex

Figure 1.4 Nucleotide excision repair in *E. coli.* A. Represents damaged DNA. B. Represents dimerization of two UvrA proteins. C. UvrA dimer associates with UvrB to form the $UvrA_2B$ complex. D. UvrB is delivered to the damaged DNA via this complex. This step requires energy in the form of ATP. Following this, UvrA, which acts only to help in the tight binding of UvrB and the damaged DNA, is then released. E. Next, UvrC binds to the UvrB-DNA complex. Binding of UvrC forms an active nuclease that functions to incise the damaged DNA strand on either side of the lesion. F. UvrC and the damaged containing lesion is released from the DNA by UvrD. G. The resultant gap is filled in by DNA polymerase I and ligase (modified from Bootsma 1993).


A
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ADP+Pi
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C
E
OH
C
P
B
D
F
OH
D
P
C
B
Pol I
+
Ligase
D
+
B
G

genomic makeup. Nucleotide excision repair processes are quite similar in yeast, *Drosophila* sp., *Xenopus* sp., and in mammalian cells. This high degree of conservation has helped in rapid advancement in this field.

Among the lower eukaryotes, *S. cerevisiae* is well characterized regarding its response to DNA damaging agents. Over 30 radiation-sensitive (*RAD*) loci which control resistance to killing by UV and ionizing radiation have been detected (Friedberg et al. 1995). These loci have been classified into three (largely) non-overlapping groups (Haynes and Kunz 1981). Loci belonging to each group control different steps in a specific biochemical pathway, consequently among the loci of a specific group, a double mutant is no more sensitive than single mutants. These groups are known as epistasis groups. Of the three epistasis groups in yeast *RAD3* group is responsible for the nucleotide excision repair of UV-induced DNA damage (Friedberg et al. 1995); *RAD6* group is involved in error prone repair (Friedberg et al. 1995) and *RAD52* group of genes are responsible for gene conversion, recombination and DNA double strand break repair (Friedberg et al. 1995).

The *RAD3* epistasis group consists of 11 genes (*RAD1, RAD2, RAD3, RAD4, RAD7, RAD10, RAD14, RAD16, RAD23, RAD25,* and *MMS19*) which control excision repair of UV-damaged DNA. Mutations in the first six genes of this group confer extreme sensitivity to UV light, and a total defect in the incision step of excision repair. Mutations in the last four members of this group confer a moderate sensitivity to UV light. Proteins encoded by those four genes may serve as accessory factors in the excision repair process (Haynes and Kunz 1981; Cooper and Kelly 1987; Friedberg et al. 1995). Both *RAD3* and *RAD25* are essential for excision repair and cell viability because they are required for transcription

(Naumovski and Friedberg 1983). *RAD3* possesses single-stranded DNA-dependent ATPase and DNA helicase activities (Sung et al. 1987). *RAD1*, *RAD10* and *RAD25* have roles in mitotic recombination, but the pathway controlled by *RAD1* and *RAD10* is distinct from *RAD25* (Schiestl et al. 1990). *RAD14* has a roles in damage recognition.

1.4.4 Excision repair in plants

1.4.4.1 Base excision repair

Several groups have partially characterized plant extracts exhibiting specificity for UV-irradiated DNA (Veleminsky et al. 1980; Doetsch et al. 1989; Murphy et al. 1993). In some of these activities, the recognition sites are [6-4] photoproducts instead of cyclobutane pyrimidine dimers. In 1991, Strikland et al. purified and characterized a plant UV-specific endonuclease. This single-stranded endonuclease apparently recognizes single-stranded region induced by [6-4] photoproducts and not cyclobutane pyrimidine dimers.

1.4.4.2 Nucleotide excision repair

The nucleotide excision repair pathway, also known as "dark repair" in plants, is very poorly characterized. Some sort of light-independent repair of UV-induced lesions has been observed in *Chlamydomonas* (Small and Greimann 1977) and also in higher plants (McClennan 1987). The excision of pyrimidine dimers from a UV-irradiated plant was first shown in the grass pea *L. sativus* (Soifer and Tsieminis 1974, 1977). Excision in the dark of UV-induced pyrimidine dimers in carrot protoplasts was demonstrated by Howland (1975). Jackson and Linskens (1978) first demonstrated that DNA synthesis was associated with

DNA repair in UV irradiated pollen of *Petunia*. Virtually all of the dimers produced in *Daucus* protoplasts by a dose of 10 J/m^2 of 254 nm radiation were removed during a 24 hour post-irradiation incubation in the dark (Howland 1975; Eastwood and McLennan 1985). However, only 60% of the dimers induced by a dose of 42 J/m^2 were excised in the same period. This percentage could not be increased by further incubation, although the remaining dimers could still be photoreactivated (Howland 1975; Howland and Hart 1977). Excision was lacking at doses above 100 J/m^2. In contrasting studies made in alfalfa (Quaite et al. 1994), and in soybean (Sutherland 1996), it was observed that excision repair was undetectable at lower initial damage levels, while easily detectable at higher doses. This contrast may be due to the differences in repair capability among plants and may also depend on different experimental setups under which the observations were made.

In *A. thaliana*, photorepair of cyclobutane dimers predominates at all damage levels (Pang and Hays 1991; Sutherland 1996). At higher damage levels cyclobutane dimers are repaired by both excision repair and photoreactivation (Quaite et al. 1994; Sutherland et al. 1996). The [6-4] photoproduct can be repaired by dark repair as well as a light dependent pathway (Chen et al. 1994). In contrast to the cyclobutane dimer specific photolyase, whose activity needs prior induction by visible light, [6-4] photoproduct specific activity is constitutively expressed.

Our understanding of the DNA repair in higher plants is limited. A detailed biochemical characterization involving the isolation of the genes and encoded proteins controlling the repair pathways is essential. Several plant genes have been isolated by functional complementation of DNA repair deficiency in *E. coli*. Pang et al. (1992, 1993) have isolated

several *A. thaliana* clones which are able to complement the *E. coli* mutant strains *recA*⁻, *uvr*⁻, *phr*⁻ (genes essential for recombination, excision repair and photoreactivation respectively) or *ruvC*⁻, and *recG*⁻ (whose products are needed for DNA recombination). Cerutti et al. (1992) have isolated a clone that has significant similarity to the *E. coli recA* gene. Along with isolation and biochemical characterization of genes another approach taken to initiate characterization of repair systems is the isolation and characterization of repair defective mutants. In this regard, the plant which is highly rated and used for classical and molecular genetic studies is the angiosperm, *A. thaliana* (discussed in section 1.5).

1.4.5 Radiation sensitive *A. thaliana* mutants isolated so far

Working with *A. thaliana*, Britt et al. (1993) identified a UV-hypersensitive mutant, *uvr*1. *uvr*1 was isolated by a procedure which screened for mutants on the basis of hypersensitivity of root tissue to UV (Britt et al. 1993). Harlow et al. (1994) isolated *uvh* (*UV-hypersenitive*)-1, by screening for *A. thaliana* mutants whose leaves were hypersensitive to UV. The two screening procedures can potentially produce different types of mutants because recombinational repair occurs predominantly in dividing (meristematic) cells of the root. Mature, quiescent cells of the leaves on the other hand can rely on damage tolerance mechanisms along with repair processes. As both, *uvr*1 and *uvh*1 show sensitivity of roots as well as leaves to UV, both mutations affect processes occurring in both dividing and quiescent cells (Chasan 1994). Upon biochemical characterization, *uvr*1 was found to be defective in dark repair of [6-4] photoproducts. The *uvh*1 mutant is yet to be characterized biochemically.

Davies et al. (1994) have isolated 12 pure lines of *A. thaliana* hypersensitive to γ-radiation. These mutants were not sensitive to UV. Genetic complementation of five of those mutants confirmed the involvement of five genes, named *RAD*1-*RAD*5. In contrast to UV, ionizing radiation is highly penetrating and causes DNA damage by double-strand breaks (Hall et al. 1992), base damage, and single-strand breaks (Dizdaroglu and Bergtold 1986; McLennan 1988). Mutants sensitive to ionizing radiation and not to UV are probably defective in recombinational repair. Mutants showing sensitivity to both types of radiation may be defective in a process that confers resistance to damage caused by both UV and ionizing radiation.

Jenkins et al. (1995) isolated five *A. thaliana* mutants on the basis of leaf hypersensitivity to UV radiation. Each mutant was assigned to a separate complementation group, based on the results of complementation analyses and also on the basis of their characteristic UV and ionizing radiation sensitivity. The mutants were named *uvh*1 and *uvh*3-6. Possible defects were suggested based on their tissue specificity and sensitivity of the mutants to UV and/or ionizing radiation (Jenkins et al. 1995).

1.5 *A. thaliana* as an experimental organism

A. thaliana (Meyerowitz 1987, 1989), a small flowering plant, is widely used as a model organism for molecular genetic studies. It belongs to the family *Brassicaceae* (mustard family). At maturity, the plant consists of a rosette of small leaves, with the main stem topped by raceme type inflorescence (youngest flowers at the top with successively older flowers below). It can grow to a maximum height of 30-40 cm. *A. thaliana* flowers

(description in section 1.5.1) normally self-fertilize with less than 0.1% chances of cross-fertilization. Flowers can also be cross-fertilized when required. After fertilization, the ovary elongates and gives rise to the fruit, called silique. Each silique contains 30-60 seeds in the mature stage. Each seed weighs less than 20 µg. It is possible to collect over 10,000 seeds from a healthy plant. With minimal loss of the ability to germinate, seeds can be stored under dry conditions for years.

The average generation time of this diploid plant (2n=10) is 6 weeks. The rate of development, and consequently the generation time, is influenced by various factors, including nutrition (for example, poor nutrition leads to rapid flowering and a very short generation time with low seed count), temperature (optimum 22-25°C), day length (*A. thaliana* flowers rapidly in long days or continuous light while flowering time is comparatively delayed under short days), and genetic makeup of an individual plant.

1.5.1 Flowering and pollination in *A. thaliana*

A. thaliana is self-compatible member of the family *Brassicaceae*. Mature flowers are approximately 3 mm long and 1 mm wide. The floral organ arrangement is typical of members of the mustard family consisting of four sepals surrounding and alternating with four petals. The color of *A. thaliana* petals is white. Inside the whorl of petals are six stamens, two short and four long, each consisting of a filament capped by a pollen bearing anther. In the floral center is the pistil with two carpels. Ovules are borne along the ovary walls.

The process of pollination involves a series of interactions between two distinct individuals: the male gametophyte, or pollen grain, and the female sporophyte. These interactions take place at many different levels during the pollination process. The pollen grain and the stigma undergo an initial recognition process that allows the pollen to hydrate on the stigma of the plants of the same (or closely related) species. The subsequent growth of the pollen tube through the transmitting tissue of the style must also involve cell-cell communication. If the pollen grain is from an incompatible pollen parent, it will fail to germinate or its tube will be unable to enter the walls of stigmatic papillar cells. The stigma is receptive from 3 days before to 3 days after the flower opens (Mohammad 1935). At anthesis flowers spontaneously self-pollinate, shedding trinucleate pollen from neighbouring stamens onto the receptive stigmatic surface. If pollen contacts any epidermal surface other than that of the stigma, the pollen grains may adhere but hydration and germination rarely occur. Fertilization occurs within about 24 hours after pollination (Akhtar 1932; Khanna and Chowdhury 1974).

1.5.2. *A. thaliana* as a molecular biology tool

The small size of the mature plant (and thus the ability to grow large number of plants in a small space), its short generation time, large seed count, and flexibility of self- and cross-fertilization have made *A. thaliana* a model organism for classical genetic studies (Laibach 1943; Redèi 1975). In addition, *A. thaliana* possesses the smallest haploid plant genome known (70,000 kb, only 15- and 5- fold larger than the *E. coli* and haploid yeast genomes, respectively) (Leutwiler et al. 1984; Pruitt and Meyerowitz 1986). Approximately 25% of

its nuclear genome consists of repeated DNA sequences, which is quite low in comparison with other angiosperms (Meyerowitz and Pruitt 1985). Out of these repeated sequences, 10-15% are highly repeated DNA, 7.5% are tandemly repeated rRNA coding sequences, and 1% consist of dispersed repeats. Due to this low content of dispersed repetitive DNA, the mean length of uninterrupted single copy DNA has been estimated to be about 120 kb. A small genome with none or a negligible amount of dispersed repetitive sequences is highly advantageous for genomic library screening and subsequent analysis of the overlapping clones to define a chromosomal region. Thus, in the case of *A. thaliana*, only 16,000 random *lambda* clones of 20-kb average insert size need to be screened (compared to tobacco, pea and wheat where 370,000; 1,000,000 and 1,400,000 *lambda* clones, respectively, have to be screened for similar purposes) to have a 99% probability of finding a particular genomic fragment.

The above mentioned characteristics of *A. thaliana* make it an uniquely suited model plant for molecular genetic studies unlike other plants, which are characterized by longer generation time and larger genomes with many dispersed repeat elements situated close together.

1.6 Purpose of this study

The aim of this project was to isolate and characterize UV-sensitive mutants of higher plants. The flowering plant *A. thaliana* was used as the experimental organism. UV-sensitive mutants were isolated by screening 49,000 seeds from 4,900 independent T-DNA transformed lines. The choice of T-DNA transformed seeds was based on the rationale that

T-DNA might insert and inactivate the gene of insert, there by producing a "loss of function" phenotype.

Mutants were further classified into two distinct groups based on their UV-sensitivity in the presence of light or in darkness. Each class was further characterized by:

I. determining their response to a series of UV doses.

II. assessing sensitivity to ionizing radiation

III. complementation analysis, where mutants were crossed to each other, and the UV sensitivity of the F_1 progeny was determined.

In the final part of this study, cosegregation analysis was done to determine whether the mutation and the T-DNA were linked together. In cases where linkage was observed, the copy number of T-DNA inserts was determined by Southern hybridization.

2 Materials and Methods

2.1 Experimental organism

A. thaliana (Meyerowitz 1987, 1989), a small flowering plant, was chosen as the experimental organism. General descriptions of the plant, floral characteristics, type of pollination, genomic constitution and the reasons for choosing *A. thaliana* as a molecular genetic tool have been given in the Introduction section of this thesis.

2.2 Ecotypes of *A. thaliana* used in this study

Ecotypes are locally adapted variant of an organism. In this study, the Wassilewskija (Ws) ecotype of *A. thaliana* was mainly used. In comparison to other ecotypes, *Agrobacterium* mediated transformation efficiency is high in Ws. Two other ecotypes have also been referred to and/or used in the course of this study. They are Landsberg *erecta* (Ler) and Columbia (Col), the latter being a derivative of Ler (Table 2.1).

2.3 Strains of *A. thaliana* used in this study

The seed strains used in this study were for most part obtained from the *Arabidopsis* Biological Resource Centre (ABRC) at Ohio State University, Columbus, Ohio, USA. *uvr*1 and its progenitor Landsberg *erecta* wild type strain were generous gifts of Dr. Ann Bagg Britt (U.C. Davis, USA). Complete descriptions of all the seed stocks used are given in Table 2.2.

Table 2.1 Ecotypes of *A. thaliana* used in this study

Ecotype	Source	Original Collector	Origin	Marker	Comments
Columbia	Somerville'85	G.P. Redei	Columbia, Missouri,USA	None	widely used because of high fertility and vigor.
Landsberg *erecta*	Meyerowitz'88	G.P. Redei	Columbia Missouri, USA.	*erecta (er)*	widely used. Mature inflorescence is short with relatively few side branches.
Wassilewskija	Feldmann'89	Langridge & Griffing'59	Russia	None	*Agrobacterium* transformation efficiency is highly rated.

Table 2.2 Seed stocks used or mentioned in this study

ABRC stock#	Allele	Gene name	Background source	Chromosomal map position	Reference
cs-2696 - cs-2654		T-DNA inser- -tion mutants.	Ws		Feldmann (1992)
	*uvh*1	ultraviolet hypersensitive 1	Col	Chromosome 3	Harlow/Mount (1993)
	*uvh*3	ultraviolet hypersensitive 3	Col	Chromosome 3	Harlow/Mount (1993)
	*uvh*6	ultraviolet hypersensitive 6	Col	Chromosome 3	Harlow/Mount (1993)
	*uvr*1	ultraviolet repair defective	Ler	Chromosome 3 position 52.5	Britt (1993)

Note:
Ws= Wassilewskija ecotype. All T-DNA transformed lines used in this study, to isolate UV-sensitive mutants, belong to this ecotype and were generated by Feldmann (1992) using 3850:1003 Ti plasmid (Velten and Schell 1985).
Col= Columbia ecotype. Markers present are *glabra* (*gl*1), the absence of trichomes on leaf and stem surfaces.
Ler= Landsberg *erecta* ecotype. Markers present *erecta* (*er*) (phenotype: compact rosette, short petioles, blunt fruits).

2.4 Growth conditions for *A. thaliana*

Temperature: An optimum temperature of 23°C is recommended, but plants can grow well at room temperature. Plants flower poorly if temperature does not drop to 23°C or below for a few hours. For this study, a temperature of 19-21°C was maintained.

Light: Continuous light, with a minimum of 150-200 microEinsteins/m^2/s PAR (400-700 nm) is recommended. Light is an absolute requirement for seed germination.

Photoperiod: Day/night cycle is not essential for most ecotypes. Continuous illumination is maintained in most growth chambers, as under this condition, most ecotypes of *A. thaliana* grow and mature relatively faster. Plants also grow well in 12 to 16 hours of daylight. In this study, plants were grown under continuous light.

Relative humidity: 50%-70% is recommended. In this study, relative humidity was maintained at a range of 65-85%. >85% relative humidity inhibits seedling and vegetative growth and affects pollination by lowering pollen release from the anther.

Vernalization: Vernalization is the process of induction of flowering by cold treatment. The seeds were vernalized by chilling them at 4°C for 48 hours before transferring to growth chambers. Vernalization also improves the uniformity and vigour of germination.

2.5 Growth chamber

For this study, *A. thaliana* plants were grown either in pots of soil or in petri dishes containing nutrient media. Both plates and pots were incubated in a growth chamber

(Conviron TC30; Controlled Environments Limited, Winnipeg, Manitoba, Canada) maintained at the specific conditions described in Materials and Methods, Section 2.4.

2.6 Seed sterilization

Before planting *A. thaliana* seeds in soil or in nutrient media it is essential to thoroughly surface sterilize the seeds. Seeds were shaken vigorously for ~1 min in microfuge tubes containing 1 ml of a 1.25% (v/v) NaOCl (Bleach) and 0.02% (v/v) TritonX-100 solution. After ensuring proper mixing, the sterilization solution was removed with a sterile pipette. The seeds were then washed 4-5 times with 1 ml of distilled water. After washing, the water was drawn off as much as possible. Following this, the seeds were dried on a sterile filter paper and then transferred to either nutrient medium or soil.

2.7 Sowing seeds in soil

For this study, Terra-lite ready earth soil (Speers Seed Store, Saskatoon, Saskatchewan, Canada) was used. Sterilized soil was transferred to pots, which had holes at the bottom for drainage. The soil was then soaked in water until there was a steady drainage of excess water from the bottom of the pot. The soil surface was smoothened and sterilized seeds were then sprinkled on it. The pots were then covered with a layer of Saran Wrap, to maintain humidity above ambient and to prevent the surface layer of soil from drying out. Pots were then kept on trays and transferred to the growth chamber. The trays were filled with enough water to cover the holes at the bottom of the pots. When the seedlings began to

emerge, i.e. roughly on the seventh day, a few slits were made in the Saran Wrap. This was done to allow the seedlings to adjust slowly to a lower humidity level. By the following day, the Saran Wrap was removed.

2.8 Sowing seeds onto nutrient media

Arabidopsis thaliana (AT) medium.

per liter: 5.0 ml 1M KNO_3
2.5 ml 1M KH_2PO_4
2.0 ml 1M $MgSO_4$
2.0 ml 1M $Ca(NO_3)_2$
2.5 ml 0.02M Fe-EDTA
1.0 ml Micronutrient Mix (Composition per litre):
70 mM H_3BO_3
14 mM $MnCl_2$, $4H_2O$
0.5 mM $CuSO_4$
1 mM $ZnSO_4$, $7H_2O$
0.2 mM $NaMoO_4$, $2H_2O$
10 mM NaCl
0.01 mM $CoCl_2$

The pH of the medium was adjusted to 5.7 using NaOH or HCl. For solid medium, 7 g of agar per litre was added.

For preparing AT medium supplemented with the antibiotic kanamycin (Kan plates), kanamycin (final concentration 50 mg/ml) was added to the autoclaved and cooled (approximately 55°C) medium which was then poured into petri dishes.

Seeds were transferred to nutrient media in a Laminar-flow hood. The seeds were placed on the surface of the agar medium with the help of a sterile 23G needle. The petri dishes,

containing seeds, were stored at 4°C for 48 hours before transferring them to the growth chamber.

2.9 Cross pollinating *A. thaliana*

A. thaliana plants were often cross pollinated using a pollination kit consisting of:

I. A pair of very fine, straight, dissecting scissors (From Boreal; 91-92 Catalogue # 68533-05).

II. A pair of fine point ended forceps (From Bioquip Products, California, USA; Catalogue # 4524).

III. A pair of magnifying viewers; Power 3.5X. (From Thomas Scientific, New Jersey, USA; Catalogue # 6386-F40).

A. thaliana plants are mainly self-pollinating; pollination is complete by the time the flower buds open up. For cross-pollination, the flower to be pollinated was selected at the bud stage (before petals open up). All other flowers near the location of the recipient were removed. Using sterile forceps, all sepals, petals and stamens from the chosen flower were gently removed. At that point, the remaining pistil was ready for pollination.

For a source of pollen (donor), an open flower was chosen which was in the stage of pollination. Gently, the petals and sepals from the donor flower were removed. A stamen which had recently undergone dehiscence of its bi-lobed anther sac to release pollen, was removed from the floral whorl. Carefully holding the filament portion of the stamen with

forceps, the released pollen sticking to the dehisced anther sacs was immediately brushed on to the stigmatic surface of the recipient carpel. Following this, the pollinated pistil was covered with a small piece of paper and kept in that condition for 3 days. Covering of the pistil with paper is done to prevent pollination from an unknown pollen source; because the stigmatic surface of the flower is receptive to pollen from 3 days before to 3 days after opening of the flower (Mohammad, 1935). Thus, protecting the freshly cross pollinated flower avoids undesired pollination by pollen in the atmosphere of the growth chamber. If pollination was successful, a healthy silique, bearing 30-40 seeds developed. Damage to the stigmatic surface while transferring pollen or selecting stamens from old, unhealthy flowers with few or dead pollen, led to abortion.

2.10 T(transfer)-DNA transformed lines

The seeds used in this study for isolating UV-sensitive mutants, carry T(transfer)-DNA insertions. T-DNA is a specific part of the Ti (tumor inducing) plasmid present in *Agrobacterium tumefaciens*. T-DNA is transferred to the plant genome during an infection process (Koukolikova-Nicola et al. 1987; for reviews see Binns and Thomashow 1988; Zambryski 1988). The T-DNA itself does not encode the enzyme responsible for its transposition but virulence (*vir*) genes present on the Ti plasmid outside the T-DNA border regions are responsible for T-DNA transposition. Because the T-DNA does not encode the enzyme responsible for its transposition, hence, unlike transposons once a T-DNA is integrated it does not jump around in the genome. The transformed lines used in this study

were obtained by Dr. Ken Feldmann (1992). These lines were constructed by the cocultivation of *A. thaliana* seeds with *A. tumefaciens* containing the Ti plasmid 3850:1003 (Velten and Schell 1985). Figure 2.1 depicts the seed infection process and the transformation and selection protocol adopted by Feldmann (1992). T-DNA from the Ti plasmid 3850:1003 is a 17 kb artificial construct. It contains the antibiotic resistance marker kanamycin, which allows for screening of T-DNA transformed seeds on kanamycin media, and pBR322 sequences containing the origin of replication, which is helpful in isolation of plant sequences flanking the T-DNA border ends by plasmid rescue. Figure 2.2 shows the map of T-DNA (modified from Behringer and Medford 1992) which was used for transforming the *A. thaliana* seeds used in this study. Only the restriction sites of enzymes relevant to the Southern hybridization performed in this study (details in section 2.20) have been marked in the T-DNA map.

2.10.1 Selection of plants containing T-DNA

T-DNA carries the kanamycin resistance marker. It is a dominant selective marker which is present once with prokaryotic and once with plant regulatory sequences. In order to select for the presence of T-DNA, plants were grown in AT medium supplemented with kanamycin (final concentration 50 mg/ml). All seeds (irrespective of kanamycin sensitivity or resistance) are able to germinate and form the first pair of leaves. By 7-10 days of age, kanamycin resistant (Kan^R) seedlings still remained green, inititated the growth of more

Figure 2.1 Seed infection and transformation protocol (modified from Feldmann 1992)

Seed transformation involved co-cultivation of *A. thaliana* seeds with *A. tumefaciens* carrying a Ti plasmid. The infected T_1 seedlings (where T-DNA can be present in both homozygous and heterozygous condition) were collected and grown to maturity. T_2 seeds collected from T_1 plants were tested on kanamycin media (T-DNA carries a kanamycin resistance marker) to confirm the presence of T-DNA. T_3 seeds were collected from kanamycin resistant (Kan^R) T_2 plants. The T_3 plants were grown to maturity and T_4 seeds were collected. T_4 seeds consist of a population of Kan^R and Kan^S seedlings in the ratio of 3:1 (Kan^R:Kan^S). Among the T_4 progeny, in two-thirds of the cases the kanamycin marker was in the homozygous condition and in one-third of the cases the kanamycin marker was in the heterozygous condition.

In this study, UV-sensitive mutants were isolated from T_4 seeds.

Figure 2.1 Seed infection and transformation protocol (modified from Feldmann 1992).

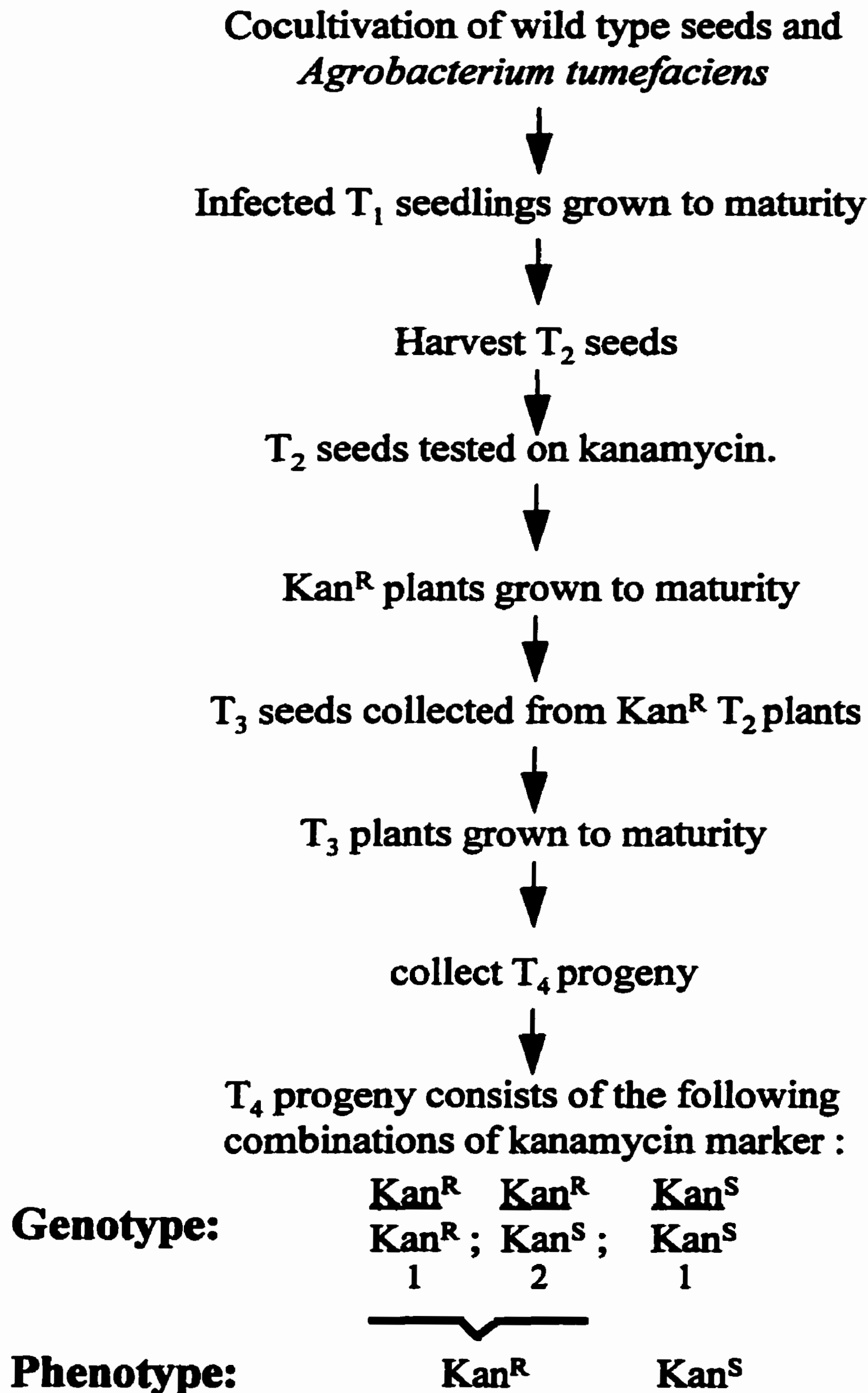


Figure 2.2 Restriction map of 3850:1003 T-DNA (Modified from Behringer and Medford 1992).

T-DNA (used in this study) is a 17 kb construct. It consists of T-DNA left and right border (3.0 and 2.2 kb respectively), two kanamycin resistant markers, Ka1 and Ka2. Ka1 is driven by a plant promoter and Ka2 by a prokaryotic promoter. T-DNA also carries two pBR322 sequences both containing the origin of replication.

The map on the following page shows the cutting sites of the restriction enzymes used for determining the copy number of T-DNA.

LB = 3.0 kb left border

RB = 2.2 kb right border

Ka1 = kanamycin resistance gene driven by plant promoter.

Ka2 = kanamycin resistance gene with prokaryotic promoter.

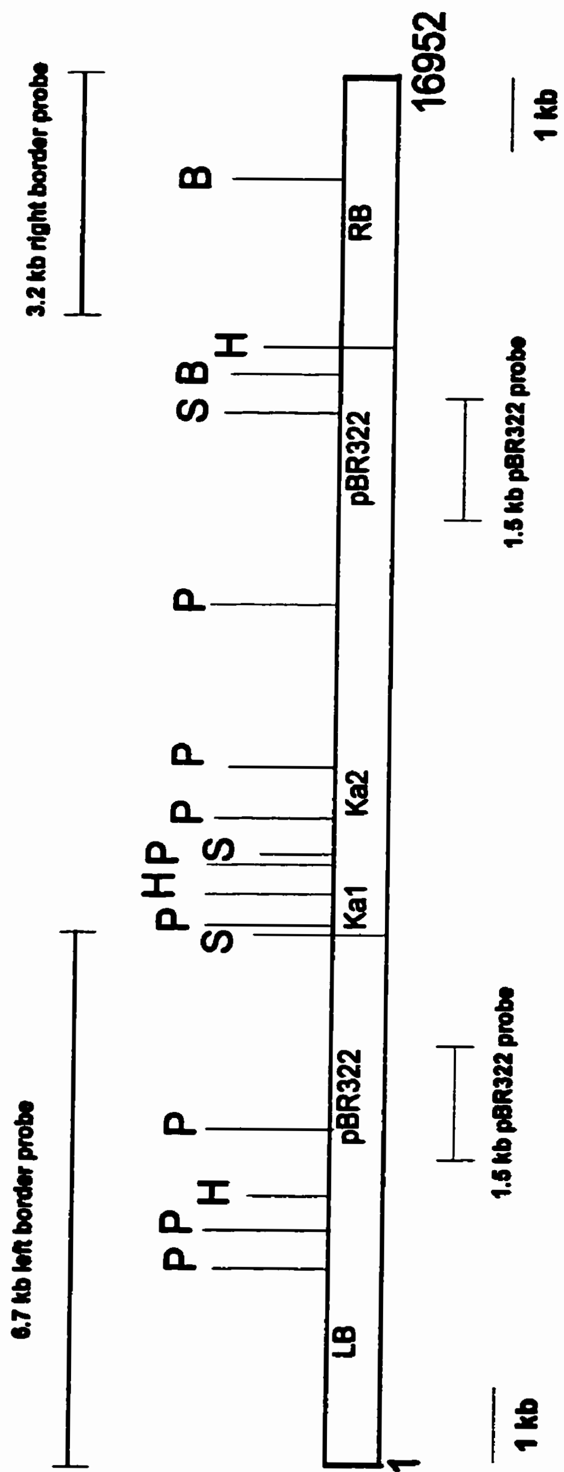
B = *Bam*H1

P = *Pst*1

S = *Sal*1

H = *Hind*III

Probes used for hybridization are marked as 6.7 kb left border probe; 3.2 kb right border probe and 1.5 kb pBR322 probe.



3.2 kb right border probe
6.7 kb left border probe
1.5 kb pBR322 probe
1.5 kb pBR322 probe
16952
1
RB
pBR322
Ka2
Ka1
pBR322
LB
1 kb
1 kb
B
S B H
P
P
P
P H P S
S
P
H
P P

leaves and showed normal root growth. In contrast, the kanamycin sensitive (Kan^S) seedlings began to bleach 3-4 days after germination and were considerably bleached 7-10 days after germination; no more leaves were initiated, the roots failed to elongate and the seedlings eventually died.

2.11 Bacterial strain and plasmids used in this study

E. coli strain JF1754 (*lac gal metB leuB hisB436 hsdR rpsL)* (Pierce et al. 1987), was used to maintain the plasmids pBSH23 and pBSH10 which contain the T-DNA right and left border sequences, respectively. Complete genotypes of the plasmids used are given in Table 2.3.

2.12 UV-Radiation sources

The UV-C source was a Sylvania G30T8 bulb with >98% of its output at 254 nm (manufacturer's specifications) and the incident dose rate was set to 190 $J/m^2/s$ with an Ultraviolet Products UVX-radiometer fitted with a UVX-25 shortrange sensor.

The UV-B source was two 15-Watt midrange bulbs (Ultraviolet Products, San Gabriel, CA) having >95% of their output between 270-400 nm with the peak output at 325 nm (manufacturer's specifications). Filtering through a plastic petri dish lid removed all wavelengths below 285 nm (determined with a Milton Roy (Rochester, NY) 3000 spectrophotometer). Using the spectrophotometric data and the bulb specifications, 31% of the transmitted radiation was calculated to lie between 285-320 nm and 69% between 320-

Table 2.3 Plasmids used in this study

ABRC[1] stock#	**Plasmid**	**Antibiotic resistance marker**	**Vector**	**Inserts**
CD3-12	pBSH23	ampicillin	pBluescript	3.2 kb *Hind*III fragment of T-DNA right border.
CD3-13	pBSH10	ampicillin	pBluescript	6.5 kb *Hind*III fragment of T-DNA left border.

[1] *Arabidopsis* Biological Resource Centre, Ohio State University, Columbus, Ohio, USA.

400 nm. When all wavelengths below 316 nm were eliminated with a Mylar D clear plastic filter (Dupont), the fluence used did not induce mutations in yeast (Armstrong 1993). Thus, for the purpose of this study, the lamp was considered to be primarily a UV-B source. The incident dose rate was set to 500 $J/m^2/s$ with an Ultraviolet Products UVX radiometer fitted with a UVX-31 midrange sensor.

2.12.1 UV irradiation procedures

2.12.1.1 Irradiation at seedling stage (Harlow et al. 1994)

UV-blocking mixture (Harlow et al. 1994)

10% (w/v) bovine serum albumin (BSA)
0.5% (w/v) *para*-aminobenzoic acid (PABA)
BSA and PABA were mixed in water and vortexed vigorously before use

Seedlings were irradiated roughly 15 days after germination (6-8 leaves stage). Just before irradiation, the UV-blocking mixture was applied as a small blob using a 5 cc syringe to the apical meristem of each plant. This was done to protect the apical meristem so that the plants could grow and produce seeds after irradiation. Care was taken so that the blob of UV-blocking mixture did not cover the leaf surfaces. After irradiation, the foam dried and flaked off within one hour, allowing light to reach the protected area.

2.12.1.2 Root bending assay (Britt et al. 1993)

Approximately 22 seeds were placed on a nutrient agar plate (Kranz and Kirchheim 1987). The plates were kept at 4°C for 2 days to synchronize germination and were then

transferred to the growth chamber. In the growth chamber the plates were incubated on edge (vertically). In this position, rather than bending into agar, the roots grew downwards along the surface of agar. After 3 days of growth, the seedlings were exposed to UV light and the agar plates were rotated by 90° and incubated on edge either in complete darkness (to isolate potential dark repair defective mutants) or in light (to isolate light sensitive mutants). Because the plates were rotated, any new growth of the irradiated roots, was at a right angle to the old growth.

2.13 Ionizing irradiation procedure

Seeds were surface sterilized (see section 2.6) and kept overnight in water. It has been demonstrated (Robbelen 1964), that imbibition of *A. thaliana* seeds increases the mutagenic effectiveness of ionizing radiation by 12-fold. The next morning, seeds were dried, plated on agar plates and irradiated with ^{60}Co generated gamma-rays using a Gammacell 220 machine (Atomic Energy of Canada Ltd.) at a dose rate of 284 rad/min. After irradiation the plates were incubated at 4°C for a minimum of 48 hours, to synchronize germination, and then transferred to the growth chamber.

2.14 Plant DNA isolation

DNA extraction buffer: 100 mM Tris-HCL, pH 8.0
50 mM Na_2EDTA, pH 8.0
500 mM NaCl
10 mM β-mercaptoethanol

TE-X buffer: 50 mM Tris-HCl, pH 8.0
10 mM Na_2EDTA, pH 8.0

TE buffer: 10 mM Tris, pH 8.0
1 mM Na_2EDTA, pH 8.0

This plant DNA isolation procedure is a modification of the procedure described by Dellaporta et al. (1983).

Approximately 0.8 g of fresh leaves were quick frozen in liquid nitrogen and ground to a fine powder using a pestle and mortar. Using a spatula pre-cooled in liquid nitrogen, the powdered tissue was transferred quickly (without allowing thawing of the tissues) to a sterile microfuge tube containing 485 µl of extraction buffer plus 35 µl 20% (w/v) SDS. The contents were mixed vigorously using a vortex mixer, for 1 min and incubated at 65°C for 10 min. Further, 161 µl of 5 M potassium acetate was added. The contents were mixed well by shaking and then incubated on ice for 20 min. This was followed by centrifugation of the sample at 12,000 x g for 15 min at 4°C in Brinkman 5415C centrifuge. The supernatant was then transferred to a fresh microfuge tube and then re-centrifuged at 12,000 x g for 5 min at 4°C in Brinkman 5415C centrifuge. This was done to pellet the debris that could not be removed with the first microcentrifugation step. The clear supernatant was then transferred to microfuge tubes containing 325 µl cold isopropanol. The contents were mixed well and incubated at -20°C for 30 min. Following this, the tubes were centrifuged (Brinkman 5415C, 12,000 x g, 1.5 min, 4°C), the supernatant was discarded, and the nucleic acid pellet was dried by aspiration. The nucleic acid pellet was dissolved in 0.7 ml TE-X buffer. 10 µl of 1 mg/ml RNaseA was added and incubated at 37°C for 30 min. Following RNase

treatment, 0.7 ml TE-X saturated phenol was added to the microfuge tubes, the contents were mixed well by shaking vigorously and centrifuged (Brinkman 5415C, 12,000 x g, 2 min, 4°C). The aqueous upper layer was transferred to fresh microfuge tubes. 0.7 ml of TE-X saturated chloroform : isoamyl alcohol (24:1) was added, contents were mixed well by shaking and then centrifuged (Brinkman 5415C, 12,000 x g, 2 min, 4°C). The aqueous upper layer was transferred to fresh microfuge tubes, 75 µl of 3 M sodium acetate and 500 µl of isopropanol were added and mixed well by inverting the tubes repeatedly until swirls disappeared. The tubes were then held at -20°C for 30 min. Following this, the tubes were centrifuged (Brinkman 5415C, 12,000 x g, 3 min, 4°C), the supernatant was aspirated off, and the DNA pellets were dried. Finally the DNA pellet was dissolved in 100 µl TE buffer and stored at -20°C.

2.15 Media recipes

A. LB (Luria-Bertani) Medium, pH 7.0

per litre:
- 10 g Bacto tryptone (Difco)
- 5 g Bacto yeast extract (Difco)
- 10 g NaCl

When required, ampicillin (final concentration: 100 µg/ml) was added after autoclaving and cooling the medium to 45°C. The medium was designated LB+amp.

For solid media, 15 g/l of agar was added to LB medium.

B. 2 x LB

per litre: 20 g Bacto tryptone (Difco)
10 g Bacto yeast extract (Difco)
20 g NaCl

2.16 Plasmid DNA isolation

GTE buffer: 50 mM glucose
25 mM Tris-HCl, pH 8.0
10 mM Na_2EDTA, pH 8.0

NaOH/SDS: 200 mM sodium hydroxide (NaOH)
1% (w/v) sodium dodecyl sulfate (SDS)
Prepared just prior to use.

Ammonium acetate: 6.1 M ammonium acetate dissolved in glacial acetic acid while being heated to 65°C, to achieve a pH of 5.8.

TE buffer: 10 mM Tris-HCl, pH 8.0
1 mM Na_2EDTA, pH 8.0

This rapid alkaline procedure for plasmid DNA isolation is a modification of the procedure by Morelle (1989).

Bacteria were grown overnight (10-12 hrs) in 5 ml LB+amp at 37°C with shaking. The next morning, the cells were collected by centrifugation (1,850 x g, 10 min, room temperature). The cells were resuspended in 500 µl GTE buffer, transferred to a microfuge tube, and pelleted by centrifugation (Brinkman 5414C, room temperature, 30 s). The pellet was resuspended in 190 µl GTE buffer. The tube was incubated at room temperature for 5 min. The tube was then transferred to ice. 400 µl NaOH/SDS was added drop-wise and the

mixture was incubated on ice for 5 min. Ammonium acetate (300 µl) was then added and the mixture was incubated on ice for 10 min. The precipitate was pelleted by centrifugation at 4°C for 15 min and the supernatant was transferred to a fresh microfuge tube. This procedure was repeated twice more. 500 µl isopropanol (room temperature) was added, the contents were mixed by inversion and the tube was kept at room temperature for 20 min. This was followed by centrifugation for 2 min at room temperature. The nucleic acid pellet was washed with ice cold 70% (v/v) ethanol, dried by aspiration and dissolved in 70 µl TE buffer. DNA samples were stored at -20°C.

2.17 Bacterial transformation

Buffer A: 100 mM NaCl
5 mM Tris-HCl, pH 7.5
5 mM $MgCl_2$

Buffer B: 100 mM $CaCl_2$
5 mM Tris-HCl, pH 7.5
5 mM $MgCl_2$

Bacterial cells were transformed using a modification (Pierce et al. 1987) of the calcium chloride procedure of Mandel and Higa (1970).

E. coli strain JF1754 was grown overnight in 5 ml LB. The cells were then diluted 1:100 in the same medium (40 ml of medium for every 10 transformations), grown for 1 h 45 min at 37°C with shaking and chilled on ice for 10 min. The culture was centrifuged (3,020 x g, 10 min, 4°C) to pellet the cells and for every 40 ml of culture, the pellet was washed and

resuspended in 10 ml ice-cold buffer A and then chilled on ice for 20 min. The cells were collected by centrifugation (3,020 x g, 10 min, 4°C) and, (for every 40 ml of original culture), 10 ml of Buffer B was used to resuspend the pelleted cells. The cells were kept on ice for 1 h, pelleted by centrifugation (3,020 x g, 10 min, 4°C) and resuspended in 2 ml of Buffer B. For each transformation, a 200 µl aliquot of the cell suspension was transferred to a sterile microfuge tube.

DNA used to transform the *E. coli* cells was added to the microfuge tube containing the transformation competent cells. Along with positive and negative controls, these microfuge tubes, containing the transformed cells were incubated on ice for 1 h. The tubes were heat-shocked by keeping them at 42°C for 2 min and then immediately chilling them by transferring them to ice for 2 min. 2x LB (200 µl) was added to each tube and the tubes were then incubated at 37°C for 1 h. The cell suspensions were plated on LB+amp medium and incubated overnight at 37°C.

2.18 Agarose gel electrophoresis

Enzyme digest: 1x restriction enzyme buffer (10x buffer supplied with enzyme by BRL)
1 µg DNA
10 units of enzyme per µg of DNA.

Stop Buffer: 50 mM Na_2EDTA
50% (w/v) sucrose
0.1% (w/v) bromophenol blue
Stored at 4°C.

λ DNA: 1x buffer 3 (10x buffer supplied with BRL)
50 µg DNA

200 units *Hind*III

The enzyme digest was incubated for 2.0 h at 37°C and the reaction was terminated by adding stop buffer (one-fourth of the reaction volume). The DNA was stored at 4°C and heated at 65°C for 5 min before use

Loening's buffer: 400 mM Tris base
200 mM sodium acetate
10 mM Na_2EDTA

Adjusted to pH 8.0 with glacial acetic acid.
Stored at room temperature.

Plasmid DNA was digested with the appropriate enzyme for 2.0 h at 37°C. Stop buffer (1/4 of the reaction volume) was added to terminate the reaction and the resulting DNA fragments were separated by agarose gel electrophoresis (0.7% w/v agarose (BRL) dissolved in Loening's buffer), for 18 hrs at 1 volt/cm. A 1 kb DNA ladder accompanied the DNA samples each time. The gel was stained with ethidium bromide (final concentration: 10 mg/ml) and destained for 10 min in distilled water.

2.19 Isolation of DNA fragments

TE buffer: 10 mM Tris-HCl, pH 7.4
1 mM Na_2EDTA, pH 7.4

Silanized glass-wool: Glass-wool was silanized with dimethyldichlorosilane in a 500 ml glass beaker by shaking a few times and rinsing thoroughly with sterile double-distilled H_20. The silanized glass-wool was stored at room temperature in sterile double-distilled H_2O.

Ammonium acetate: 10 M ammonium acetate was dissolved in glacial acetic acid while being heated to 65°C to achieve a pH of 7.8.

DNA fragments were isolated from agarose gels by a silanized glass-wool method (Heery et al. 1990).

The caps of two 1.5 ml microfuge tubes were cut off. A small hole was made at the base of a 0.6 ml microfuge tube with a syringe needle. A small amount of silanized glass wool was cut and packed in the bottom of the 0.6 ml tube (3-4 mm in depth). The 0.6 ml tubes were placed inside the 1.5 ml tubes. The 1.5 ml tubes were then centrifuged (Brinkman 5415C, 12,000 x g, 20 min, room temperature) to remove any excess dimethyldichloro-silane from the glass wool. After centrifugation, the 1.5 ml outer tube was discarded and replaced with a fresh tube. The band containing the DNA fragment was excised from the agarose gel and trimmed. The agarose slice was placed in the 0.6 ml tube, which was placed inside 1.5 ml tube, and the tubes were centrifuged (Brinkman, 12,000 x g, 20 min, room temperature) until all the liquid had been forced out of agarose gel slice. After centrifugation, the 0.6 ml tube was discarded and the supernatant in the 1.5 ml outer tube was transferred to a fresh 1.5 ml tube without transferring any of the pellet. One-third the volume of ammonium acetate and an equal volume of isopropanol was added to the eluate. The contents of the tube were mixed well by inversion and the tube was incubated at room temperature for 10 min. After incubation, the nucleic acids were pelleted by centrifugation (Brinkman 5415, 12000 x g, 15 min, room temperature), washed with 0.5 ml ice-cold 70% (v/v) ethanol, dried by aspiration, dissolved in 10-20 µl TE buffer, and then stored at -20°C.

2.20 Southern hybridization

2.20.1 Preparation of probes

The probes used for determining the copy number of T-DNA inserts within the *A. thaliana* genome were made of segments from T-DNA right and left border ends and the pBR322 origin of replication sequence which is also present in T-DNA (see map of T-DNA, Figure 2.2). Plasmids containing the T-DNA border ends were received from ABRC as DNA samples (CD3-12, CD3-13, details in Table 2.3). The DNA samples were transformed (see section 2.17) into the *E. coli* strain JF1754. From overnight cultures of the transformed *E. coli* JF1754 (with CD3-12 and with CD3-13), plasmids were isolated (see section 2.16). The plasmids (with the 3.2 kb and 6.5 kb *Hin*dIII fragments of T-DNA right and left borders respectively), were then digested with the enzyme *Hin*dIII and the fragments were separated by agarose gel electrophoresis (see section 2.18). From the electrophoresed gel, bands corresponding to the 3.2 kb (right border) fragment and 6.5 kb (left border) fragment were cut out; DNA was isolated from those bands (see section 2.19) and used to generate probes.

For the preparation of the probe containing the bacterial origin of replication, pBR322 (4.3 kb) (New England, Biolabs catalog 1995), was first digested with enzyme *Pvu*II. The linearized plasmid was isolated by gel electrophoresis. The linear product was then digested with *Pst*I and the fragments were separated by gel electrophoresis. Of the two fragments (2.8 kb and 1.5 kb) produced, DNA from the 1.5 kb fragment, containing the origin of replication sequence, was isolated and used for probe synthesis.

2.20.2 Capillary blotting

Denaturing solution:	1.5 M NaCl 0.5 M NaOH
Neutralizing solution:	1.5 M NaCl 0.5 M Tris-HCl, pH 7.2 0.001 M Na_2EDTA
20 x SSC:	3 M NaCl 0.3 M Na_3citrate

After electrophoresis, a picture of the gel was taken on Polaroid film using a 302 nm UV transilluminator for later sizing of the bands. The gel was soaked in denaturing solution at room temperature with slow shaking. After 45 min, the denaturing solution was replaced with neutralizing solution. After 45 min, the neutralizing solution was removed. The excess liquid from the gel was blotted off with tissues.

A sheet of Hybond-N (Amersham) membrane was cut to the exact size of the gel. The membrane was wetted in distilled water and then transferred to 2 x SSC. A sheet of 3MM Whatman filter paper was cut to the same width as the gel, but long enough to form a wick between the buffer reservoir and the transfer apparatus. The filter paper was carefully placed onto the gel, so that the gel was centrally positioned on the filter paper. It was ensured that no air bubbles were present between the filter paper and the gel. The gel and the filter paper were inverted and placed on a bridge over the buffer reservoir such that the ends of the wick dipped into the reservoirs. Very carefully, the Hybond membrane was placed onto the gel. It was ensured that no air bubbles were trapped between the gel and the Hybond

transfer membrane. Three to four Whatman filter papers were cut to the same size as the gel and placed over the Hybond membrane. A stack of paper towels which were the same size as the gel was placed on top of the Whatman filter. The stack of paper towels was compressed using a glass plate and a 1-1.5 kg weight. 20 x SSC was added to the buffer reservoir such that the ends of the filter paper wick were immersed in the buffer. Transfer was allowed to proceed for 4-16 hrs. After blotting, the Hybond membrane was carefully removed, washed in 2 x SSC to remove any adhering agarose and allowed to dry. The dried Hybond membrane was wrapped in Saran Wrap and placed, DNA side down, on a standard UV Transilluminator for 2-5 min to cross link the transferred DNA.

2.20.3 Random primer labelling

The Random Primer Buffer Mixture, dNTPs, stop buffer and DNA polymerase were supplied by Gibco-BRL in a Random Primer Labelling Kit.

Random Primer Buffer Mixture: 670 mM HEPES
170 mM Tris, pH 7.2
17 mM $MgCl_2$
33 mM ß-mercaptoethanol
1.3 mg/ml Bovine Serum Albumin
18 OD_{260} units/ml oligodeoxyribonucleotide primers (hexamer fraction), pH 6.8

[^{32}P]dATP: 3000 Ci/mmol α-[^{32}P]dATP (DuPont). Stored at -60°C.

dCTP solution: 0.5 mM dCTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA.

dGTP solution: 0.5 mM dGTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA.

dTTP solution: 0.5 mM dTTP in 3 mM Tris-HCl (pH 7.0), 0.2 mM Na_2EDTA.

Reaction mix: 5 ml Random Primer Buffer Mixture
20 mM dCTP
20 mM dGTP
20 mM dTTP
50 mCi [^{32}P]dATP

Stop solution: 200 mM Na_2EDTA, pH 7.5

DNA polymerase: 6U/µl *E. coli* DNA polymerase I Klenow fragment (BRL). Stored at -20°C.

Yeast tRNA: Yeast tRNA (Boehringer Mannheim) was dissolved in double distilled H_2O (20 mg/ml) and stored at -20°C.

Sodium acetate: 3M sodium acetate was dissolved in glacial acetic acid being heated to 65°C to achieve a pH of 4.8.

[^{32}P]-labelled probes for hybridization were prepared using the BRL Random Primer DNA Labelling Kit. DNA (25 ng), dissolved in 5-20 µl of distilled water in a sterile screw cap microfuge tube, was denatured by heating in boiling water for 5 min and then immediately cooled by keeping on ice. The reaction mixture was added to the tube on ice and the final volume of 49 µl was made by adding sterile double-distilled H_2O. Next, DNA polymerase (1 µl) was added and the tube was incubated at 25°C for 2 hrs. The reaction was stopped by adding 5 µl stop buffer. Successively, 5 µl yeast tRNA, 7 µl $MgCl_2$ (100 mM), 7 µl sodium acetate and 200 µl ice-cold ethanol (95%) was added. The tube was kept at -60°C for 1 hr and the precipitate was pelleted by centrifugation (Brinkman 5415C, 12,000 x

g, 15 min, 4°C). The supernatant was removed with a Pasteur pipette. The pellet was washed with 1 ml ice-cold ethanol (70%), dried and dissolved in 100 μl sterile double-distilled H_2O. The tube was heated at 100°C for 5 min and then rapidly transferred to ice-water.

2.20.4 Hybridization procedure

20x SSC buffer: see section 2.20.2

Salmon sperm DNA: Salmon sperm DNA (Sigma) was dissolved in sterile double-distilled H_2O (10 mg/ml) and then passed through a needle (22 gauge) several times to shear the DNA. Aliquots were stored at -20°C.

50x Denhardt's solution: 10 mg/ml Ficoll
10 mg/ml polyvinylpyrolidone
10 mg/ml Bovine Serum Albumen (BRL)
Stored at -20°C.

Prehybridization solution: 6x SSC
5x Denhardt's solution
0.5% (w/v) SDS
0.2 mg/ml salmon sperm DNA
Prepared just before use and heated to 65°C.

Hybridization solution: 10 mM Na_2EDTA
6x SSC
5x Denhardt's solution
0.5% (w/v) SDS
Prepared just before use and heated to 65°C.

Solution I: 2x SSC
0.5% (w/v) SDS

Solution II: 2x SSC
0.1% (w/v) SDS

Solution III: 0.1x SSC
0.5% (w/v) SDS

DNA hybridization was carried out as described by Maniatis et al. (1989).

The DNA immobilized onto the Hybond membrane (see section 2.20.2 for capillary blotting), was rolled into a tight spiral and placed in a 35 mm x 150 mm glass hybridization bottle (BIO/CAN Scientific). Prehybridization solution (20 ml) was added and the bottle was sealed and incubated for 10 min at 65°C in a BIO/CAN Scientific Turbo Speed Rotary Hybridization Oven. After 10 min, the cap of the bottle was opened to release the pressure build-up, refastened, and was further incubated for 2-4 h, at 65°C, in the oven. The prehybridization solution was removed and replaced with 20 ml of hybridization solution, the labelled DNA probe was added, the bottle was sealed and incubated overnight at 65°C. The Hybond membrane was removed carefully from the bottle and the following washes were performed: **a.** 20 ml of solution I for 5 min at room temperature (twice); **b.** 20 ml of solution II for 15 min at room temperature (twice); **c.** 20 ml of solution III for 2 h, 1 h and 0.5 h (0.5 h, was done twice) at 65°C. The membrane was then placed on 3 MM Whatman Chromatography paper, air-dried, covered with a single layer of Saran Wrap and exposed to Kodak XAR-5 film with an intensifying screen at -60°C (the exposure time varied according to the radioactive intensity of the membrane).

2.21 Statistical analysis

Chi-square (X^2) test, employing Yates' correction for continuity, was used to evaluate differences in a variety of parameters (Strickberger 1976).

X^2 result was calculated based on the following formula:

$$\frac{[\,|\text{Observed} - \text{Expected number}| - 1/2]^2}{\text{Expected number}}$$

The reduction of ½ from the absolute value of |observed - expected| number is known as the *Yates correction term* and adds to the accuracy of the chi-square determinations.

2.21.1 Calculation of genetic ratios

Genetic ratios were calculated based on the Mendelian laws of segregation and independent assortment. To determine the probability of finding each progeny class resulting from a cross, a device of a checkerboard (Punnett square) was used. The gametes of each parent were placed on one side of the checkerboard based on their probability of being formed. If two gametes resulting from a parent are formed with equal probability, then each is formed with a probability of one-half. Each box of the checkerboard represents a zygote which is formed by multiplying the frequency of two gametes.

2.21.2 Level of significance

Genetic ratios calculated from the probability values of the gametic constitution of each parent is hypothetical or expected. Actual observed ratios depart to a greater or lesser extent from the expected values. The size of the discrepancy between the expected and observed

values determines whether a hypothesis should be rejected or accepted. In order to accept a hypothesis, the discrepancy between the expected and observed values should be small (or "not significant"); to reject a hypothesis, the discrepancy should be large (or "significant").

In this study, a 5% level of significance was used to accept or reject a hypothesis.

3 Results

3.1 Isolation of UV-sensitive mutants of *A. thaliana*

The purpose of this project was to isolate and characterize UV-hypersensitive and/or DNA repair defective mutants from the angiosperm *A. thaliana*. Approximately, 49,000 *A. thaliana* seeds derived from 4,900 independent T-DNA transformed lines were used for mutant screening (Figure 2.1 depicts the seed infection and transformation protocol). Mutants were isolated based on the hypersensitivity of their roots to UV.

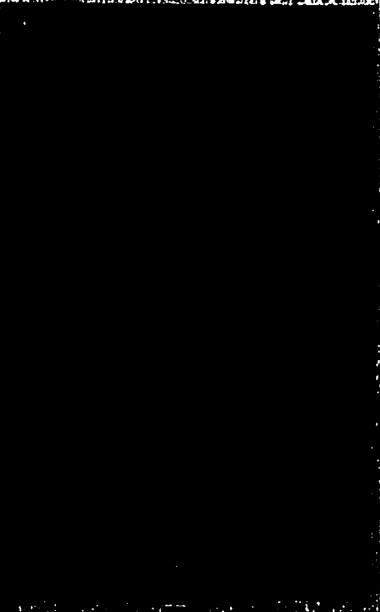
3.1.1 Isolation procedure

The isolation procedure involved a root bending assay (Britt et al. 1993) (described in Materials and Methods, section 2.12.1). In this assay, seeds were sown on nutrient plates and incubated on edge (vertically) to facilitate the downward growth of roots along the surface of agar. After 3 days of growth, the seedlings were irradiated. Following irradiation, the Petri plates were rotated by 90° and incubated in the growth chamber for an additional 3 days. Because the plates were rotated, any new growth of root occurred at right angles to the old growth. Three different responses were observed in the root bending assay: 1. A perfect right angle was formed, as growth after irradiation was not affected by UV (i.e. wild type condition); 2. Right angle was imperfect, as after irradiation the root grew but was slow in comparison to the unirradiated control (i.e. mutant showing intermediate hypersensitivity to UV); 3. No right angle was formed, as there was no growth of root after irradiation (i.e. mutant showing extreme hypersensitivity to UV) (Figure 3.1).

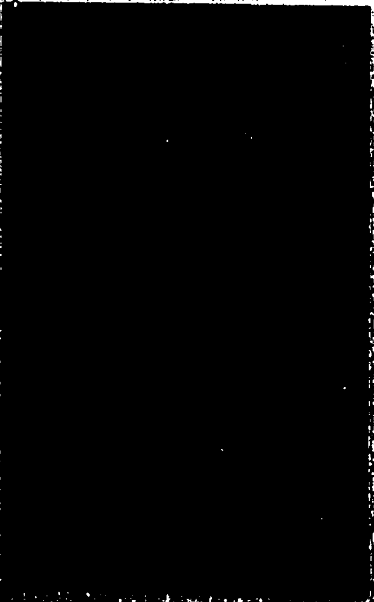
Figure 3.1 Root bending assay (Britt et al. 1993). Approximately 40 seeds were sown on nutrient plates. The plates were incubated at 4°C for 2 days to synchronize germination. Following this, the plates were transferred to growth chamber and incubated vertically. In that condition the roots grew along the surface of agar. After 3 days of growth, the plates were irradiated with UV. After irradiation, the plates were turned by 90° and incubated either in dark (to isolate mutants potentially defective in dark repair) or in light (to isolate mutants potentially defective in photoreactivation or UV-protective mechanism.

Three different responses were observed. The roots grew normally and a perfect right angle was formed by the root growth before and after irradiation (as shown in A); roots were moderately sensitive to UV and an imperfect right angle was formed as root growth was slow in comparison to wild type (shown in B), no right angle formed as there was no root growth (as shown in C).

A



B



C

3.1.2 Determination of an appropriate UV-C dose for mutant screening

As mentioned previously (see Introduction, section 1.6), the purpose of this study was to isolate and characterize UV-sensitive mutant plants which are potentially defective either in photoreactivation or dark repair mechanisms (which includes excision repair as well as DNA damage tolerance mechanisms). In light, the process of photoreactivation, which requires visible light, and dark repair which is independent of light, can both operate to repair UV-induced damage to DNA. In absence of visible light only the dark repair machinery is functional. Thus, a higher UV dose is required to cause damage in cases where the plants are grown in light as opposed to plants grown in non-photoreactivating conditions.

UV-C radiation was used to isolate the UV-sensitive mutants. The choice of UV-C was based on the rationale that, i. DNA has a strong absorption maximum in the UV-C range (260 nm), and ii. the highly energetic UV-C photons can cause high levels of DNA damage at a very short time (section 1.2.1.1). In order to determine an appropriate UV-C dose for isolating UV-sensitive mutant seedlings, root tests were done with the Ws wild type seeds which had been irradiated with a series of UV-C doses. After irradiation, the plates were rotated by 90° and incubated either in complete darkness (to determine the dose at which the damage caused by UV radiation exceeds the "dark repair" capacity of roots), or in light (to determine the dose at which the damage caused by UV radiation surpasses the capacity of photoreactivation or other UV-protective mechanisms of the roots). The roots were irradiated at a dose rate of 190 $J/m^2/s$. It was observed that, in the dark, wild type roots grew normally until a final dose of almost 500 J/m^2. Root growth

slowed down at doses ~500 J/m^2 and eventually, at a dose of 2000 J/m^2, root growth ceased. Based on these results, the dose for screening mutants potentially defective in dark repair was set at 750 J/m^2, which was less than one-half the dose at which the wild type was affected. In light, root growth of wild type was unaffected until 2000 J/m^2. At doses above 2000 J/m^2, the root growth gradually slowed down and the growth completely stopped at a dose of 3400 J/m^2. The dose for screening mutants was set at 1000 J/m^2.

3.1.3 Isolation of T-DNA induced UV-sensitive mutants

In addition to isolation and characterization of UV-sensitive and/or DNA repair defective mutants, the aim of this project was also to isolate mutants where the UV-sensitive mutation was potentially the result of T-DNA insertion(s) and thus, the mutation will be co-segregating with the T-DNA insert. Information gained from such linkage studies would be instrumental in isolating plant sequences flanking the T-DNA and eventually provide a clue regarding the gene(s) affecting UV-sensitivity. Therefore, in the first step of the screening procedure, all 49,000 seeds from 4,900 independent transformed lines were screened on nutrient plates supplemented with kanamycin (procedure for screening plants with T-DNA is described in section 2.10.1), a dominant antibiotic marker present in the right border region of T-DNA (Figure 2.2). The kanamycin resistant (Kan^R) plants (considered in this study, as the primary mutagenised or M_1 population) thus isolated, were allowed to grow and produce seeds. The principle objective of this selection was to limit the population of seeds to those of interest. Thus,

no account of the total number of kanamycin resistant and kanamycin sensitive seedlings was noted at this point. The M_2 seeds were collected and subjected to the root bending assay (Britt et al. 1993), once in complete darkness (to isolate mutants defective in dark repair), and once in visible light (to isolate mutants defective in photoreactivation and/or UV-protective mechanisms). However, potential mutants thus isolated could be either a protection mutant with a defect in protective pigment synthesis or its induction by UV radiation or, a repair mutant with defects in repairing UV-induced DNA damage, with either a defect in photoreactivation or a defect in dark repair (Chasan 1994).

After screening the M_2 Kan^R progeny (where the T-DNA can be present in either homozygous or heterozygous condition) (for seed transformation protocol, see Figure 2.1), 553 putative mutant plants, showing UV-sensitive roots were isolated. The plants were allowed to grow and self-pollinate. M_3 seeds were collected. Out of those 553 potential mutants, roots of only 55 mutants met the criteria of UV-sensitive phenotype in the next generation. Out of these, 44 mutants were potentially defective in dark repair and the remaining 11 mutants were sensitive in white light.

3.1.4 Nomenclature of UV-sensitive *A. thaliana* mutants isolated in this study

Working with *A. thaliana*, ecotype Landsberg *erecta*, Britt et al. (1993) have isolated a UV-sensitive mutant, *uvr*1. Six more radiation sensitive mutants have been isolated from the Colombia ecotype by Harlow et al. (1994) and Jenkins et al. (1995). These mutants have been referred to as *uvh*1-6. The *A. thaliana* seeds used in this study belong to the transformation efficient Ws ecotype. The 11 UV-sensitive mutants with UV-sensitive

roots in light have been named *uvs*1-11. The 44 UV-sensitive mutants with UV-sensitive roots in dark have been named *uvs*12-55.

3.2. Characterization of UV-sensitive mutants

UV-sensitive mutants isolated from the T-DNA transformed lines (section 3.1) were characterized by: i. classifying the mutants based on the response of root growth to a series of UV-C doses, ii. analyzing the segregation pattern of the responsible mutated gene, iii. determining the total number of complementation groups involved for each of the two mutant categories (light sensitive and dark sensitive), iv. studying the response of leaf tissue to UV-C and UV-B radiation, v. assessing the sensitivity of the mutants to ionizing radiation, and vi. determining if the UV-sensitivity was possibly due to T-DNA insertion and finding the copy number of T-DNA in those cases.

3.2.1 Classification of *uvs* mutants based on sensitivity of roots to UV

The *uvs* mutants were isolated using two appropriate doses of UV-C (section 3.1.2); one for isolating the potential photoreactivation mutants (1000 J/m^2) and one for isolating the potential dark repair defective mutants (750 J/m^2). In order to compare the UV-sensitivity pattern of the *uvs* mutants, the mutants were subjected to the root bending assay using series of UV-C doses. For each mutant, approximately 40 roots were used for each dose. The individual root growth after irradiation for each seedling, if any, was measured (in cm) and added up. Then the average root growth was calculated. For a particular dose, the average root growth in the irradiated plate was divided by the average

root growth in an unirradiated (control) plate to give an estimation of the percentage of root growth after irradiation. To allow a comparison of UV-sensitivity of *uvs* mutants with that of the already isolated mutant *uvr*1 (Britt et al. 1993) both *uvr*1 and its progenitor strain Landsberg *erecta* were also included in the root test.

In presence of light, root growth of both *uvr*1 (Britt et al. 1993) and its progenitor strain Landsberg *erecta* were more sensitive than the root growth of Ws wild type. Both *uvr*1 and Landsberg are homozygous for the mutation *tt*5 (transparent testa), which is defective in UV absorptive flavonoid pigment production (Koornneff 1990). Thus, increased penetration of UV light in *tt*5 strains may account for their inherent increased UV-sensitivity in light. The roots of potential photoreactivation defective *uvs* mutants (*uvs*1-11) were sensitive at different doses. Table 3.1 shows the percentage of roots which continued growing after UV-irradiation. Figure 3.2 is a graphical representation of root growth of Ws wild type, Landsberg *erecta* and *uvr*1, based on the values of Table 3.1. For the *uvs* mutants sensitive in light (*uvs*1-11), root growth stopped at a series of UV-C doses (data shown in Table 3.1). The most sensitive mutant was *uvs*7, which stopped growing at a UV-C dose of 1000 J/m^2. Roots of *uvs*3 and *uvs*8 were the most resistant and they were still able to grow at a dose of 3000 J/m^2. Thus, unlike the potential dark repair defective mutants (*uvs*12-55) (as mentioned in the next paragraph), the mutants sensitive in light (*uvs*1-11) (whose roots were not sensitive in dark) could not be classified (based on 100% inhibition of root growth) into different sub-classes.

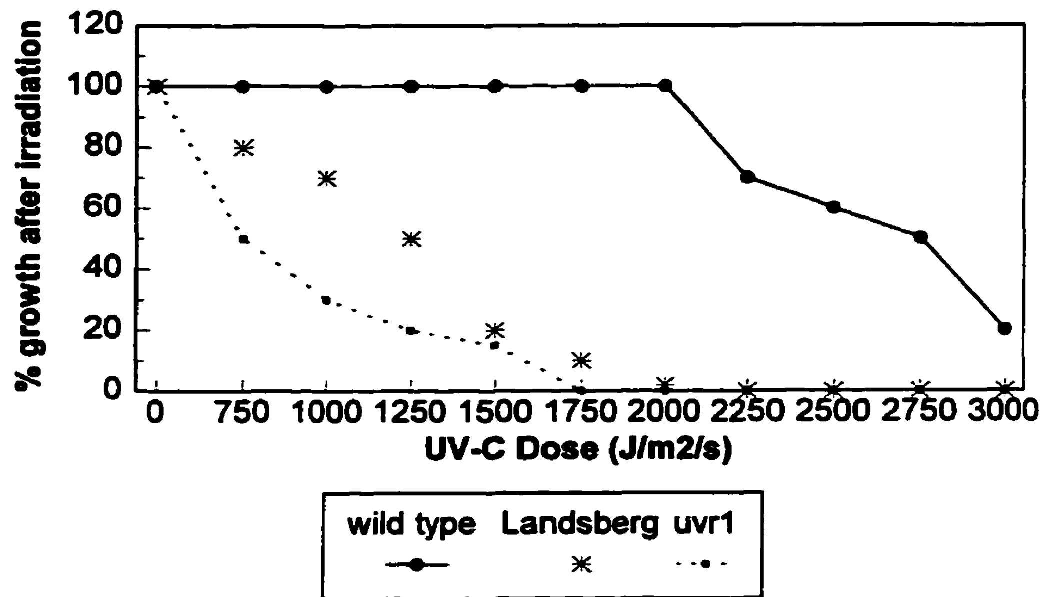
In absence of photoreactivation, wild type strains of both Ws and Landsberg *erecta* ecotypes were observed to have similar pattern of sensitivity to UV-C and both strains

Table 3.1. Sensitivity of (potential) photoreactivation defective *uvs* mutant roots to UV-C radiation

	UV-C doses in J/m²								
	0	750	1000	1200	1600	1800	2000	2500	3000
Wild type	100±0	98.66±0.66	99.33±0.33	99.66±0.33	96.33±1.76	95±2.51	99±0.57	48.33±1.76	18.33±0.88
Landsberg	100±0	77.33±1.45	70±0.57	48.66±0.88	20±0.57	10.33±1.45	2±0.57	0.33±0.33	0
*uvr*1	100±0	48.66±1.20	30±0.57	18.33±0.88	13.33±1.20	0.5±0.28	0	0	0
*uvs*1	100±0	46±0.57	40±0.57	36.66±0.88	27±1.15	16±0.57	2.5±0.28	1.16±0.16	0
*uvs*2	100±0	97.33±0.88	98±1	27±0.57	0	0	0	0	0
*uvs*3	98±0.33	95±1.45	97±0.66	96.66±0.88	94.66±0.88	98±0.57	14.66±1.20	8.33±0.66	3.66±0.33
*uvs*4	97.66±0.33	33±0.57	35±0.57	25±1.52	11±0.57	5.66±0.88	0	0	0
*uvs*5	99±0.57	99.33±0.66	30±0.57	13±0.57	0	0	0	0	0
*uvs*6	99±0.57	99.33±0.33	35±0.57	22±0.57	11±0.57	1.83±0.16	0	0	0
*uvs*7	99.66±0.33	55±0.57	0	0	0	0	0	0	0
*uvs*8	99.33±0.33	98.66±0.38	96.33±1.06	96.33±0.33	59±0.57	31±0.57	17±0.57	12±0.57	10.33±0.33
*uvs*9	99±0.33	99.33±0.66	53±0.57	22±0.57	20±0.57	0	0	0	0
*uvs*10	98±0.33	99.33±0.33	99.66±0.33	96.33±1.76	26±0.57	12±0.57	0	0	0
*uvs*11	98.66±0.38	96.33±1.06	21±0.57	10±0.57	0	0	0	0	0

Root growth was measured using the root bending assay (Britt et al. 1993) described in section 3.1.1. For each dose tested response of approximately 40 roots was analyzed. Values are percentages of irradiated roots that continued growing as indicated by root bending. The mean and the standard error are shown. (n=3)

Figure 3.2 Comparison of root growth in Ws wild type, Landsberg *erecta* wild type and *uvr1* (Britt et al. 1993) in relationship to increasing UV-C doses and in presence of photoreactivation.



Ws, Landsberg *erecta* and *uvr1* seeds were subjected to the root bending assay (in light) using a series of UV-C doses. New root growth (which grew at right angles to the root growth before irradiation) was measured 3 days after irradiation at the indicated UV-C doses. The results indicate an average of measurements made on between 35-40 seedlings.

stopped growing at 2000 J/m^2. The root growth of *uvr*1 (Britt et al. 1993) was completely inhibited at 300 J/m^2. The potential dark repair defective *uvs* mutants (*uvs*12-55) were sensitive at different doses. Root growth at each dose was more sensitive to UV-C than observed for the Ws wild type seedlings. Table 3.2 shows the percentage of roots which continued growing after UV-irradiation. Figure 3.3 is a graphical representation of the root growth of Ws wild type, Landsberg *erecta* and *uvr*1 based on those values. Based on 100% inhibition of root growth (according to values shown in Table 3.2), the potential dark repair defective *uvs* mutants (whose roots were not sensitive in light) fell into three distinct classes:

(a) mutants where roots stopped growing at ~ 600 J/m^2 UV-C (which includes, *uvs*12-13).

(b) mutants where roots stopped growing at ~ 800 J/m^2 UV-C (which includes, *uvs*14-30).

(c) mutants where roots stopped growing at ~ 1,200 J/m^2 UV-C (which inciudes, *uvs*31-55).

3.2.1.1 Sensitivity levels based on 50% inhibition of root growth

The *uvs* mutants thus isolated and classified, were characterized based on 50% inhibition of root growth. This characterization was based on the response of root growth to a series of UV-C doses. On that basis, the mutants were characterized as extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-). Table 3.3 and 3.4 show the resistance levels of *uvs* mutants sensitive in light and in dark,

Table 3.2 Sensitivity of (potential) dark repair defective *uvs* mutant roots to UV-C radiation

	UV-C doses in J/m²								
	0	300	500	600	700	800	1,000	1,200	1,500
Wild type	100±0	99.66±0.33	88.66±0.88	76±0.57	65.66±0.33	56±0.57	45±0.57	36±0.57	25.33±0.33
Landsberg	99±0.33	98±0.66	89±0.57	75±0.57	64.66±0.88	53.33±0.88	44±0.57	32±0.57	20.66±0.33
*uvr*1	100±0	0	0	0	0	0	0	0	0
*uvs*12	100±0	98±0.33	99.66±0.66	0	0	0	0	0	0
*uvs*13	99.66±0.33	99±0.57	25±0.57	0	0	0	0	0	0
*uvs*14	100±0	99±0.33	46±0.57	22.66±0.88	17±0.57	0	0	0	0
*uvs*15	100±0	100±0	47±0.57	18.33±0.33	12±0.57	0	0	0	0
*uvs*16	99±0.33	100±0	23.33±0.33	14.66±0.33	0	0	0	0	0
*uvs*17	100±0	98±0.33	89±0.57	14.33±0.33	8.66±0.33	0	0	0	0
*uvs*18	100±0	99±0.66	87±0.57	98±0.33	100±0	0	0	0	0
*uvs*19	100±0	100±0	87±0.33	22±0.33	17±0.66	0	0	0	0
*uvs*20	100±0	37.33±0.33	31±0.57	17.66±0.33	0	0	0	0	0
*uvs*21	100±0	100±0	87±0	34±0.57	18±0.57	0	0	0	0
*uvs*22	100±0	100±0	81±0	72±0.33	66±0.66	0	0	0	0
*uvs*23	100±0	98±0.66	27±0.57	18±0.57	12.33±0.33	0	0	0	0
*uvs*24	99±0.66	41±1.52	35±0.57	15.33±0.33	13.33±0.33	0	0	0	0
*uvs*25	99±0.33	100±0	28±1.15	27±0.57	6±1.52	4.33±0.33	0	0	0
*uvs*26	100±0	99±0.33	100±0	100±0	89±0.57	9.33±0.33	0	0	0
*uvs*27	100±0	100±0	28±0.57	18±0.57	7.66±0.66	2.33±0.33	0	0	0
*uvs*28	100±0	35±1.15	24.66±1.45	14.66±1.20	9±1.15	0	0	0	0
*uvs*29	100±0	100±0	100±0	20±0.57	12.66±0.88	0	0	0	0

Table 3.2 Continued.......

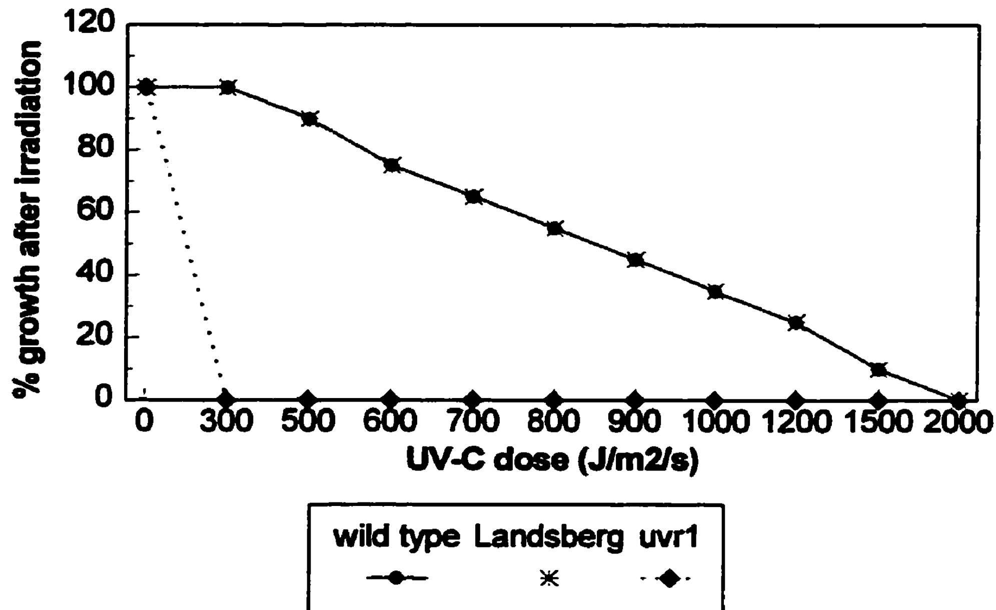
	UV-C doses in J/m^2								
	0	300	500	600	700	800	1,000	1,200	1,500
*uvs*30	100±0	100±0	100±0	18±0.57	12±0.57	3.33±0.33	0	0	0
*uvs*31	100±0	100±0	100±0	55.33±1.20	37.33±1.20	9±0.57	5.66±1.20	0	0
*uvs*32	100±0	99±0.33	98±0.57	100±0	100±0	45.33±1.45	38.33±0.88	0	0
*uvs*33	100±0	100±0	100±0	38±1.52	33±0.66	22±1.73	18.33±1.20	0	0
*uvs*34	100±0	100±0	44.66±1.20	22±0.57	16±0.57	12.66±1.20	7.33±0.33	5±1.15	3.66±0.88
*uvs*35	100±0	100±0	100±0	100±0	29.33±0.88	22.66±1.20	10±0.57	0	0
*uvs*36	100±0	100±0	100±0	47±0.57	37.33±0.88	17.33±0.88	10.66±0.88	0	0
*uvs*37	100±0	100±0	100±0	44.66±0.88	22.33±0.88	14.66±1.20	5.66±1.20	0	0
*uvs*38	99±0.33	100±0	100±0	36.66±1.85	27.33±1.33	18±0.57	3±0.57	0	0
*uvs*39	100±0	100±0	100±0	39±0.57	27±1.15	12±0.57	7.66±0.66	0	0
*uvs*40	100±0	100±0	100±0	100±0	34.8±0.91	25.66±1.20	12±0.57	0	0
*uvs*41	100±0	100±0	35.66±0.88	27±0.57	18±0.57	11±1.15	8.66±0.33	0	0
*uvs*42	100±0	100±0	100±0	100±0	100±0	21.66±1.45	11.33±1.20	0	0
*uvs*43	100±0	100±0	100±0	49.33±0.88	36±0.57	26±0.57	20.66±0.88	6.66±0.88	3±0.57
*uvs*44	100±0	100±0	100±0	100±0	49.33±0.88	36.66±1.20	16.33±1.45	8.66±0.88	0
*uvs*45	100±0	100±0	100±0	38±0.57	25.66±0.88	18.66±0.33	10.33±0.88	0	0
*uvs*46	100±0	100±0	100±0	100±0	36.66±1.45	18.33±0.88	10±0.57	8±0.57	2±0.66
*uvs*47	100±0	100±0	100±0	41±1.15	32.66±0.88	18±0.57	8.33±0.66	4±0.57	0.66±0.33
*uvs*48	100±0	100±0	100±0	100±0	100±0	20.33±0.88	8.66±0.88	0	0
*uvs*49	100±0	100±0	100±0	100±0	23.66±1.15	16±0.33	3.33±0.66	0	0
*uvs*50	100±0	100±0	49±1.15	33±1.15	27.66±0.88	21.33±1.20	12±0.57	7±0.57	3±0.57

Table 3.2 Continued.......

	UV-C doses in J/m²								
	0	**300**	**500**	**600**	**700**	**800**	**1,000**	**1,200**	**1,500**
*uvs*51	100±0	100±0	100±0	47±0.57	35±0.57	18.66±0.33	9±0.57	0	0
*uvs*52	100±0	100±0	100±0	100±0	37.33±1.20	15.66±0.88	8.66±0.88	4.33±0.88	3±0.66
*uvs*53	100±0	100±0	99±0.66	100±0	35±1.15	17±0.66	9±1.12	0	0
*uvs*54	100±0	100±0	100±0	39.66±1.12	22±0.66	13.99±1.52	7±1.98	4.66±0.66	2±0.33
*uvs*55	100±0	100±0	41±1.15	32±1.52	28.66±0.88	17.66±0.88	8.33±2.40	5.66±1.20	1.16±0.44

Root growth was measured using the root bending assay (Britt et al. 1993) described in section 3.1.1. For each dose tested response of approximately 40 roots was analyzed. Values are percentages of irradiated roots that continued growing as indicated by root bending. Mean and standard error are shown. (n=3)

Figure 3.3 Comparison of root growth in Ws wild type, Landsberg *erecta* wild type and *uvr*1 (Britt et al. 1993) in relationship to increasing UV-C doses and in absence of photoreactivation.



Ws, Landsberg *erecta* and *uvr*1 seeds were subjected to the root bending assay (in dark) using a series of UV-C doses. New root growth (which grew at right angles to the root growth before irradiation) was measured 3 days after irradiation at the indicated UV-C doses. The results indicate an average of measurements made on between 35-40 seedlings.

Table 3.3 Categorization of (potential) photoreactivation defective *uvs* mutants

	Lethal dose 50% kJ/m^2	extremely hypersensitive (++)	moderately hypersensitive (+)	wild type level of resistance (-)
Wild type	2.5			
Landsberg	1.2		+	
*uvr*1	0.75	++		
*uvs*1	~0.75	++		
*uvs*2	1-1.2	++		
*uvs*3	1.8-2.0		+	
*uvs*4	0-0.75	++		
*uvs*5	0.75-1.0	++		
*uvs*6	0.75-1.0	++		
*uvs*7	~0.75	++		
*uvs*8	1.2-1.6		+	
*uvs*9	~1.0	++		
*uvs*10	1.2-1.6		+	
*uvs*11	0.75-1.0	++		

Based on 50% inhibition of root growth the *uvs* mutants were categorized into extremely hypersensitive (++), moderately hypersensitive (+) and wild type level of resistance (-).
The dose (kJ/m^2) at which there was 50% inhibition is shown. For details see Table 3.1 and text (section 3.2.2)

Table 3.4 Categorization of (potential) dark repair defective *uvs* mutants.

	Lethal dose 50% kJ/m^2	extremely hypersensitive (++)	moderately hypersensitive (+)	wild type level of resistance (-)
Wild type	~0.8			-
Landsberg	~0.8			-
*uvr*1	0-0.3	++		
*uvs*12	0.5-0.6		+	
*uvs*13	0.3-0.5	++		
*uvs*14	~0.5	++		
*uvs*15	~0.5	++		
*uvs*16	0.3-0.5	++		
*uvs*17	0.5-0.6		+	
*uvs*18	0.7-0.8			-
*uvs*19	0.5-0.6		+	
*uvs*20	0-0.3	++		
*uvs*21	0.5-0.6		+	
*uvs*22	0.7-0.8			-
*uvs*23	0.3-0.5	++		
*uvs*24	0-0.3	++		
*uvs*25	0.3-0.5	++		
*uvs*26	0.7-0.8			-
*uvs*27	0.3-0.5	++		
*uvs*28	0-0.3	++		
*uvs*29	0.5-0.6		+	
*uvs*30	0.5-0.6		+	

Table 3.4 Continued.......

*uvs*31	~0.6		+	
*uvs*32	~0.8			-
*uvs*33	0.5-0.6		+	
*uvs*34	~0.5	++		
*uvs*35	0.6-0.7		+	
*uvs*36	~0.6		+	
*uvs*37	~0.6		+	
*uvs*38	~0.6		+	
*uvs*39	~0.6		+	
*uvs*40	0.6-0.7		+	
*uvs*41	0.3-0.5	++		
*uvs*42	0.7-0.8			-
*uvs*43	~0.6		+	
*uvs*44	~0.7		+	
*uvs*45	~0.6		+	
*uvs*46	~0.7		+	
*uvs*47	~0.6		+	
*uvs*48	0.7-0.8			-
*uvs*49	0.6-0.7		+	
*uvs*50	~0.5	++		
*uvs*51	~0.6		+	
*uvs*52	0.6-0.7		+	
*uvs*53	0.6-0.7		+	
*uvs*54	~0.6		+	
*uvs*55	~0.5	++		

Based on 50% inhibition of root growth the *uvs* mutants were categorized into extremely hypersensitive (++), moderately hypersensitive (+) and wild type level of resistance (-).For details see Table 3.2 and text (section 3.2.2).

respectively, based on 50% inhibition of root growth. These tables also show the dose at which 50% of the mutant growth were affected. Among the mutants sensitive to light (*uvs*1-11) (results in Table 3.3), with the exception of *uvs*3, *uvs*8 and *uvs*10, all are extremely hypersensitive (++). Among the subclass of mutants whose roots stopped growing at ~600 J/m^2 in dark (*uvs*12-13), *uvs*13 is extremely hypersensitive (++) and *uvs*12 is moderately hypersensitive (results in Table 3.4). Among *uvs*14-30, whose roots stopped growing at ~800 J/m^2, in cases of *uvs*14, *uvs*15, *uvs*16, *uvs*20, *uvs*23, *uvs*24, *uvs*25, *uvs*27 and *uvs*28 the roots were extremely hypersensitive (++); in cases of *uvs*18, *uvs*22 and *uvs*26, the roots showed wild type level of resistance; and in rest of the cases (*uvs*17, *uvs*19, *uvs*21, *uvs*29 and *uvs*30), the roots were moderately sensitive to UV (+). Among *uvs*31-55, where the roots stopped growing at ~1,200 J/m^2, in cases of *uvs*34, *uvs*41, *uvs*50 and *uvs*55, the roots were extremely hypersensitive to UV (++); in cases of *uvs*32, *uvs*42 and *uvs*48, the roots showed wild type level of resistance (-); and in the rest of the cases, the roots were moderately sensitive to UV (+) (see Table 3.4).

Thus, UV-sensitive mutants thus isolated showed a broad spectrum of sensitivity. The number of mutants to be chosen for future characterization could have been reduced (from a total of 55) at this point, but instead all 55 mutants were characterized (as shown in succeeding chapters).

3.2.2 Segregation pattern of UV-sensitive mutations

In order to analyze the segregation pattern of the UV-sensitive mutations, each *uvs* mutant (M_2) was crossed to the Ws wild type parent line. Approximately 30 F_1 progeny arising from each cross were tested for their UV-sensitivity. In every case, roots of all F_1 progeny, when subjected to the root bending assay, showed wild type level of UV resistance (data not shown). This confirmed that the mutation conferring UV-hypersensitivity to the roots in all 55 mutants isolated from this study was recessive. The F_1 heterozygotes were selfed and the ratio of wild type : mutant phenotype in the F_2 was determined (Table 3.5 and 3.6). A ratio of 3:1 (wild type : mutant) indicates that the mutant phenotype is recessive and due to a mutation in single gene.

From analyzing the segregation ratios of the *uvs* mutants (Table 3.5 and 3.6), it was determined that UV-sensitive mutations in 38 mutant lines (out of a total of 55 mutants), were single gene mutations. For the remaining 16 mutant lines, the ratio of the mutant and wild type phenotype deviated considerably from the 3:1 ratio. Deviations from a 3:1 ratio can be explained by chromosomal aberrations, polygenic interactions (Strickberger 1976). In addition, to providing knowledge about the segregation pattern of the mutation in each line, this experiment was focused on screening for mutants where the mutation segregates in the ratio of 3:1 because these mutants are potential candidates with which cosegregation analysis of the mutation and the T-DNA were performed (section 3.2.6).

Among the mutations sensitive in light (*uvs*1-11) (data shown in Table 3.5), in all lines except *uvs*1, *uvs*6 and *uvs*10, the mutation segregated in the ratio of 3:1 at 5% level of

Table 3.5 Segregation pattern of UV-sensitive phenotype in F_2 progeny from crosses of (potential) photoreactivation defective *uvs* mutants and wild type plants.

Cross	# UV^R	# UV^S	X^2 (3:1)	P^a
*uvs*1 x WT	276	34	31.8	<0.001
*uvs*2 x WT*	190	48	2.7	0.1
*uvs*3 x WT*	195	48	3.29	0.05-0.1
*uvs*4 x WT*	206	74	0.22	0.5-0.7
*uvs*5 x WT*	140	60	2.4	0.1-0.2
*uvs*6 x WT	233	28	23.4	<0.001
*uvs*7 x WT*	206	74	0.22	0.5-0.7
*uvs*8 x WT*	142	58	1.49	0.2-0.3
*uvs*9 x WT*	172	70	1.77	0.1-0.2
*uvs*10 x WT	286	60	10.41	0.001-0.01
*uvs*11 x WT*	252	70	1.65	0.1-0.2

*** indicates mutants in which the UV-sensitive mutation is segregating in a ratio of approximately 3:1.**

WT= wild type.

 # UV^R = total number of plants with UV-resistant roots.

 # UV^S = total number of plants with UV-sensitive roots.

P^a Calculated using Yates's correction factor.

For 1 degree of freedom, the X^2 value is 3.84 at p=0.05 (or 5% level of significance).

Table 3.6 Segregation pattern of UV-sensitive phenotype in F_2 progeny from crosses of (potential) dark repair defective *uvs* mutants and wild type plants.

Cross	# UV^R	# UV^S	X^2 (3:1)	P^a
*uvs*12 x WT*	172	64	0.45	0.5-0.7
*uvs*13 x WT*	324	94	1.26	0.2-0.3
*uvs*14 x WT*	163	44	1.34	0.2-0.3
*uvs*15 x WT*	129	43	0.0	>0.95
*uvs*16 x WT	102	57	9.4	0.001-0.01
*uvs*17 x WT	106	98	56.52	<0.001
*uvs*18 x WT	260	58	7.38	0.001-0.01
*uvs*19 x WT	200	40	8.44	0.001-0.01
*uvs*20 x WT	288	68	6.29	0.01-0.05
*uvs*21 x WT*	138	62	3.52	0.05-0.1
*uvs*22 x WT*	122	48	0.77	0.3-0.5
*uvs*23 x WT*	140	48	0.0	>0.95
*uvs*24 x WT*	170	42	2.77	0.05-0.1
*uvs*25 x WT*	143	46	0.02	0.7-0.9
*uvs*26 x WT*	118	50	1.77	0.1-0.2
*uvs*27 x WT*	206	84	2.21	0.1-0.2
*uvs*28 x WT*	108	48	2.46	0.1-0.2
*uvs*29 x WT	102	12	11.97	<0.001
*uvs*30 x WT*	138	44	0.02	0.7-0.9
*uvs*31 x WT	128	68	9.3	0.001-0.01
*uvs*32 x WT	123	60	5.64	0.01-0.05
*uvs*33 x WT*	178	50	0.98	0.3-0.5
*uvs*34 x WT*	124	49	0.84	0.3-0.5
*uvs*35 x WT	186	82	4.17	0.01-0.05
*uvs*36 x WT*	126	38	0.2	0.5-0.7
*uvs*37 x WT	190	116	26.42	<0.001
*uvs*38 x WT	152	19	16.85	<0.001
*uvs*39 x WT	123	57	5.64	0.01-0.05
*uvs*40 x WT*	200	75	0.64	0.3-0.5
*uvs*41 x WT*	190	68	0.17	0.5-0.7
*uvs*42 x WT*	112	42	0.3	0.5-0.7
*uvs*43 x WT*	129	38	0.33	0.5-0.7
*uvs*44 x WT*	196	70	0.17	0.5-0.7
*uvs*45 x WT*	103	38	0.18	0.5-0.7
*uvs*46 x WT*	106	34	0.0	>0.95
*uvs*47 x WT*	120	36	0.21	0.5-0.7
*uvs*48 x WT*	124	30	2.21	0.1-0.2

Table 3.6 Continued....

Cross	# UV^R	# UV^S	X^2	P^a
*uvs*49 x WT*	136	33	2.41	0.05-0.1
*uvs*50 x WT*	134	40	0.26	0.5-0.7
*uvs*51 x WT*	104	26	1.46	0.2-0.3
*uvs*52 x WT*	162	44	1.26	0.2-0.3
*uvs*53 x WT	407	92	11.1	<0.001
*uvs*54 X WT	133	20	10.97	<0.001
*uvs*55 X WT*	130	49	0.41	0.5-0.7

*** indicates mutants in which the UV-sensitive mutation is segregating in a ratio of approximately 3:1.**

WT= wild type.

 # UV^R = total number of plants with UV-resistant roots.

 # UV^S = total number of plants with UV-sensitive roots.

P^a Calculated using Yates's correction factor.

For 1 degree of freedom, the X^2 is 3.84 at p=0.05 (or 5% level of significance).

significance. Among the mutants sensitive in dark (*uvs*12-55) (data shown in Table 3.6), in all lines except, *uvs*16, *uvs*17, *uvs*18, *uvs*19, *uvs*20, *uvs*29, *uvs*31, *uvs*32, *uvs*35, *uvs*37, *uvs*38, *uvs*39, *uvs*53 and *uvs*54, the mutation segregates in the ratio of 3:1.

Based on the results of the segregation analysis, the *uvs* mutants where the UV-sensitivity was found to segregate in the ratio of 3:1 (at 5% level of significance) were chosen to determine whether the mutations in those cases are the result of T-DNA insertion (see result section 3.2.6).

3.2.3 Complementation (or allelism) test

A complementation test determines whether two independently isolated monogenic mutants, displaying an identical or very similar phenotype, have resulted from mutations in the same gene (allelic) or from mutations in two different genes (non-allelic). When two allelic mutants are crossed, their F_1 hybrid has a mutant phenotype. When two non-allelic mutants are crossed, the mutations complement each other and their F_1 hybrid has a non-mutant (wild type) phenotype. Figure 3.4 shows the F_1 generation of crosses when mutants are either allelic or non-allelic.

As mentioned in section 3.2.2, the UV-sensitive mutants were classified broadly (based on 100% inhibition of the response of root growth with UV-C) into: a) Sensitive in light, and b) Sensitive in dark. The latter class was again subdivided into: i. roots sensitive at ~600 J/m^2 UV-C; ii. roots sensitive at ~800 J/m^2 UV-C; and iii. roots sensitive at ~1200 J/m^2 UV-C.

Figure 3.4 Complementation (or allelism) test.

Complementation test determines whether or not two recessive mutants are allelic.

A. When two allelic mutants (*a-1/a-1* and *a-2/a-2*) are crossed, their F_1 hybrid has a mutant phenotype (*a-1/a-2*).

B. When two non-allelic mutants (a/a,B/B and A/A,b/b) are crossed, their F_1 hybrid has a non-mutant phenotype (*A/a,B/b*).

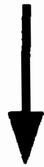
A.

a-1/a-1* x *a-2/a-2

F_1 ➜ *a-1/a-2*

B.

a/a, B/B* x *A/A, b/b



F_1 ➜ *A/a, B/b*

In this study, complementation tests were restricted to each of the (above mentioned) four classes of mutants. Mutants were crossed to each other in all possible combinations. 25-30 F_1 seeds were analyzed for UV sensitivity using the root assay. In this study, determination of complementation groups was strictly based on 100% inhibition of root growth in the F_1. Even in cases where the F_1 roots grew very slowly, mutants were considered to be non-allelic. Each cross was repeated by using one mutant as a female parent in one cross and then using the same mutant as a male parent in the same cross. For example, for mutants A and B, once the cross was done using A as female parent and the cross was repeated using B as the female parent. Crosses were repeated three to four times.

3.2.3.1 Results of complementation analysis

For detailed results of complementation test refer to the appendix section following the discussion section of the thesis. Among the potential photoreactivation mutants (*uvs*1-11), only in the crosses between *uvs*2 x *uvs*9 and *uvs*6 x *uvs*11, did the F_1 progeny uniformly displayed a UV-sensitive phenotype indicating that in both cases (*uvs*2 x *uvs*9 and *uvs*6 x *uvs*11), the mutants were not complementing each other's phenotype and thus they were allelic.

Among the potential dark repair defective mutants (*uvs*12-55), in most cases the mutations complemented and the F_1 were uniformly UV-resistant. However, in the crosses mentioned below, the root sensitivity did not complement as determined by

uniform UV-sensitivity of the roots of the F_1 progeny. Thus, in those cases the mutants are not complementing each others UV-sensitivity and are thus allelic.

i. Mutants whose roots stopped growing at ~800 J/m^2 UV-C :

*uvs*19 x *uvs*17

*uvs*30 x *uvs*23

ii. Mutants whose roots stopped growing at ~1,200 J/m^2 UV-C :

*uvs*44 x *uvs*40

*uvs*45 x *uvs*32

*uvs*35 x *uvs*49

Based on the root sensitivity analysis, the potential photoreactivation mutants can be grouped into 9 complementation groups and the potential dark repair defective mutants into 39 complementation groups. As mentioned before, complementation test was restricted among each class of mutants into which they have been classified. On that basis, the total number of complementation groups were determined to be 48 (39 + 9). This number may reduce further when the complementation test is done with mutants from different classes. In addition, detailed study of allelism test by considering the class of F_1 progeny where the root grows slowly (considered wild type phenotype for the experiment done for this study), might help in isolating those cases where the mutants are partially complementing each others' phenotype. This might help in reducing the number of complementation groups further. Also complementation analysis based on exposing

the leaves of F_1 to UV might provide information whether root and shoot sensitivity are uniform as mutants complementing for root sensitivity may or may not (depending on uniformity of UV-sensitivity in the mutant line) complement for leaf sensitivity.

3.2.4 Sensitivity of *uvs* seedlings to UV light

Isolation of *uvs* mutants was based on the ability of the root tip cells to divide and elongate after UV irradiation. Root tips are mainly composed of dividing (meristematic) cells. In contrast to roots, leaves of mature plants consist of dividing and non-dividing cells. In addition, unlike leaves, roots are normally not exposed to UV. Thus, different types of repair mechanisms may be prevalent in leaves and in roots. An example being the induction of photolyase mRNA in leaf but not in the root tissue of *Sinapis alba* (Batschauer 1993). Thus, in order to determine whether the leaves of *uvs* mutants are also uniformly UV-sensitive, and to compare the sensitivity of the mutants to UV-B and UV-C, the *uvs* mutants were irradiated with different doses of both UV-C and UV-B.

Irradiation of plant leaves with UV was based on the seedling irradiation procedure described in section 2.12.1. In *A. thaliana*, photolyase activity has been found to be absent in seeds, submaximal at 2 leaf stage and appears to be constant from 4 (7 days old) to 12 leaf (15 days old) stage, and persists for at least 30 days (Pang and Hays 1991). Thus, 15 day old seedlings (6-8 leaf stage) were used for all irradiation experiments. UV-C experiments were performed by allowing the seeds to germinate in soil. For irradiation with UV-B, the seedlings were grown in nutrient media contained in plastic petri plates. UV-B bulb used for irradiation (section 2.12) had >95% of output in between 270-400 nm with

a peak output at 325 nm. Thus, during irradiation with UV-B, the plastic petri dish cover, which filtered all wavelengths below 285 nm, was not removed and therefore the seedlings were prevented from being exposed to UV-C wavelengths. Approximately, 40 seeds were allowed to germinate and grown for 15 days (6-8 leaf stage). The seedlings were then exposed to a series of UV-C or UV-B doses.

In order to choose the appropriate doses for UV-irradiation, at first the wild type seedlings were exposed to a series of UV doses. The dose at which all wild type seedlings die, i.e. unable to form apical meristem and produce seeds, was determined. For irradiation with UV-C (at a dose rate of 190 $J/m^2/s$), 100% of the wild type was affected and died at a final dose of 12.8 kJ/m^2; with UV-B (at a dose rate of 500 $J/m^2/s$), the dose was 108.80 kJ/m^2. For irradiating the *uvs* mutant leaves, doses between 0 and 12.8 kJ/m^2 were used for UV-C; and doses between 0 and 108.80 kJ/m^2 were used for UV-B. The doses at which the leaf tissue is affected is much higher than the dose at which the roots are affected. This may be due to the fact that leaves which are exposed to sunlight are inherently more resistant to the effects of UV than roots which grow under soil.

After irradiation, the plants were transferred to growth chamber and incubated for ~11 days before scoring results. Unlike irradiation of root which was done both in light and in darkness, exposure of leaves to UV was restricted only to light due to the unavailability of appropriate filters. Thus, from this experiment it was not possible to assess which mutants were defective in photoreactivation. Sensitivity of mutant leaves to light could be the result of either defective photoreactivation or protection mechanism or dark repair. UV-sensitivity was measured by the degree of yellowing and shriveling of leaves. Figure

3.5 shows the phenotypic changes in seedlings before and after exposure to UV. In this particular situation shown, a high dose of UV-B was used (108.80 kJ/m^2). Phenotypic changes of leaves due to UV-B and UV-C were observed to be quite similar. The sensitivity of the *uvs* mutants and Ws wild type were compared with *uvr*1 (Britt et al. 1993) and its progenitor Landsberg *erecta*.

Results for UV-C dose response are found in Tables 3.7 and 3.8. Results of UV-B dose response are found in Tables 3.9 and 3.10. Values given in these tables are percentages of undamaged seedlings after UV irradiation. Depending on the results of UV-dose response, the mutants were characterized (based on 50% reduction in growth) as extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-). For UV-C irradiation, among the mutants sensitive in light (*uvs*1-11), *uvs*4 and *uvs*10 are classified as extremely hypersensitive (++); *uvs*5, *uvs*7 and *uvs*8 are moderately hypersensitive (+) and rest of the mutants have wild type level of resistance (results in Table 3.7). When the same mutants (*uvs*1-11) were exposed to UV-B, response pattern of *uvs*5 and *uvs*11 classified them as extremely hypersensitive (++), *uvs*1, *uvs*2, *uvs*3, *uvs*6,*uvs*7, *uvs*9 and *uvs*10 were found to be moderately sensitive to UV (+), and the rest of the mutants showed wild type level of resistance (results in Table 3.9).

When the dark repair defective mutants (*uvs*12-55), were irradiated with UV-C, only three mutants *uvs*13 (belonging to the subclass, where the root growth stopped growing at ~ 600 J/m^2), *uvs*39 and *uvs*52 (both belonging to the subclass, where the root stopped growing at ~ 1200 J/m^2) were categorized as extremely sensitive to UV-C (based on 50%

Figure 3.5 UV-sensitive phenotype of *A. thaliana* seedlings. The picture shows the difference in phenotype before (A) and after (B) UV-B irradiation of 15 day old wild type *A. thaliana* seedlings. Seeds were sown in two nutrient agar plates and incubated at 4°C to synchronize germination. The plates were transferred to growth chamber. After 15 days of growth (6-8 leaf stage), one plates was irradiated with UV-B. In this particular situation, a dose of 108.80 kJ/m^2 UV-B was used. UV-sensitive phenotypes include yellow-brown leaves and is also associated with curling of the leaf edge.

A

B

Table 3.7 Sensitivity of (potential) photoreactivation defective *uvs* mutant seedlings to UV-C

	UV-C dose in J/m²							
	0	800	1,600	3,200	6,400	12,800	Lethal dose 50% kJ/m²	Sensitivity
Wild type	100	100	100	100	52	0	~6.4	-
Landsberg[a]	100	100	100	100	33	0	3.2-6.4	+
uvr1[b]	100	100	100	68	0	0	3.2-6.4	+
uvs1	100	100	100	100	47	0	~6.4	-
uvs2	100	100	100	100	39	0	~6.4	-
uvs3	100	100	100	100	43	0	~6.4	-
uvs4	100	100	100	17	0	0	1.6-3.2	++
uvs5	100	100	100	77	0	0	3.2-6.4	+
uvs6	100	100	100	98	50	0	~6.4	-
uvs7	100	100	100	77	0	0	3.2-6.4	+
uvs8	100	100	100	83	0	0	3.2-6.4	+
uvs9	100	100	100	100	57	0	~6.4	-
uvs10	100	100	100	17	25	0	1.6-3.2	++
uvs11	100	100	100	100	59	0	~6.4	-

Table values are percentages of undamaged seedlings after UV irradiation. Experiment was repeated thrice with the similar results. Bases on the UV-sensitivity of the mutants, the mutants have been categorized into extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-).

[b] isolated by Britt et al. (1993)

[a] progenitor strain of *uvr1* (Britt et al. 1993).

Table 3.8 Sensitivity of (potentially) dark repair defective *uvs* mutant seedlings to UV-C

	UV-C dose in J/m^2							
	0	**800**	**1,600**	**3,200**	**6,400**	**12,800**	**Lethal dose 50% kJ/m^2**	**Sensitivity**
Wild type	100	100	100	100	52	0	~6.4	-
Landsberg[a]	100	100	100	100	33	0	3.2-6.4	+
*uvr*1[b]	100	100	100	68	0	0	~3.2	+
Roots sensitive at ~ 600 J/m^2								
*uvs*12	100	100	100	98	47	0	~6.4	-
*uvs*13	100	100	96	17	0	0	1.6-3.2	++
Roots sensitive at ~ 800 J/m^2								
*uvs*14	100	100	100	100	41	0	~6.4	-
*uvs*15	100	100	100	73	11	0	3.2-6.4	+
*uvs*16	100	100	100	81	7	0	3.2-6.4	+
*uvs*17	100	100	100	100	46	0	~6.4	-
*uvs*18	100	100	100	100	41	0	~6.4	-
*uvs*19	100	100	100	100	51	0	~6.4	-
*uvs*20	100	100	100	71	37	0	~6.4	-
*uvs*21	100	100	100	98	39	0	~6.4	-
*uvs*22	100	100	100	100	22	0	3.2-6.4	+
*uvs*23	100	100	100	100	50	0	~6.4	-

Table 3.8 Continued....

	UV-C dose in J/m^2							
	0	800	1,600	3,200	6,400	12,800	Lethal dose 50% kJ/m^2	Sensitivity
*uvs*24	100	100	100	100	39	0	~6.4	-
*uvs*25	100	100	100	82	0	0	3.2-6.4	+
*uvs*26	100	100	100	100	48	0	~6.4	-
*uvs*27	100	100	100	100	26	0	3.2-6.4	+
*uvs*28	100	100	100	100	0	0	3.2-6.4	+
*uvs*29	100	100	100	100	0	0	3.2-6.4	+
*uvs*30	100	100	100	97	0	0	3.2-6.4	+
Roots sensitive at ~1,200 J/m^2								
*uvs*31	100	100	100	100	31	0	3.2-6.4	+
*uvs*32	100	100	100	100	23	0	3.2-6.4	+
*uvs*33	100	100	100	100	0	0	3.2-6.4	+
*uvs*34	100	100	100	100	39	0	~6.4	-
*uvs*35	100	100	100	100	47	0	~6.4	-
*uvs*36	100	100	100	91	0	0	3.2-6.4	+
*uvs*37	100	100	100	100	0	0	3.2-6.4	+
*uvs*38	100	100	100	100	34	0	3.2-6.4	+
*uvs*39	100	100	100	33	21	0	1.6-3.2	++
*uvs*40	100	100	100	100	43	0	~6.4	-
*uvs*41	100	100	100	100	47	0	~6.4	-
*uvs*42	100	100	100	91	37	0	~6.4	-
*uvs*43	100	100	100	100	17	0	3.2-6.4	+
*uvs*44	100	100	100	100	31	0	3.2-6.4	+

Table 3.8 Continued....

	UV-C dose in J/m^2							
	0	800	1,600	3,200	6,400	12,800	Lethal dose 50% kJ/m^2	sensitivity
*uvs*45	100	100	100	100	9	0	3.2-5.4	+
*uvs*46	100	100	100	100	46	0	~6.4	-
*uvs*47	100	100	100	100	0	0	3.2-6.4	+
*uvs*48	100	100	100	92	27	0	3.2-6.4	+
*uvs*49	100	100	100	100	12	0	3.2-6.4	+
*uvs*50	100	100	100	100	41	0	~6.4	-
*uvs*51	100	100	100	100	35	0	~6.4	-
*uvs*52	100	100	100	37	0	0	1.6-3.2	++
*uvs*53	100	100	100	97	41	0	~6.4	-
*uvs*54	100	100	100	96	0	0	3.2-6.4	+
*uvs*55	100	100	100	100	0	0	3.2-6.4	+

Table values are percentages of undamaged seedlings after UV irradiation. Experiment was repeated thrice with the similar results. Bases on the UV-sensitivity of the mutants, the mutants have been categorized into extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-).

[b] isolated by Britt et al. (1993)

[a] progenitor strain of *uvr*1 (Britt et al. 1993).

Table 3.9 Sensitivity of (potential) photoreactivation defective *uvs* mutant seedlings to UV-B

	UV-B dose in kJ/m^2							Lethal dose 50% kJ/m^2	Sensitivity
	0	3.77	7.6	15.11	31.08	54.40	108.80		
Wild type	100	100	100	100	77	42	0	~54.40	-
Landsberg[a]	100	100	100	100	78	31	0	31.08-54.40	-
*uvr*1[b]	100	91	67	59	43	27	0	~15.11	+
*uvs*1	100	100	89	87	51	23	0	~31.08	+
*uvs*2	100	89	97	61	0	0	0	~15.11	+
*uvs*3	100	100	83	78	10	0	0	15.11-31.08	+
*uvs*4	100	97	98	77	69	33	0	31.08-54.40	-
*uvs*5	100	89	75	31	0	0	0	7.6-15.11	++
*uvs*6	100	100	81	79	53	10	0	~31.08	+
*uvs*7	100	100	86	73	52	9	0	~31.08	+
*uvs*8	100	100	92	76	68	0	0	31.08-54.40	-
*uvs*9	100	100	96	98	15	0	0	15.11-31.08	+
*uvs*10	100	100	83	66	51	22	0	~31.08	+
*uvs*11	100	100	100	17	9	0	0	7.6-15.11	++

Table values are percentages of undamaged seedlings after UV irradiation. Experiment was repeated thrice with the similar results. Bases on the UV-sensitivity of the mutants, the mutants have been categorized into extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-).

[b] isolated by Britt et al. (1993)

[a] progenitor strain of *uvr*1 (Britt et al. 1993).

Table 3.10 Sensitivity of (potentially) dark repair defective *uvs* mutant seedlings to UV-B

	UV-B dose in kJ/m²							Lethal dose 50%	
	0	3.77	7.6	15.11	31.08	54.40	108.80	kJ/m²	Sensitivity
Wild type	100	100	100	100	77	42	0	~54.40	-
Landsberg [a]	100	100	100	100	78	31	0	31.08-54.40	-
uvr1 [b]	100	91	67	59	43	27	0	~15.11	+
Roots sensitive at ~ 600 J/m²									
uvs12	100	100	100	88	60	0	0	~31.08	+
uvs13	100	96	76	63	47	21	0	~15.11	+
Roots sensitive at ~ 800 J/m²									
uvs14	100	87	63	40	11	0	0	7.6-15.11	++
uvs15	100	96	87	71	43	33	0	15.11-31.08	+
uvs16	100	85	93	75	21	12	0	15.11-31.08	+
uvs17	100	83	87	24	10	0	0	7.6-15.11	++
uvs18	100	89	73	67	21	13	0	~15.11	+
uvs19	100	100	91	79	65	7	0	~31.08	+
uvs20	100	100	100	64	59	31	0	~31.08	+
uvs21	100	100	87	89	45	12	0	15.11-31.08	+
uvs22	100	100	63	37	17	10	0	7.6-15.11	++

Table 3.10 Continued.....

	UV-B dose in kJ/m^2							Lethal dose 50% kJ/m^2	Sensitivity
	0	3.77	7.6	15.11	31.08	54.40	108.80		
*uvs*23	100	100	100	83	64	39	0	31.08-54.40	-
*uvs*24	100	98	87	69	52	24	0	~31.08	+
*uvs*25	100	100	79	73	69	57	0	31.08-54.40	-
*uvs*26	100	100	100	72	56	34	0	~31.08	+
*uvs*27	100	89	56	29	13	2	0	~7.6	++
*uvs*28	100	76	51	59	25	11	0	7.6	++
*uvs*29	100	100	100	100	42	28	0	~31.08	+
*uvs*30	100	100	100	78	55	23	0	~31.08	+
Roots sensitive at ~1,200 J/m^2									
*uvs*31	100	100	100	73	63	42	0	31.08-54.40	-
*uvs*32	100	100	86	71	50	28	0	31.08	+
*uvs*33	100	100	100	83	61	33	0	31.08-54.40	-
*uvs*34	100	100	100	93	34	17	0	15.11-31.08	+
*uvs*35	100	100	100	100	63	39	0	31.08-54.40	-
*uvs*36	100	100	100	100	57	31	0	~31.08	+
*uvs*37	100	100	100	100	58	27	0	~31.08	+
*uvs*38	100	87	81	59	40	22	0	~15.11	+
*uvs*39	100	100	100	84	35	9	0	15.11-31.08	+
*uvs*40	100	100	100	78	33	16	0	15.11-31.08	+

Table 3.10 Continued.....

	UV-B dose in kJ/m²							Lethal dose 50%	
	0	**3.77**	**7.6**	**15.11**	**31.08**	**54.40**	**108.80**	**kJ/m²**	**Sensitivity**
*uvs*41	100	100	88	49	37	15	0	15.11	+
*uvs*42	100	100	86	39	43	27	0	~15.11	+
*uvs*43	100	100	91	33	48	16	0	~15.11	+
*uvs*44	100	100	100	86	61	22	0	~31.08	+
*uvs*45	100	100	100	81	67	38	0	~31.08	+
*uvs*46	100	92	83	44	23	6	0	~15.11	+
*uvs*47	100	100	88	86	51	29	0	31.08	+
*uvs*48	100	100	93	55	42	27	0	~15.11	+
*uvs*49	100	91	84	77	51	30	0	~31.08	+
*uvs*50	100	100	97	81	75	40	0	~54.40	-
*uvs*51	100	100	100	88	69	39	0	~54.40	-
*uvs*52	100	88	55	34	24	10	0	3.77-7.6	++
*uvs*53	100	100	91	73	56	33	0	~31.08	+
*uvs*54	100	83	71	63	41	22	0	~15.11	+
*uvs*55	100	92	77	59	39	7	0	~15.11	+

Table values are percentages of undamaged seedlings after UV irradiation. Experiment was repeated thrice with the similar results. Bases on the UV-sensitivity of the mutants, the mutants have been categorized into extremely hypersensitive (++), moderately hypersensitive (+), and wild type level of resistance (-).

[b] isolated by Britt et al. (1993)
[a] progenitor strain of *uvr*1 (Britt et al. 1993).

inhibition of growth). *uvs*15, *uvs*16, *uvs*22, *uvs*25, *uvs*27-33, *uvs*36-38, *uvs*43-45, *uvs*47-49, *uvs*54 and *uvs*55 were moderately sensitive (+) to UV-C. The rest of the mutants showed wild type level of resistance (Table 3.8). When the same mutants were irradiated with UV-B *uvs*14, *uvs*17, *uvs*22, *uvs*27, *uvs*28 (all belonging to the subclass, where the root stopped growing at ~ 800 J/m^2), and *uvs*52 (included in the subclass where the root stopped growing at ~ 1,200 J/m^2) were classified as extremely hypersensitive (++) to UV-B. In all other mutants (except, *uvs*23, *uvs*25, *uvs*31, *uvs*33, *uvs*35, *uvs*50 and *uvs*51 where the mutants showed wild type level of resistance), the moderate sensitivity (+) to UV-B was observed.

Both *uvr*1 and its progenitor were moderately sensitive to both UV-B and UV-C in light. This might be due to the defective flavonoid production. It is worth noting that some of the mutants which were sensitive in the dark in the root test, have leaves which are sensitive in light, even though the roots of these mutants are not sensitive in light.

A comparison of the response of the mutants to UV-C and UV-B indicates that (based on 50% inhibition of growth) the *uvs* mutant leaves are generally more sensitive to the effects of UV-B than UV-C. Unlike UV-C, which damages DNA mostly by direct interaction with DNA bases, the ecologically relevant UV-B radiation causes DNA damage by both direct and indirect interaction with the DNA bases. In *A. thaliana* as well as in other plants like alfalfa and soybean, it has been observed that photorepair predominates at all damage level but excision repair is measurable only at high damage levels (Pang and Hays 1991, Quaite et al. 1992; Sutherland et al. 1996). Thus, even in the case of severe ozone depletion, cyclobutane pyrimidine dimers will be monomerized as

long as the photolyase enzyme is available (Beggs et al. 1985). This implies that direct non-cyclobutane pyrimidine dimer and indirect DNA damage are more ecologically relevant. Results of this experiment where the *uvs* mutants are more sensitive to UV-B than UV-C probably substantiates the above statement.

3.2.5 Sensitivity of *uvs* mutants to ionizing radiation

In contrast to UV, ionizing radiation is highly penetrating and damages DNA mainly by producing double- and single-strand breaks (McLennan 1988; Ager et al. 1990). A mutant defective in protection against UV light (e.g. defective in flavonoid biosynthetic pathway) or repair of UV-specific photoproducts should not be hypersensitive to ionizing radiation. Sensitivity to both types of radiation indicates a defect in the mechanism that confers resistance to both UV and ionizing radiation. Thus, assessing the sensitivity of the *uvs* mutants to ionizing radiation helped in categorizing the mutants, primarily by distinguishing the potential DNA repair defective mutants from UV-protection mutants. Ionizing radiation has no effect on the germination of seeds and expansion of cotyledons both are processes which do not require cell division. However, the production of the first pair of true leaves requires cell division and consequently is affected (Ivanov and Sanina 1967; Fershat and Stepanenko 1973).

Sensitivity of the *uvs* mutants to ionizing radiation (procedure described in section 1.10) was assessed by irradiating approximately 100 seeds to final doses of 10 krad, 20 krad and 40 krad at a dose rate of 8.25 rads/sec. Sensitivity to ionizing radiation was scored 9 days after irradiation based on the formation of the total number of first pair of true leaves. Three different responses to leaf growth were observed: 1. both of the first true leaves were produced (wild type); 2. one of the first true leaves was produced and a small pigmented structure appeared at the position of the missing leaf (mutant phenotype); or 3. none of the first true leaves were produced (mutant phenotype). Figure

3.6 shows the different types of responses of the seedlings to ionizing radiation based on the formation of the first pair of true leaves.

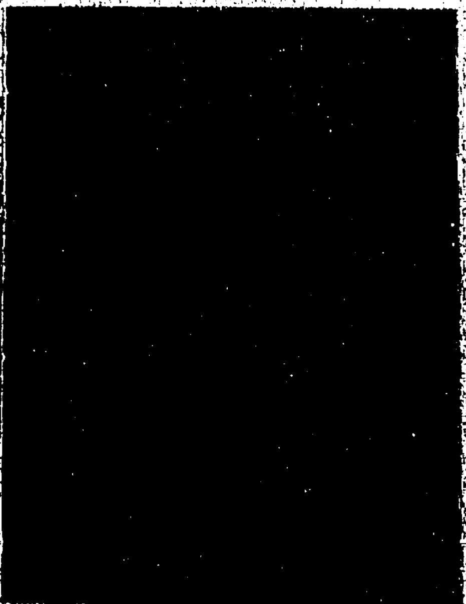
Table 3.11 shows the results of treatment of *uvs* mutants with ionizing radiation. In almost all cases (exceptions are Ws wild type, *uvs*1, *uvs*15, *uvs*16 and *uvs*24) no leaves appeared at doses of 40 Krad. Based on the average number of first pair of true leaves (Table 3.11), the *uvs* mutants were classified into extremely hypersensitive (++), moderately hypersensitive (+) and wild type level of resistance (-). In cases, where average number of first pair of true leaves were less than 0.5 at dose of 20 krad, the mutants were considered to be extremely hypersensitive (++) to ionizing radiation. On that basis, *uvs*2-4, *uvs*33, *uvs*35, *uvs*37, *uvs*50, *uvs*53, *uvs*54 can be considered as extremely hypersensitive to ionizing radiation.

Mutants where the average leaf number was ~1 at doses of 10 krad and 20 krad (*uvs*6, *uvs*17, *uvs*19, *uvs*25, *uvs*41 and *uvs*46, Landsberg *erecta* wild type and *uvr*1), or between 0.5 - 1 at 20 krad (*uvs*1, *uvs*5, *uvs*12, *uvs*13, *uvs*14, *uvs*15, *uvs*18, *uvs*21, *uvs*31, *uvs*32, *uvs*34, *uvs*36, *uvs*38, *uvs*47-49, *uvs*52 and *uvs*55) were considered to be moderately hypersensitive (+) to ionizing radiation. The rest of the mutants were considered to have wild type level of resistance (-).

Figure 3.6 Assessing sensitivity of *A. thaliana* to ionizing radiation. Approximately 100 seeds were sterilized and imbibed by presoaking in 200 µl of water for 24 hours. Imbibation increases the mutagenic effectiveness of ionizing radiation 12-fold (Robbelen, 1964). The seeds were then plated on nutrient medium and irradiated from above by ^{60}Co γ -rays at a dose of 8.25 rads/sec. Irradiated seeds were incubated at 4°C for ~48 hours to synchronize germination and then transferred to growth chamber. Sensitivity to ionizing radiation was scored 9 days after irradiation based on the formation of the total number of first pair of true leaves. Three different responses were observed: 1. both of the first true leaves were produced (wild type phenotype); 2. one of the first true leaves was produced and a small pigmented structure appeared at the position of the missing leaf; or 3. none of the first true leaves was produced.

A. shows the wild type, where leaves are not affected by ionizing radiation (wild type phenotype); B. shows a mixed response, where in some cases one or two leaves are produced and in few cases no leaf is produced. C. is the extreme condition where no leaf is produces. Both B amd C represent mutant phenotypes.

A


B

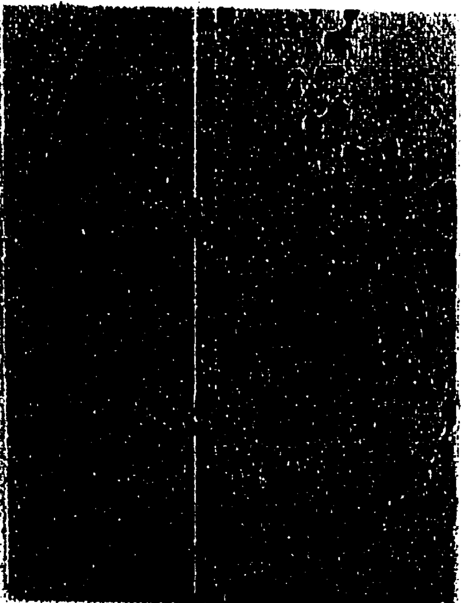

C

Table 3.11 Sensitivity of *uvs* mutants to ionizing radiation

	0 Krad	10 Krad	20 Krad	40 Krad	Sensitivity [a]
Wild type	2.0	2.0	1.00	0.03	-
Landsberg *erecta*	2.0	1.4	0.92	0.0	+
*uvr*1	2.0	1.06	0.72	0.0	+
Mutants sensitive in light					
*uvs*1	2.0	1.25	0.54	0.063	+
*uvs*2	2.0	1.75	0.47	0.0	++
*uvs*3	2.0	1.94	0.11	0.0	++
*uvs*4	2.0	1.11	0.48	0.0	++
*uvs*5	2.0	1.29	0.56	0.0	+
*uvs*6	2.0	0.9	0.7	0.0	+
*uvs*7	2.0	1.54	1.22	0.0	-
*uvs*8	2.0	1.86	0.99	0.0	-
*uvs*9	2.0	2.0	1.1	0.0	-
*uvs*10	2.0	1.82	1.16	0.0	-
*uvs*11	2.0	1.72	1.57	0.0	-
mutants sensitive at ~ 600 J/m^2 in dark					
*uvs*12	2.0	2.0	0.66	0.0	+
*uvs*13	2.0	1.47	0.75	0.0	+
mutants sensitive at ~ 800 J/m^2 in dark					
*uvs*14	2.0	1.30	0.55	0.0	+
*uvs*15	2.0	1.35	0.77	0.03	+
*uvs*16	2.0	2.0	0.85	0.03	-
*uvs*17	2.0	1.32	1.24	0.0	+
*uvs*18	2.0	1.60	0.56	0.0	+
*uvs*19	2.0	0.9	1.01	0.0	+
*uvs*20	2.0	1.66	0.82	0.0	-
*uvs*21	2.0	1.42	0.79	0.0	+
*uvs*22	2.0	1.7	1.25	0.0	-
*uvs*23	2.0	1.67	0.70	0.0	-
*uvs*24	2.0	1.45	1.30	0.004	-
*uvs*25	2.0	1.06	0.80	0.0	+

Table 3.11 Continued...

	0 Krad	10 Krad	20 Krad	40 Krad	Sensitivity [a]
*uvs*26	2.0	1.67	0.88	0.0	-
*uvs*27	2.0	1.78	1.18	0.0	-
*uvs*28	2.0	1.57	1.34	0.0	-
*uvs*29	2.0	1.5	1.07	0.0	-
*uvs*30	2.0	1.68	0.84	0.0	-
mutants sensitive at ~ 1200 J/m^2 in dark					
*uvs*31	2.0	2.0	0.69	0.0	+
*uvs*32	2.0	2.0	0.59	0.0	+
*uvs*33	2.0	2.0	0.22	0.0	++
*uvs*34	2.0	2.0	0.73	0.0	+
*uvs*35	2.0	1.95	0.45	0.0	++
*uvs*36	2.0	2.0	0.66	0.0	+
*uvs*37	2.0	1.62	0.48	0.0	++
*uvs*38	2.0	1.7	0.50	0.0	+
*uvs*39	2.0	1.66	1.43	0.0	-
*uvs*40	2.0	2.0	1.30	0.0	-
*uvs*41	2.0	1.33	1.06	0.0	+
*uvs*42	2.0	1.62	1.63	0.0	-
*uvs*43	2.0	1.56	0.69	0.0	-
*uvs*44	2.0	2.0	1.08	0.0	-
*uvs*45	2.0	1.56	0.21	0.0	++
*uvs*46	2.0	1.31	0.98	0.0	+
*uvs*47	2.0	2.0	0.50	0.0	+
*uvs*48	2.0	1.77	0.50	0.0	+
*uvs*49	2.0	1.71	0.70	0.0	+
*uvs*50	2.0	1.26	0.44	0.0	++
*uvs*51	2.0	1.7	0.84	0.0	-
*uvs*52	2.0	1.17	0.76	0.0	+
*uvs*53	2.0	1.84	0.10	0.0	++
*uvs*54	2.0	1.71	0.40	0.0	++
*uvs*55	2.0	1.88	0.60	0.0	+

Values indicate the total number of true leaves produced, 9 days after irradiation. Total number of leaves produced was calculated based on the formula:

Table 3.11 Continued...

$$\frac{(n1 \times 2) + (n2 \times 1) + (n3 \times 0)}{n1 + n2 + n3}$$

where, n1 = # of plants with 2 leaves
n2 = # of plants with 1 leaf
n3 = # of plants with no leaf

Sensitivity[a] Criteria for determining the sensitivity level is explained in text.

3.2.6 Cosegregation analysis

The *uvs* mutants were isolated from T-DNA transformed lines. Thus, it was necessary to determine whether or not the identified mutant phenotype was linked to T-DNA. If both the mutation and the kanamycin marker (present in T-DNA) segregate in the ratio of 3:1 and all mutant plants are kanamycin resistant, then, there is a high probability (>80%) that a single T-DNA insert has caused the mutation (Feldmann 1992). In cases of more than one T-DNA insert, the kanamycin marker will segregate in a ratio which is greater than 3:1. Thus, if among the F_2 population of a cross between a mutant line (where the mutation is potentially caused by T-DNA) and its wild type progenitor, the mutation segregates in the ratio of 3:1 and the kanamycin marker in the ratio of ≥3:1 and all mutant plants are kanamycin resistant, then there is a high probability that the T-DNA has caused the mutation. The ideal, simplest situation, where a monogenic mutation is linked to T-DNA is explained in Figure 3.7.

For cosegregation analysis, the *uvs* mutants were crossed to the progenitor wild type strain. The resulting F_2 progeny were analyzed for the segregation pattern of the UV-sensitive mutation and the kanamycin marker.

3.2.6.1 Segregation pattern of the kanamycin marker

Before screening for UV-sensitive mutants, all M_1 seeds (~49,000) from the T-DNA transformed lines were grown on kanamycin medium to isolate plants containing T-DNA (section 3.1.3). UV-sensitive mutants were isolates from kanamycin resistant plants, but at that point it was not determined whether the T-DNA was in the heterozygous or

Figure 3.7 Ideal situation of single (recessive) mutation linked to T-DNA.

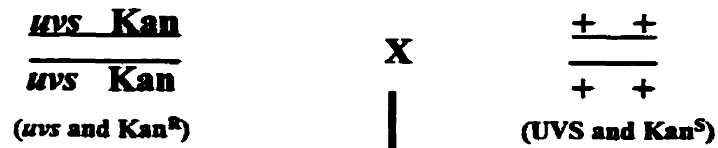
Expected results of a genetic cross where a homozygous recessive mutation (*uvs*) linked to T-DNA, is crossed with a wild type parent are shown.

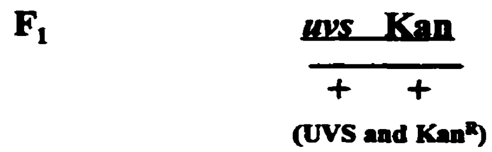
Expected phenotypes of the F_2 progeny are indicated in brackets underneath each genotype. In case of complete linkage between the kanamycin marker and the *uvs* mutation, a ratio of 3:1 (wild type : mutant) is expected for the segregation pattern of both the *uvs* mutation and Kan marker.

'+' indicates wild type condition and 'Kan' represents the kanamycin marker (present on T-DNA).

uvs is the UV-sensitive allele and UVS is its wild type allele.

Kan^R and Kan^S represents the kanamycin resistant and sensitive phenotype respectively.





F_2

	uvs Kan	+ +
uvs Kan	*uvs* Kan / *uvs* Kan (*uvs* and Kan^R)	+ + / *uvs* Kan (UVS and Kan^R)
+ +	*uvs* Kan / + + (UVS and Kan^R)	+ + / + + (UVS and Kan^S)

homozygous state in the mutants as the Kan^R phenotype of the resulting plants T-DNA transformed seeds can be due to either a heterozygous and homozygous situation (Figure 2.1). In order to determine whether the kanamycin marker was in the homozygous or heterozygous condition in the mutants, approximately 100 F_3 seeds, collected by growing UV-sensitive F_2 progeny (from the cross *uvs* x wild type) were grown on nutrient plates containing the antibiotic kanamycin. After 7-10 days the ratio of Kan^R : Kan^S was determined (for procedure of isolating plants with T-DNA, see section 2.10.1). In most cases, all seedlings were kanamycin resistant, confirming the homozygous nature of T-DNA (if there is a single T-DNA insert) and the probability of at least one or potentially more than one T-DNA in the homozygous condition. Presence of both kanamycin resistant and sensitive seedlings was an indication that T-DNA was present in the heterozygous condition. Table 3.12, lists the few *uvs* mutants where the kanamycin marker was present in heterozygous condition. The total number of Kan^S and Kan^R progeny arising from growing approximately 100 mutant seeds on kanamycin plates is also indicated in Table 3.12.

3.2.6.2 Cosegregation of T-DNA and the mutation

In section 3.2.2, the segregating pattern of each mutation isolated was determined. A ratio of 3:1 (wild type:mutant) indicated that the mutation was recessive and due to a single gene. Results where the null hypothesis (stating, that the observed and the expected frequencies have no difference) are not significantly different were analysed further for the

Table 3.12 *uvs* mutants with kanamycin marker in the heterozygous condition.

Mutants sensitive in light	# Kan^R	# Kan^S	Kan^R:Kan^S
*uvs*6	3	86	0.034:1
*uvs*8	9	112	0.080:1
*uvs*9	3	89	0.033:1

Mutants sensitive in dark	# Kan^R	# Kan^S	Kan^R: Kan^S
*uvs*12	7	106	0.066:1
*uvs*32	70	22	3.18:1
*uvs*33	34	26	1.30:1
*uvs*45	78	18	4.33:1
*uvs*53	80	26	3.07:1
*uvs*54	4	126	0.03:1

In the rest of the *uvs* mutants (not listed), all seedlings germinated as Kan^R individuals. If those mutants are due to a single insert, then the T-DNA is most likely present in the homozygous condition. In cases of multiple inserts, at least one T-DNA is present in the homozygous condition.

Kan^R = total number of kanamycin resistant seedlings.
Kan^S = total number of kanamycin sensitive seedlings.

cosegregation pattern of the T-DNA and the mutation. For this analysis, three different experiments were done with F_2 progeny of each cross (mutant x WT) :

1. Roots of ~200 F_2 progeny were subjected to root test on AT medium. This was done to determine the segregation pattern (UV^R:UV^S) of each mutation.

2. Seedlings of ~200 F_2 progeny were grown on AT medium supplemented with the antibiotic kanamycin. This was done to determine the ratio of Kan^R:Kan^S.

3. Roots of ~200 F_2 progeny were subjected to root test on AT medium supplemented with the antibiotic kanamycin. From this result, the ratio of kanamycin resistant, UV-resistant (Kan^R;UV^R) and kanamycin resistant, UV-sensitive (Kan^R:UV^S) progeny was obtained.

In Experiments 1 and 2 mentioned above, the segregation pattern of the UV-sensitive mutation and the T-DNA were analysed independently of each other. A ratio of 3:1 for UV^R:UV^S (in Experiment 1) and a ratio of $\geq$3:1 for Kan^R:Kan^S (in Experiment 2) were the expected ratios. In Experiment 3, as the root test was done on kanamycin medium, the kanamycin sensitive (Kan^S) plants died. In the case of an ideal situation of single T-DNA insert causing the mutation (Figure 3.7), elimination of the Kan^S class would exclude the homozygous wild type progeny from the F_2 population being analysed. This would change the expected ratio from 3:1 (homo- and heterozygous wild type:mutant) to 2:1 (heterozygous wild type:mutant). For results of cosegregation analysis see Tables 3.13 and 3.14.

For each mutant, results of all three experiments were compared. The mutants used to perform the co-segregation analysis were confirmed to be segregating in the ratio of 3:1

Table 3.13 Cosegregation analysis of mutants with roots sensitive in light.

mutants x WT

	Experiment I[a]			Experiment II[a]				Experiment III[a]		
Cross	**Kan^R**	**Kan^S**	**$K^R:K^S$**	**UV^R**	**UV^S**	**X^2 (3:1)**	**P^b**	**$K^RU^R:K^RU^S$**	**X^2 (2:1)**	**P^b**
*uvs*2*	192	15	12.8:1	190	48	2.7	0.01	129:63	0.004	0.95
*uvs*3	180	20	9:1	195	48	3.29	0.05-0.1	144:36	13.8	<0.001
*uvs*4	128	34	3.76:1	206	74	0.22	0.5-0.7	156:27	27.58	<0.001
*uvs*5*	138	12	11.5:1	140	60	2.4	0.1-0.2	105:70	3.19	0.05-0.1
*uvs*7*	140	31	4.51:1	206	74	0.22	0.5-0.7	103:37	2.69	0.1-0.2
*uvs*11	108	24	4.5:1	252	70	1.65	0.1-0.2	100:16	19.05	<0.001

X^2 results are based on the formula $\frac{(|\text{Observed data - Expected data}| - 1/2)^2}{\text{Expected data}}$

[a] **see text for explanation.**

P^b was calculated using *Yates*'s correction factor.

For 1 degree of freedom, the X^2 is 3.84 for p=0.05 (or 5% level of significance).

* **Cases where the T-DNA and the mutation are cosegregating.**

Table 3.14 Cosegregation analysis of mutants with roots sensitive in dark.

mutant x WT

	Experiment I[a]			Experiment II[a]				Experiment III[a]		
Cross	**Kan^R**	**Kan^S**	**K^R:K^S**	**UV^R**	**UV^S**	**X^2 (3:1)**	***P*** [b]	**K^RU^R:K^RU^S**	**X^2 (2:1)**	***P*** [b]
*uvs*13	132	20	6.6:1	324	94	1.26	0.2-0.3	120:12	33.82	<0.001
*uvs*14*	174	64	2.7:1	163	44	1.34	0.2-0.3	117:57	0.004	0.95
*uvs*15	118	46	2.56:1	129	43	0.0	>0.95	180:56	9.36	0.001-0.01
*uvs*21	186	10	18.6:1	138	62	3.52	0.05-0.1	152:24	29.84	<0.001
*uvs*22	76	24	3.1:1	122	48	0.77	0.3-0.5	96:24	9.00	0.001-0.01
*uvs*23	180	34	5.2:1	140	48	0.0	>0.95	146:20	32.9	<0.001
*uvs*24*	123	45	2.73:1	170	42	2.77	0.05-0.1	116:56	0.01	0.9-0.95
*uvs*25*	122	41	2.97:1	143	46	0.02	0.7-0.9	155:65	1.24	0.2-0.3
*uvs*26	198	20	9.9:1	118	50	1.77	0.1-0.2	126:24	19.5	<0.001
*uvs*27*	110	8	13.75:1	206	84	2.21	0.1-0.2	116:68	0.92	0.3-0.5
*uvs*28	70	31	2.25:1	108	48	2.46	0.1-0.2	57:76	32.86	<0.001
*uvs*30	130	33	3.93:1	138	44	0.02	0.7-0.9	151:19	36.56	<0.001

Table 3.14 (Continued..)

Cross	Experiment I[a]			Experiment II[a]				Experiment III[a]		
	Kan^R	Kan^S	$K^R:K^S$	UV^R	UV^S	$X^2(3:1)$	P^b	$K^RU^R:K^RU^S$	$X^2(2:1)$	P^b
*uvs*34*	134	40	3.35:1	124	49	0.84	0.3-0.5	120:72	1.3	0.2-0.3
*uvs*36	116	24	4.83:1	126	38	0.2	0.5-0.7	99:75	7.03	0.01-0.05
*uvs*40*	160	42	3.80:1	200	75	0.64	0.3-0.5	115:60	0.03	0.7-0.9
*uvs*41*	89	30	2.96:1	190	68	0.17	0.5-0.7	156:72	0.24	0.5-0.7
*uvs*42*	141	41	3.43:1	112	42	0.3	0.5-0.7	155:70	0.4	0.5-0.7
*uvs*43*	125	15	8.33:1	129	38	0.33	0.5-0.7	120:63	0.04	0.7-0.9
*uvs*44*	139	38	3.65:1	196	70	0.17	0.5-0.7	122:59	0.16	0.5-0.7
*uvs*46	102	27	3.77:1	106	34	0.0	>0.95	72:132	88.93	<0.001
*uvs*47	111	29	3.82:1	120	36	0.21	0.5-0.7	91:69	5.48	0.05-0.1
*uvs*48	80	15	5.33:1	124	30	2.21	0.1-0.2	76:8	20.37	<0.001
*uvs*49	144	30	4.8:1	136	33	2.41	0.05-0.1	94:26	6.82	0.01-0.05
*uvs*50	130	85	1.52:1	134	40	0.26	0.5-0.7	123:33	9.87	0.001-0.01
*uvs*51*	84	35	2.4:1	104	26	1.46	0.2-0.3	163:77	0.1	0.7-0.9
*uvs*52	101	70	1.44:1	162	44	1.26	0.2-0.3	122:14	32.48	<0.001
*uvs*55*	230	67	3.43:1	130	49	0.41	0.5-0.7	106:50	0.10	0.7-0.9

X^2 results are based on the formula $\frac{(|\text{Observed data - Expected data}| - 1/2)^2}{\text{Expected data}}$

[a] see text for explanation.

P^b was calculated using *Yates*'s correction factor.

For 1 degree of freedom, the X^2 is 3.84 for p=0.05 (or 5% level of significance).

*** Cases where the T-DNA and the mutation are cosegregating.**

(wild type:mutant), at 5% level of significance for the mutant phenotype. Among these mutants only those which were segregating in the ratio of 2:1 for the kanamycin resistant, UV-resistant : kanamycin resistant, UV-sensitive ($Kan^R UV^R$: $Kan^R UV^S$) and ≥3:1 for kanamycin resistant : kanamycin sensitive class (Kan^R:Kan^S) at 5% level of significance, were identified and characterized as the mutants where the T-DNA and the mutation are potentially linked. On that basis, among the mutants sensitive in light, in the case of *uvs*2, *uvs*5 and *uvs*7 the UV-sensitivity and the T-DNA were found to be linked (results in Table 3.13). In the case of *uvs*2 and *uvs*5, the kanamycin marker was found to segregate in the ratio of >3:1 (12.8:1 for *uvs*2 and 11.5:1 for *uvs*5). In those cases the T-DNA copy number is potentially more than one (e.g. when 2 copies of T-DNA are present in the genome, the kanamycin marker segregates in the ratio of 15:1; for 3 T-DNA copies the segregation pattern of the kanamycin marker is 63:1). Among the mutants with roots sensitive in dark (results in Table 3.14), in cases of *uvs*14, *uvs*24, *uvs*25, *uvs*27, *uvs*34, *uvs*40, *uvs*41, *uvs*42, *uvs*43, *uvs*44, *uvs*51 and *uvs*55 the mutation was found linked to the T-DNA. Out of these, in the case of *uvs*27 and *uvs*43 the Kan^R : Kan^S ratio was observed to be 13.75:1 and 8.33:1, respectively; indicating, the possibility of multiple T-DNA insertion. The mutants where the mutation and the T-DNA are potentially proven to be linked by co-segregation analysis are marked in asterisk (*) in the tables showing the results (Table 3.13 and 3.14).

3.2.6.3. Estimates of the copy number of T-DNA

In 15 out of 55 UV-sensitive mutants isolated in this study, the mutation was found to be linked to T-DNA (as determined by genetic analysis). These mutants are potential candidates for isolation of flanking plant sequences which might provide clue to the gene involved. In the final step of this study, I determined the copy number of T-DNA insertions in those mutants. The process by which T-DNA integrates into the plant genome is not quite clear (Mayerhofer et al. 1991). It is documented that T-DNA integration is associated with rearrangements of both T-DNA and plant sequences (Gheysen et al. 1987). In order to determine the possible number of T-DNA insertions in each of the mutants, genomic plant DNA was probed with sequences of the right and left T-DNA border and the pBR322 origin of replication (see map of T-DNA in section Figure 2.2). Hybridization was done twice with similar results. Estimation of the copy number of T-DNA in the genome was done by counting the number of bands for each sample. For any particular sample, the absence of a band in the case of one probe and the presence of a band in the case of another probe can be explained by the deletion of that particular portion of T-DNA during integration.

Each DNA sample (~1 μg) was digested with *Sal*I (for probing with 6.7 kb left border end of T-DNA), *Pst*I (for probing with 3.2 kb right border end of T-DNA) and *BamH*I (for probing with a 1.5 kb fragment of pBR322 containing the origin of replication). Digested DNA fragments were separated by agarose gel electrophoresis. The DNA was blotted onto Hybond membrane from the agarose gel by capillary action. Following this the hybrdization was done with the respective probe. As a control, DNA isolated from

untransformed Ws wild type was used. Figures 3.8 and 3.9 show the results of hybridization with portions of T-DNA as probes. An estimation of the copy number of T-DNA was done from the number of bands. Table 3.15 depicts the possible number of T-DNA bands identified in each case and a conclusion of the total number of T-DNA insertions for each analyzed mutants.

6.7 kb from the left end of T-DNA consists of the entire 3 kb T-DNA left border and the pBR322 sequence present after the left border. The probe can bind to both the left border end and also to the pBR322 sequences near the right border end of T-DNA. Thus, genomic DNA carrying a single T-DNA copy when digested with the enzyme S*alI* and probed with the 6.7 kb DNA from the left end of T-DNA is expected to show two bands: (I) the first one being no smaller than 6.7 kb (corresponding to the left T-DNA border); (ii) a 6.0 kb band is also expected if the probe binds to an internal *Sal*I fragment (6.0 kb) which contains the origin of replication sequence (which is present in 6.7 kb left end of T-DNA used as probe).

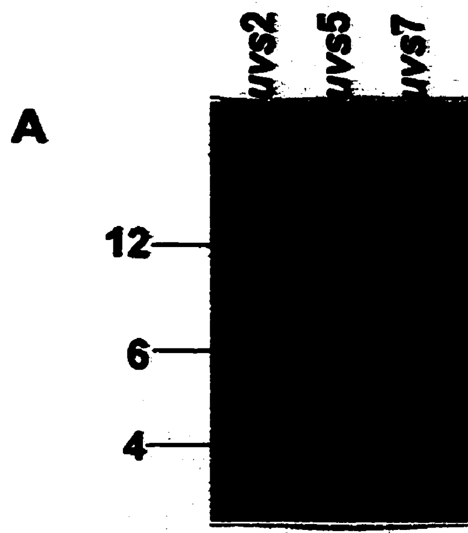
Thus, an intact single T-DNA insert can be identified by the presence of a 6.7 and 6.0 kb bands when probed with T-DNA left border end.

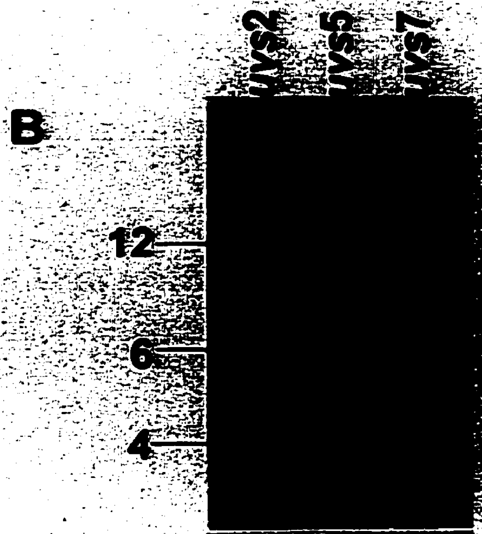
When 3.2 kb from the right border end of T-DNA was used as probe, the DNA was digested with *Pst*I, the (3.2 kb) probe can bind to the right border where it will give a band size of atleast 6 kb (depending on the P*st*I cut sites). Thus, number of bands detected, when T-DNA right border was used to probe genomic DNA digested with *Pst*I, gives an estimate of the copy number of T-DNA.

When 1.5 kb origin of replication sequence of the plasmid pBR322 was used as probe and the DNA was digested with B*amHI*, the probe will bind to both pBR322 sequences present in T-DNA. However, since there is no B*amHI* site between the pBR322 from the right end till the left end of T-DNA (see Figure 2.2 for description), there should be a single band of minimum 14 kb.

On the basis of the criteria stated in the previous paragraph, the number of bands were analyzed in each case. In cases of *uvs*7 no band was detected in any of the blots. Thus, T-DNA in these mutants must have undergone extensive amount of structural changes while integration and thus those specific regions of T-DNA, where the probes are supposed to bind, are absent. Thus the copy number of T-DNA could not be determined. As these mutants are kanamycin resistant, use of kanamycin region of T-DNA would probably serve as a better probe than the ones used. In quite a few cases differences in band intensity affected the interpretation of copy number of T-DNA. As controls, I used untransformed Ws wild type DNA. Use of proper positive control with the a knowledge about the number of T-DNA loaded in agarose gel will help to solve the confusion regarding the interpretation of results with different band intensities. Other than *uvs*7, based on number of bands present, it was interpreted that more than one T-DNA insertion was present in each case.

Figure 3.8 Hybridization analysis of potential photoreactivation defective *uvs* mutants. Genomic DNA from *uvs*2, *uvs*5 and *uvs*7 was digested with *Sal*I (A), *Bam*HI (B) and *Pst*I (C) and probed with 6.7 kb T-DNA left border (A), 1.5 kb pBR322 origin of replication (B) and 3.2 kb T-DNA right border (C) respectively. Interpretation of the possible copy number of T-DNA is summarized in Table 3.15.


A
uvs2
uvs5
uvs7
12
6
4


B
uvs2
uvs5
uvs7
12
6
4

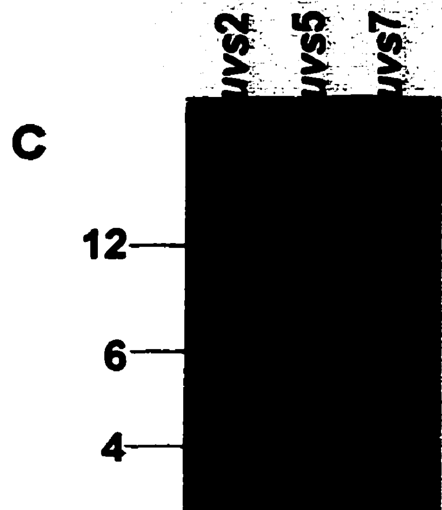
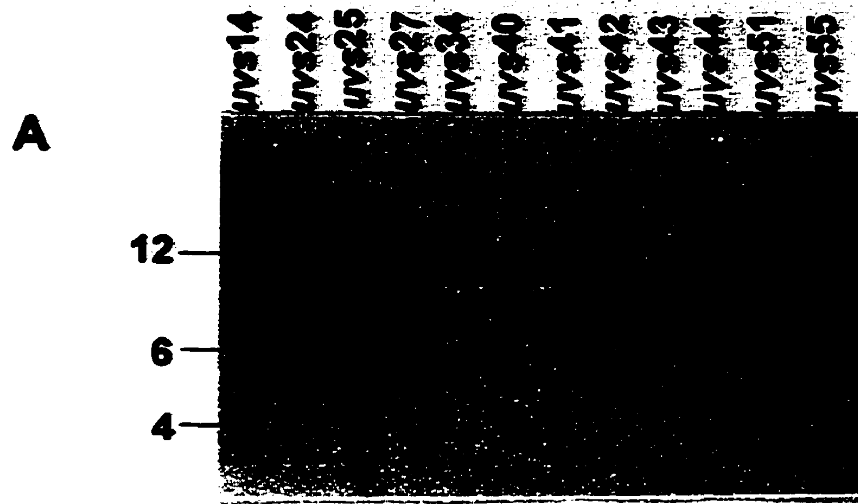
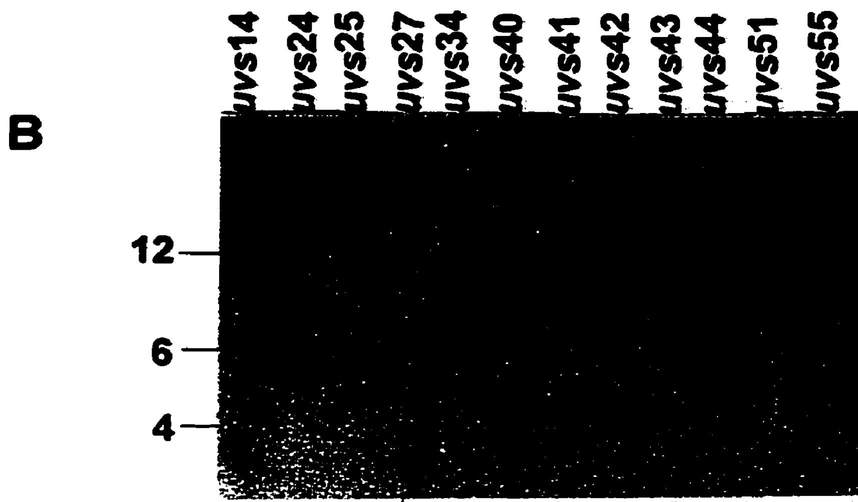

C
uvs2
uvs5
uvs7
12
6
4

Figure 3.9 Hybridization analysis of potential dark repair defective *uvs* mutants. Genomic DNA from *uvs*14, *uvs*24, *uvs*25, *uvs*27, *uvs*34, *uvs*40, *uvs*41, *uvs*42, *uvs*43, *uvs*44, *uvs*51 and *uvs*55 was digested with *Sal*I (A), *Bam*HI (B) and *Pst*I (C) and probed with 6.7 kb T-DNA left border (A), 1.5 kb pBR322 origin of replication (B) and 3.2 kb T-DNA right border (C) respectively. Interpretation of the possible copy number of T-DNA is summarized in Table 3.15.


A
uvs14
uvs24
uvs25
uvs27
uvs34
uvs40
uvs41
uvs42
uvs43
uvs44
uvs51
uvs55
12
6
4


B
uvs14
uvs24
uvs25
uvs27
uvs34
uvs40
uvs41
uvs42
uvs43
uvs44
uvs51
uvs55
12
6
4

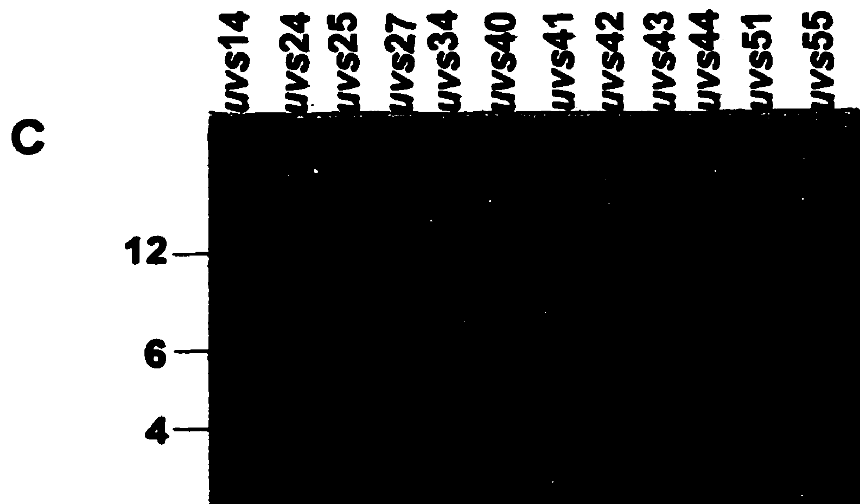

C
uvs14
uvs24
uvs25
uvs27
uvs34
uvs40
uvs41
uvs42
uvs43
uvs44
uvs51
uvs55
12
6
4

Table 3.15 Summary and interpretation of possible number of T-DNA insertions

Mutants	Probes: left border (6.7 kb)	Probes: pBR322 *ori* sequence (1.5 kb)	Probes: right border (3.2 kb)	Estimated Copy # of T-DNA
*uvs*2	1	~5	X	>1
*uvs*5	~5	X	1	>1
*uvs*7	X	X	X	?
*uvs*14	~4	X	~9	>1
*uvs*24	~2	1	~2	~2
*uvs*25	>4	1	>1	>1
*uvs*27	6	3	3	>1
*uvs*34	2	1	~1	>1
*uvs*40	1	~6	3	>1
*uvs*41	5	~1	4	>1
*uvs*42	~1	~1	4	>1
*uvs*43	~3	0	3	>1
*uvs*44	~2	X	5	>1
*uvs*51	~2	X	X	>1
*uvs*55	3	~1	~1	>1

The hybridization was repeated twice with similar results.
'X' indicates band was absent, probably because that portion of T-DNA was deleted.
Multiple bands could either indicate more than one T-DNA copy or tandem duplication of T-DNA in that region.
? indicates copy number could not determined.
~ indicates copy number could not be determined due to varoius band intensity.

4 Discussion

In relation to other eukaryotes, our understanding of the DNA repair system in higher plants is very limited. One of the primary reasons is the relative lack of DNA repair defective mutants. Thus, this study was aimed at isolation and characterization of UV-sensitive and/or DNA repair defective mutants from a population of approximately 49,000 *A. thaliana* seeds derived from 4,900 independent *A. tumefaciens* T-DNA transformed lines. The rationale for using T-DNA transformed seeds was based on the fact that T-DNA might insert and inactivate the gene of interest causing a 'loss of function' phenotype. In addition, mutants in which the T-DNA and the mutation are linked, are potential candidates for the isolation of plant sequences flanking the T-DNA insert and possible determination of actual genes disrupted.

Mutant isolation was based on the ability of the root tip cells to divide and elongate after exposure to UV-C. Out of 55 *uvs* mutants isolated in this study, 11 (*uvs*1-11) were potentially defective in photoreactivation or in other UV-protective mechanism and 44 (*uvs*12-55) were potentially defective in dark repair. For further characterizing the mutants, an attempt was made to answer the following questions:

1. Is the UV-sensitivity uniform throughout the plant or is it present only in the root?
2. Are the mutants UV-sensitive due to defect(s) in DNA repair mechanisms or in UV-protective mechanisms?
3. Are the UV-sensitive phenotypes due to a single gene mutation?
4. Are the mutations the result of T-DNA insertion?

The following approaches were taken to answer the questions:

Question 1. UV-sensitivity in roots and aerial tissues.

This study focuses on characterization of mutants defective in repairing UV-induced damage to DNA. The *uvs* mutants were isolated based on the sensitivity of the root tissue to UV-C. Root tissue consists mostly of meristematic cells which are constantly in the process of division. Unlike roots, leaves consist of both meristematic cells, which are in the process of continuous division, and quiescent cells which have stopped dividing. To determine whether the UV-sensitivity was present only in roots, or in both the roots and the aerial tissues, 15 days old seedlings were irradiated with a series of UV-B and UV-C doses. After irradiation, the plants were kept in light in the growth chamber. In this experiment, leaves of *uvs* mutants sensitive to UV, in light, could be defective either in photoreactivation or dark repair or in UV-protective mechanisms. The *uvs* mutants have been classified (section 3.2.1) into light or dark repair defective mutants based on the UV-sensitivity of the roots. In the dark, root growth is not affected until six days after germination (Britt et al. 1993). Thus, the root bending assay (Britt et al. 1993) can be performed both in the light as well as in the dark. Unlike roots, leaves cannot be grown in the dark. In such cases appropriate controls and filters can be used which absorb the shorter wavelength (450 nm and under) while transmitting the photosynthetically active radiation. In this study, biological effects of photoreactivation in leaves could not be analyzed because of the unavailability of appropriate filters. Thus, I was unable to determine and distinguish whether the leaf tissues are defective in photoreactivation or dark repair processes. Nonetheless, from this study it was possible to determine:

a. whether the root tissue was sensitive to UV-C in dark or light.

b. whether the aerial tissues of mutants were sensitive to UV-B and UV-C.

Results of (a) helped in the isolation of *uvs* mutants and classification of the mutants based on their sensitivity in light and dark. Mutants sensitive in dark were again subdivided into three subclasses based on their degree of sensitivity.

Along with interacting directly with DNA bases, UV-B also causes damage by indirectly interacting with DNA, e.g. via sensitized molecules or via oxidative damage (Farr et al. 1986). In addition to DNA damage, UV-B also damages amino acids and polypeptides (Hartman et al. 1979), causes growth delay, and affects cell membranes (Kelland et al. 1984). Thus, analysis of (b) might help in determining whether DNA damage was also inflicted upon other cellular components. In cases where the aerial tissues showed wild type levels of UV-resistance, the repair or protection defect was probably root specific.

Question 2. Repair and/or protection mutants.

The *uvs* mutants isolated in this study could be defective in UV-protective mechanisms and/or DNA repair. UV induces relatively few chromosomal aberrations compared to γ-rays and thus little loss of genetic material would result from exposure to UV; killing by UV most likely is due to direct inactivation of a necessary gene. Assessing the sensitivity of the mutants to ionizing radiation (which damages DNA mainly by single- and double-strand breaks), helped in categorizing mutants into: 1. defective in UV-protective mechanisms and/or repair of UV-specific photoproducts (mutants sensitive to UV and not ionizing radiation); 2. defective in DNA repair (mutants sensitive to ionizing radiation and

may or may not be sensitive to UV); and 3. defective in a mechanism that confers resistance to both types of radiation (mutants sensitive to both ionizing and UV radiation).

4.1 Sensitivity characteristics of *uvs* mutants to UV and ionizing radiation

The *uvs* mutants were classified into four categories: i. sensitive to UV under light conditions, ii sensitive to UV under dark conditions; and the latter class was again classified into a) mutants where the roots stopped growing at ~ 600 J/m^2, b) mutants where the roots stopped growing at ~ 800 J/m^2 and c) mutants where the roots stopped growing at ~ 1200 J/m^2. This classification was based on 100% inhibition of root growth. Later, the mutants were characterized based on 50% inhibition of root growth and 50% inhibition of population growth. On that basis each *uvs* mutant was characterized as: i. extremely hypersensitive (++), ii. moderately hypersensitive (+), or iii. wild type level of resistance (-). Extremely hypersensitive (++) mutants are >50% more sensitive than the wild type while moderately hypersensitive (+) mutants are ~50% more sensitive than wild type. Along with the Ws wild type, *uvr*1 and its progenitor Landsberg *erecta* were included in the class of controls (Tables 3.1; 3.2; 3.7; 3.8; 3.9 and 3.10). Along with UV, sensitivity of the *uvs* mutants to ionizing radiation was also assessed. Depending on the ability of the seedlings to form the first pair of true leaves, the mutants were characterized as i. extremely hypersensitive (when the average number of first pair of two leaves at a dose of 20 krad was less than 0.5); ii. moderately hypersensitive (when the average number of first pair of true leaves was at both 10 krad and 20 krad were 1.0 or if the average number at 20 krad was between 0.5 and 1; iii. wild type level of resistance .

Table 4.1 summarizes the sensitivity of each mutant to UV and ionizing radiation. Based on the sensitivity characteristics the *uvs* mutants were then divided into five classes. The UV-sensitive phenotype was either observed both in the root and leaf tissue or the damage was phenotypically distinguishable only in the leaves or in roots. Response of leaves to UV-B and/or UV-C also indicated whether the damage was due to direct and/or indirect interaction of DNA bases. Table 4.2 depicts the different classes into which the mutants have been classified based on their radiation sensitivity pattern.

Table 4.1 Summary characteristics of *uvs* mutants

Mutant	Root sensitivity		Seedling sensitivity		Seed sensitivity
	UV-C (+phr)	UV-C (-phr)	UV-B (+phr)	UV-C (+phr)	Ionizing radiation
*uvs*1	++	-	+	-	+
*uvs*2	++	-	+	-	+
*uvs*3	+	-	+	-	++
*uvs*4	++	-	-	++	+
*uvs*5	++	-	++	+	+
*uvs*6	++	-	+	-	+
*uvs*7	++	-	+	+	-
*uvs*8	+	-	-	+	-
*uvs*9	++	-	+	-	-
*uvs*10	+	-	+	++	-
*uvs*11	++	-	++	-	-
*uvs*12	-	+	+	-	+
*uvs*13	-	++	+	++	+
*uvs*14	-	++	++	-	+
*uvs*15	-	++	+	+	+
*uvs*16	-	++	+	+	-
*uvs*17	-	+	++	-	+
*uvs*18	-	-	+	+	+
*uvs*19	-	+	+	-	+
*uvs*20	-	++	+	-	-
*uvs*21	-	+	+	-	+
*uvs*22	-	-	++	+	-
*uvs*23	-	++	-	-	-
*uvs*24	-	++	+	-	-
*uvs*25	-	++	-	+	+
*uvs*26	-	-	+	-	-
*uvs*27	-	++	++	+	-
*uvs*28	-	++	++	+	-
*uvs*29	-	+	+	+	-
*uvs*30	-	+	+	+	-
*uvs*31	-	+	-	+	+
*uvs*32	-	-	+	+	+
*uvs*33	-	+	-	+	++
*uvs*34	-	++	+	-	+
*uvs*35	-	+	-	-	+
*uvs*36	-	+	+	+	+
*uvs*37	-	+	+	+	+
*uvs*38	-	+	+	+	+
*uvs*39	-	+	+	++	-
*uvs*40	-	+	+	-	-

Table 4.1 continued……

Mutant	Root sensitivity UV-C (+phr)	Root sensitivity UV-C (-phr)	Seedling sensitivity UV-B (+phr)	Seedling sensitivity UV-C (+phr)	Seed sensitivity Ionizing radiation
*uvs*41	-	++	+	-	+
*uvs*42	-	-	+	-	-
*uvs*43	-	+	+	+	-
*uvs*44	-	+	+	+	-
*uvs*45	-	+	+	+	++
*uvs*46	-	+	+	-	+
*uvs*47	-	+	+	+	+
*uvs*48	-	-	+	+	+
*uvs*49	-	+	+	+	+
*uvs*50	-	++	-	-	++
*uvs*51	-	+	-	-	-
*uvs*52	-	+	++	++	+
*uvs*53	-	+	+	-	++
*uvs*54	-	+	+	+	+
*uvs*55	-	+	+	+	+
*uvr*1[a]	+	++	+	+	+
Landsberg[b]	+	-	+	+	+

++ indicates extremely hypersensitive condition (>50% more sensitive than wild type condition).

+ indicates moderately sensitive condition (~50% more sensitive than wild type condition).

- indicates wild type level of resistance.

+phr after irradiation, recovery was in photoreactivating condition.

-phr after irradiation, recovery was in non-photoreactivating condition.

For detail of each section see text..

[a] mutant isolated by Britt et al. 1993.

[b] progenitor strain of *uvr*1.

Table 4.2 Summary of probable classes among the *uvs* mutants

1. Hypersensitive to UV and not to ionizing radiation

*uvs*7
*uvs*9 - leaves to UV-B.
*uvs*10
*uvs*11 - leaves to UV-B.
*uvs*16
*uvs*20 - leaves to UV-B.
*uvs*22 - roots (-); leaves to UV-B.
*uvs*23 - leaves (-).
*uvs*24 - leaves to UV-B.
*uvs*27
*uvs*28
*uvs*39

2. Hypersensitive to UV and moderately to ionizing radiation

*uvs*1 - leaves to UV-B.
*uvs*2 - leaves to UV-B.
*uvs*4 - leaves to UV-C.
*uvs*5
*uvs*6 - leaves to UV-B.
*uvs*13
*uvs*14 - leaves to UV-B.
*uvs*15
*uvs*17 - leaves to UV-B.
*uvs*25 - leaves to UV-C.
*uvs*34 - leaves to UV-B.
*uvs*41 - leaves to UV-B.
*uvs*52
*uvr*1[a]

3. Hypersensitive to ionizing radiation and moderately sensitive to UV

*uvs*3 - leaves to UV-B.
*uvs*32 - roots (-).
*uvs*45
*uvs*53 - leaves to UV-B.

4. Moderately sensitive to UV and not to ionizing radiation

*uvs*8 - leaves to UV-C.
*uvs*26 - roots (-); leaves to UV-B.
*uvs*29
*uvs*30
*uvs*40 - leaves to UV-B.
*uvs*42 - roots (-); leaves to UV-B.
*uvs*43
*uvs*44
*uvs*51 - leaves (-).

5. Moderately sensitive to both UV and ionizing radiation

*uvs*12 - leaves to UV-B.
*uvs*18 - root (-).
*uvs*19 - leaves to UV-B.
*uvs*21 - leaves to UV-B.
*uvs*31 - leaves to UV-C.
*uvs*32 - root (-).
*uvs*35 - leaves (-).
*uvs*36
*uvs*37
*uvs*38
*uvs*46 - leaves to UV-B.
*uvs*47
*uvs*48 - root (-).
*uvs*49
*uvs*54
*uvs*55
Landsberg *erecta*[b]

6. Hypersensitive to both UV and ionizing radiation

*uvs*50 - leaves (-).

Unless otherwise mentioned, in all cases, both leaves and roots were affected and also leaves were sensitive to both UV-B and UV-C. Deviations from this situation are mentioned beside each mutant designation.
[a] isolated by Britt et al. 1993.
[b] progenitor strain of *uvr*1.

Classification based on information in Table 4.1.

4.1.1 Comparison of *uvs* mutants with yeast epistasis groups

Among lower eukaryotes, *S. cerevisiae* is a model organism for DNA repair and mutagenesis studies. Irradiation of yeast cells with UV and ionizing radiation have resulted in the isolation of radiation sensitive (*RAD*) mutants which have been classified into three epistasis groups (Haynes and Kunz 1981). Each epistasis group contains loci commonly involved in a particular repair pathway. Therefore, in case of mutants epistatic to each other, a double mutant is much more sensitive than each of the single mutants. In yeast, there are three epistasis groups formed on the basis of radiation sensitivity of yeast cells to UV and ionizing radiation. Mutants belonging to the yeast *RAD3* epistasis group are extremely UV-sensitive but not to ionizing radiation. *rad3* mutants are found to be defective in damage specific incision of UV-irradiated DNA and as a result in the excision of pyrimidine dimers. Among the *uvs* mutants isolated from this study, the mutants belonging to classes 1 and 4 in Table 4.2 show phenotypic characteristics similar to *RAD3* mutants in being sensitive to UV and not to ionizing radiation.

Yeast mutants extremely sensitive to killing by ionizing radiation belong to the *RAD52* epistasis group (Friedberg et al, 1995) and are associated with recombinational repair of DNA double-strand breaks (Game 1983; Friedberg 1988). Among the *rad* mutants, *rad51*, *rad52* and *rad54* are completely defective in meiotic and mitotic recombination and also in repairing double-strand breaks. A few of the yeast *rad52* mutants are also moderately sensitive to ultraviolet radiation, indicating that recombination events governed by *RAD52* loci may be involved in the postreplicative repair of UV radiation damage. Among the *uvs* mutants isolated, class 3 in Table 4.2 shares phenotypic characteristics with the *RAD52*

epistasis group in being extremely sensitive to ionizing radiation and moderately sensitive to UV. As mutants were isolated on the basis of their UV sensitivity, thus, all classes of *uvs* mutants are sensitive to UV, to some degree. Thus, from this study, it was not possible to isolate mutants sensitive to ionizing radiation and not sensitive to UV. In this regard, the *A. thaliana RAD* mutants (*rad*1-5) isolated by Davies et al. (1994) may be more similar in phenotype to the *RAD52* epistasis group in being hypersensitive to ionizing radiation and not sensitive to UV.

Yeast mutants belonging to *RAD6* epistasis groups are sensitive to both UV and ionizing radiation. The *RAD6* group of genes are involved in error-prone repair in yeast (Friedberg et al. 1995). Among *uvs* mutants, classes 2, 5 and 6 are sensitive to both UV and ionizing radiation. *uvs* mutants belonging to Class 5 are moderately sensitive to UV and ionizing radiation, characteristic of the yeast *rev* (defective mutant reversion) mutants. *rev* loci are involved in translesion synthesis.

*uvr*1 (Britt et al. 1993), belongs to class 2 mutants (Table 4.2) and is hypersensitive to UV and moderately sensitive to ionizing radiation. *uvr*1 has been shown to be defective in dark repair of [6-4] photoproducts. Such a mutant is not likely to be sensitive to ionizing radiation, but in this study, both *uvr*1 and its progenitor strain were found to be moderately sensitive to ionizing radiation. This may be either due to an inherent defect in DNA repair capability among Landsberg *erecta* possibly due to a defect in flavonoid biosynthesis as flavonoids have been shown to protect DNA from damage in maize (Stapleton and Walbot 1994) and consequently may also have a similar role in DNA protection in *A. thaliana*.

Differences in ionizing radiation sensitivity can also be explained by variation in DNA repair capabilities among different ecotypes (Smith 1942).

Question 3. Segregation pattern of UV-sensitive mutations

To determine the inheritance pattern of the mutation, the mutants were crossed to wild type. UV-sensitivity of the F_1 generation confirmed whether the mutation was recessive or dominant. The F_1 progeny was selfed. The wild type : mutant ratio of the F_2 were analyzed. A ratio of 3:1 confirmed the monogenic nature of the trait. In all *uvs* mutants, the mutation has been determined to be recessive. Among the mutants sensitive in light, in all cases except *uvs*1, *uvs*6 and *uvs*10, the mutation segregates in a 3:1 ratio. Among the potential dark repair defective mutants, segregation patterns of mutations in *uvs*16, *uvs*17, *uvs*18, *uvs*19, *uvs*20, *uvs*29, *uvs*31, *uvs*32, *uvs*37, *uvs*38 and *uvs*39, deviate from the expected 3:1 ratio. Ratios which are less than 3:1 can be explained by deletion or inversion of chromosomes. Ratios more than 3:1 indicate interaction among genes (Strickberger 1986).

Question 4. Mutations caused by T-DNA

In order to determine whether the mutation was the result of T-DNA insertion, segregation patterns of the UV-sensitive phenotype and kanamycin marker for the *uvs* mutants were analyzed. If both UV-resistant:UV-sensitive and kanamycin resistant:kanamycin sensitive traits segregates in the ratio of 3:1 and all UV-sensitive mutants are kanamycin resistant, the linkage of the mutation to the T-DNA is confirmed (see Figure 3.7 for an ideal situation of a single gene mutation linked to T-DNA). Only 15

out of 33 *uvs* mutants, where the mutation is segregating in the ratio of 3:1, were found to be linked to the T-DNA. Other mutations were found to be independent of T-DNA insertion and may have been formed spontaneously.

In cases where the T-DNA and the mutation were linked, an attempt was made to determine the copy number of T-DNA by Southern hybridization. There were certain discrepancies associated with this experiment. The basic strategy used for finding out the copy number of T-DNA involves digesting genomic DNA with appropriate restriction enzymes (the enzyme should not have a restriction site in the probe used), separating the digested fragments by gel electrophoresis, blotting it onto a nylon membrane, and hybridizing it with probes made from different segments of T-DNA. The rationale for using different T-DNA segments is based on the fact that T-DNA integration in the plant genome is associated with rearrangement and deletion of T-DNA segments and also at times incomplete transfer of T-DNA from *A. tumefaciens*.

The long term goal of this project is to isolate plant sequences flanking T-DNA regions in cases where the mutation and the T-DNA are linked. These flanking sequences may provide clues regarding the gene involved. Thus, in this situation my main aim of performing the Southern hybridization was not to elucidate the complete structure of T-DNA for each insertion event but only to determine the copy number of T-DNA present. In case of mutants with more than one copy of T-DNA, it is not possible to ascertain which T-DNA has caused the mutation. Such mutants can be back crossed to its wild type parent for few successive generations to reduce the number of T-DNA insertions. Thus, mutants where the T-DNA has inserted only once can be chosen for isolating the flanking sequences.

Thus, in this experiment, I used a single enzyme to digest genomic DNA in each case. The probes used were 6.7 kb T-DNA left border, 3.2 kb T-DNA right border, and 1.5 kb pBR322 fragment. The hybridization experiment was performed twice with similar results. An interpretation of the probable copy number of T-DNA was done in each case. As shown in Table 3.18, in many cases the number of bands were not easily determined. Two reasons can be attributed to this. Either the bands were deleted during T-DNA insertion or the bands are actually missing due to technical reasons. In the former situation, more probes can be used to repeat the hybridization process. As all the mutants are resistant to kanamycin, it would be better to use the kanamycin resistance gene as a probe. The problem of not being able to confirm the copy number can also be due to technical reasons. First, if an assumption is made based on band intensity and different amounts of DNA was loaded in each lane, it could have very well affected the interpretation of results. But in this case, ~1μg of DNA was used for each lane and same spectrophotometer was used to measure the concentration of DNA. The probes were also made fresh each time they were used. Moreover as mentioned previously, the experiment was repeated twice with very similar results obtained each time. The T-DNA insertion event is quite complex, however by using probes from different regions of T-DNA, such as the kanamycin resistance gene, may provide more concrete and reliable information about insert number.

4.2 Concluding remarks

This project is an initial step in the long term characterization of DNA repair and mutagenesis in *A. thaliana,* and eventually in higher plants. This study has resulted in the

identification of 55 UV-sensitive mutants and classified these mutants based on their sensitivity to UV and ionizing radiation. Some of the mutants may be defective in UV-specific repair or may be protection mutants, others are potentially defective in DNA repair. Irrespective of whether the UV-sensitive mutations are linked or unlinked to T-DNA, each mutant should be characterized both phenotypically and more importantly, biochemically to provide more information on the mode of UV-damage involved.

4.3 Future work

Future work needs to be done in order to phenotypically and genotypically characterize the mutants.

For determining the UV-sensitivity of the aerial tissue, the plants were transferred to light after irradiation. Thus, it was not possible to isolate aerial tissues deficient in photoreactivating activity. This was mainly due to the unavailability of appropriate filters. Thus, the first step in the future study should involve isolating plants with leaf tissue defective in photoreactivating activity.

A comparative study of the rate of removal of the two major photoproducts induced by UV: cyclobutane pyrimidine dimer and [6-4] photoproduct is essential to biochemically characterize the mutants. This can be performed by growing seeds of the mutants and their progenitor strain on agar plates. After 5 days of growth, the seedlings should be irradiated and kept either in dark followed by immediate DNA extraction. The concentration of the UV-photoproducts can be assayed by the use of lesion-specific radioimmunoassay

(Mitchell 1985). Results of this test will confirm whether the UV-sensitivity is due to a DNA repair defect or due to an abnormality in any other mechanisms.

In this study, complementation analysis was restricted to the classes into which the mutants have been classified based on 100% inhibition of root growth, i.e., mutants belonging to each class were crossed amongst themselves. On that basis the total number of complementation groups was found to be 39. This experiment is not quite complete. In future, complementation tests between groups (inter-group) might help to reduce the number of mutants and provide an estimation of the total number of gene loci involved. In addition to doing the root test in the F_1 generation, leaves of the F_1 plants can also be irradiated. This might help in differentiating those mutants where only the UV-sensitivity of the roots complement and not the shoots.

In some cases, where the mutation and the T-DNA have been proven linked, the copy number of T-DNA in the genome could not be definitely determined with T-DNA right, left and pBR322 sequences as probes. The main reason for this may be that those parts of T-DNA might have undergone deletion or rearrangement while being inserted into the plant genome. In those cases, Southern hybridization could be repeated with the kanamycin resistance gene (present in T-DNA) as a probe. This might give better result as the mutants were all resistant when selected in nutrient medium supplemented with kanamycin.

In cases, where there is one T-DNA insert (proven by hybridization), the T-DNA and the flanking plant sequences should be isolated by plasmid rescue and used as probes to probe *A. thaliana* cDNA library. Any homolog thus isolated, should be used to probe a genomic DNA library to get the full gene sequence.

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