The Plant Cell, Vol. 4, 333–347, March 1992

Effects of Ionizing Radiation on a Plant Genome: Analysis of Two Arabidopsis *transparent testa* Mutations

Brenda W. Shirley, Susan Hanley, and Howard M. Goodman [1]

Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114

Ionizing radiation is known to cause chromosomal alterations such as inversions and deletions and has been used extensively for inducing mutations. In Arabidopsis, two methods for the isolation of genes identified on the basis of mutant phenotypes—genomic subtraction and chromosome walking—either rely on or are greatly facilitated by the availability of these types of mutations. This article gives a detailed characterization of ionizing radiation-induced mutations in plants. The Arabidopsis genes encoding chalcone flavanone isomerase (CHI) and dihydroflavonol 4-reductase (DFR) were cloned and found to correspond to two *transparent testa* loci. A CHI allele, generated by fast-neutron irradiation, consisted of an inversion within the gene. A 272-bp fragment from 38 centimorgans away on the same chromosome was transferred to one end of this inversion. A DFR allele, induced by x-irradiation, contained two deletions and an inversion of the 2.8-centimorgan intervening region. Sequence analysis of the break points in both mutants indicate that repair of radiation-induced damage involves mechanisms similar or identical to those that mediate the integration of foreign sequences into the genome. The chromosome rearrangements found in these mutants have important implications for the use of ionizing radiation-induced alleles in classical and molecular genetic experiments in plants.

INTRODUCTION

Induced mutagenesis, which has been used extensively in plant breeding programs and classical genetic studies, is gaining importance as a tool for the identification and isolation of plant genes using molecular approaches. In many plants, screening or selection of mutagenized populations has been used to identify loci of novel genes or of previously cloned sequences. These mutants can often be classified into phenotypic groups that provide the basis for dissecting complex signal transduction and biosynthetic pathways. These types of studies are providing insight into the interactions of genes and gene products that function, for example, in the light response, in flower development, and in hormone biosynthesis and function.

In plants, the isolation of genes corresponding to mutant loci relies primarily on three methods. Gene tagging by transposons or T-DNA has been used to isolate a number of genes in maize, snapdragon, and Arabidopsis (O'Reilly et al., 1985; Herman and Marks, 1989; Coen et al., 1990; Konz et al., 1990). In this approach, genes are cloned from plants exhibiting a mutant phenotype by isolating regions adjacent to inserted sequences from phage libraries or by plasmid rescue. Chromosome walking with cosmid and yeast artificial chromosome clones (Bender et al., 1983; Jordon, 1988) has also been used successfully in Arabidopsis to approach the chromosomal locations of several genes (I. Hwang, T. Kohchi, and H. Goodman, unpublished results). These genes will ultimately be identified either by locating the mutation or by complementing the mutant phenotype in transgenic plants. The utility of a third method, genomic subtraction (Straus and Ausubel, 1990), to directly clone sequences corresponding to deletions, has recently been demonstrated in Arabidopsis (Sun et al., 1992). All three of these methods allow genes that are identified by a mutant phenotype to be isolated without requiring specific information about the structure or function of the gene or gene product.

The chemical agents and radiation used for mutagenesis can cause very different types of lesions in chromosomal DNA. Mutations that delete several kilobases at the locus of interest would be particularly useful for the identification and isolation of genes by chromosome walking or genomic subtraction. It is well established that ionizing radiation can cause deletions as well as other types of chromosome alterations by double-strand breaks followed by aberrant rejoining of the ends. More than 50 years ago, McClintock showed that chromosomes broken by x-ray treatment of maize pollen were joined with extraordinary efficiency and that this often resulted in chromosome rearrangements (reviewed in McClintock, 1984). However, the cellular mechanisms that contribute to these types of rearrangements are still poorly understood. Mutations

[1] To whom correspondence should be addressed.

involving large deletions have only rarely been identified in plants, and, even in other organisms, only limited analysis of these types of lesions has been performed. No efforts have been made to identify conditions that favor the formation of specific types of mutations.

This article describes the characterization of radiation-induced mutations at two flavonoid biosynthetic loci in Arabidopsis. Flavonoids are an enormously diverse group of secondary metabolites that are unique to plants and that play a variety of roles in development, reproduction, and survival (for a review, see Stafford, 1990). Despite these numerous functions, mutations in flavonoid biosynthesis are nonlethal. This feature, together with the availability of easily scorable phenotypes such as seed and flower color, has led to detailed analyses of the genetics and biochemistry of flavonoid synthesis in maize, petunia, and snapdragon (Stafford, 1990). The flavonoid pathway has also been a model system for the isolation of structural and regulatory genes by transposon tagging (e.g., Wienand et al., 1982; Luo et al., 1991). Flavonoid loci for which genes have not yet been isolated are thus good candidates for genetic approaches to gene cloning.

Eleven flavonoid biosynthetic loci have been identified in Arabidopsis (Koornneef, 1990b). Mutations at these loci result in plants that produce yellow or ochre seeds, rather than the dark brown seeds characteristic of wild-type Arabidopsis plants. This phenotype is the result of a reduction or absence of pigments in the seed coat. The mutants are therefore named *tt* for *transparent testa* (seed coat). Many of the *tt* loci also control the production of flavonoids in other organs, indicating that at least some of the components of the pathway function throughout the plant. The only flavonoid gene previously cloned in Arabidopsis encodes the enzyme chalcone synthase, which catalyzes the first committed step in flavonoid production (Feinbaum and Ausubel, 1988). Restriction fragment length polymorphism (RFLP) mapping experiments indicate that this single-copy gene corresponds to the *TT4* locus (Meyerowitz, 1990), which is consistent with the complete absence of flavonoid compounds in *tt4* mutant plants (M. Koornneef, personal communication; B. Shirley, W. Kubasek, G. Stortz, M. Koornneef, F. Ausubel, and H. Goodman, unpublished results).

To extend the analysis of flavonoid biosynthesis in Arabidopsis, the genes encoding chalcone flavanone isomerase (CHI) and dihydroflavonol 4-reductase (DFR) were cloned. CHI and DFR catalyze the conversion of chalcones into flavanones and dihydroflavonols into flavan-3,4-diols, respectively. Two alleles—*tt3* (M318) and *tt5* (40.443)—that were isolated using x-ray or fast-neutron radiation contained rearrangements of these genes. In both cases, the mutations also affected sites located a substantial distance away on the same chromosome. Sequence analysis of the break points indicates that the rearrangements were caused by aberrant joining of double-strand breaks by nonhomologous recombination. The chromosome rearrangements found in these Arabidopsis mutants have important implications for use of radiation-induced alleles in gene cloning strategies.

RESULTS

Isolation of the Arabidopsis CHI and DFR Genes

As a first step toward extending the analysis of flavonoid biosynthesis in Arabidopsis, genes homologous to two flavonoid structural genes previously cloned in other plants were isolated. Initial attempts to use a heterologous CHI cDNA or degenerate oligonucleotides to isolate the genes from a genomic phage library were unsuccessful. As an alternative approach, degenerate oligonucleotides were used with Arabidopsis genomic DNA in the polymerase chain reaction (PCR) according to the method of Gould et al. (1989). The oligonucleotide primers, shown in Figures 1 and 2, were designed based on the sequences from bean (Blyden et al., 1991) and petunia (van Tunen et al., 1989) for CHI and from maize (O'Reilly et al., 1985), snapdragon, and petunia (Beld et al., 1989) for DFR. The products obtained using primers I and IV were reamplified with internal primers (II and III) to ensure that the correct products were synthesized. In all reactions, the primers flanked the sites of one or more introns in the genes from other species. Single products of the expected lengths (~320 and 300 bp for CHI, 1.1 and 0.9 kb for DFR, assuming introns of 100 bp) were obtained with genomic DNA from both the Landsberg and Columbia ecotypes of Arabidopsis (data not shown). In the case of CHI, the PCR-amplified products hybridized to a CHI cDNA clone from petunia (data not shown). The 320-bp CHI product and the 0.9-kb DFR product were cloned into plasmid vectors. Sequence analysis of the putative CHI clone and the ends of the DFR clone confirmed the identification of these products as novel CHI and DFR genes.

The PCR-derived clones were used to screen a Landsberg genomic library (Voytas et al., 1990). Four overlapping λ clones were obtained with each probe. Plasmid subclones were constructed and used for sequencing and DNA and RNA gel blot analyses (see Methods). Because low-stringency hybridization of genomic DNA gel blots failed to uncover additional strong bands (data not shown) and unique products were isolated in the PCR cloning experiments, it appears that, like chalcone synthase (CHS), CHI and DFR are encoded by single-copy genes in Arabidopsis.

The complete nucleotide sequences of the Arabidopsis CHI and DFR genes were determined, as shown in Figures 1 and 2. Both genes exhibited significant homology with the corresponding genes isolated in other plant species. Intron positions were deduced by comparison with the petunia CHI-B gene and the petunia and snapdragon DFR genes. The hexanucleotide CACGTG is present in the 5′ untranslated regions of the CHI gene (one copy) and the DFR gene (two copies). This motif is part of the sequence that mediates the response of the parsley CHS gene to UV light (Schulze-Lefert et al., 1989) and is identical to the putative binding site for the product of the maize *R* gene (Goff et al., 1990). Similar or identical motifs are present in the 5′ untranslated regions of other light-, hormone-, and

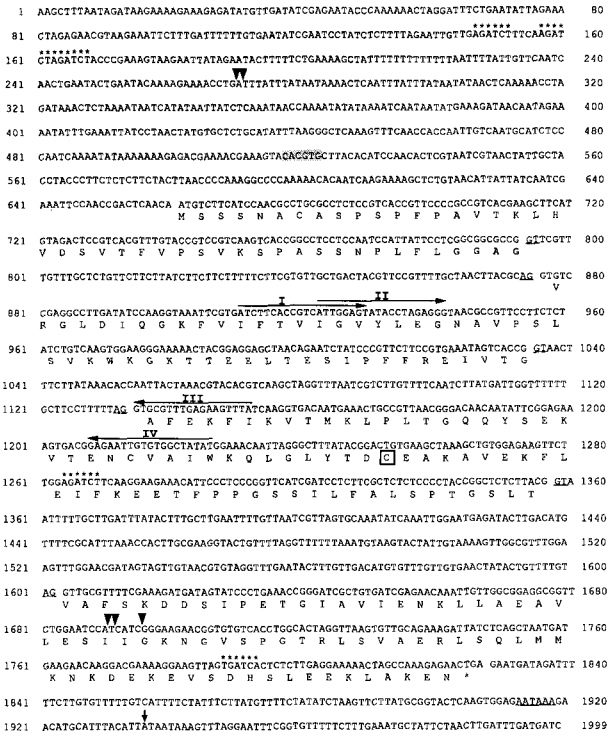

Figure 1. Sequence of the Arabidopsis CHI Gene.

The positions of three introns are inferred by comparison with the petunia CHI-B gene (van Tunen et al., 1989). The polyadenylation site found in a cDNA clone is indicated with a vertical arrow, and a putative polyadenylation signal and conserved GT and AG nucleotides at the intron boundaries are underlined. The CACGTG hexanucleotide (see text) present in the 5′ untranslated region is shaded. Asterisks above the sequence indicate the positions of BglII and BclI sites. Triangles mark the inversion break points identified in *tt5*. The active-site cysteine in the deduced amino acid sequence of the CHI enzyme is boxed. Horizontal arrows (I to IV) show the positions of the primers used for cloning the gene by PCR.

stress-regulated genes, including the Arabidopsis CHS gene (Feinbaum and Ausubel, 1988; Staiger et al., 1989; Guiltinan et al., 1990; Martin et al., 1991). The region surrounding the active-site cysteine (Bednar et al., 1989) in the deduced amino acid sequence of the Arabidopsis CHI gene is highly conserved relative to the four other published CHI sequences. The Arabidopsis, snapdragon (Martin et al., 1991), and petunia CHI genes are more similar to each other than to the gene from bean, which may reflect the additional function of the legume enzyme in the synthesis of isoflavonoids (Blyden et al., 1991). The 13 amino acids thought to define the substrate specificity of the DFR enzyme (Beld et al., 1989) are most similar between the Arabidopsis and snapdragon proteins. This is consistent with the observation that these two species synthesize the DFR substrates dihydroquercetin and dihydrokaempferol, in contrast to petunia, which makes dihydroquercetin and dihydromyrecetin (Harrison and Stickland, 1974; Meyer et al., 1987; B. Shirley, W. Kubasek, G. Stortz, M. Koornneef, F. Ausubel, and H. Goodman, unpublished results).

RFLP Mapping of the CHI and DFR Genes

To determine whether the CHI and DFR genes corresponded to any of the known flavonoid loci in Arabidopsis, the chromosomal locations of the two genes were determined by RFLP

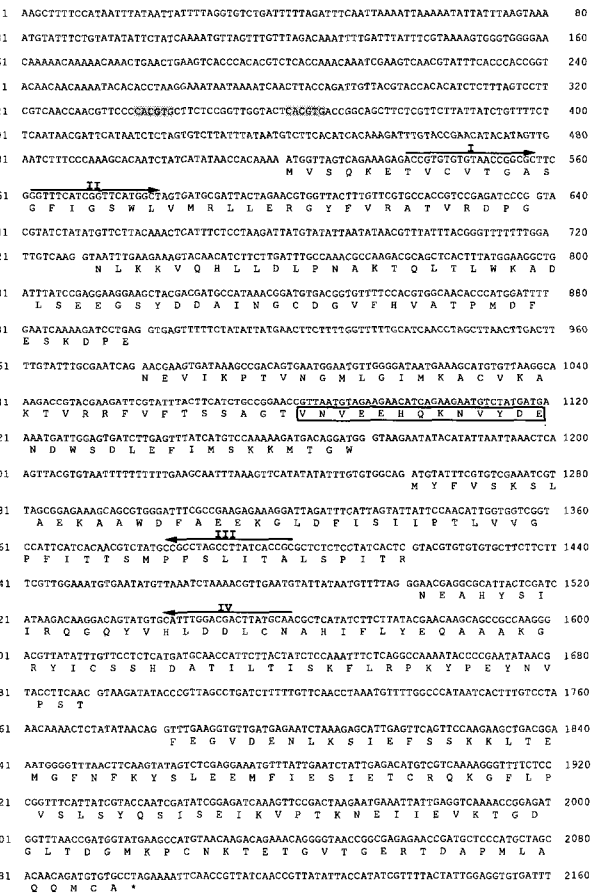

Figure 2. Sequence of the Arabidopsis DFR Gene.

The positions of five introns are inferred from the sequences of DFR genes from petunia and snapdragon (Beld et al., 1989). Two CACGTG hexanucleotides (see text) present in the 5′ untranslated region are shaded. The 13 residues in the deduced amino acid sequence of DFR that may determine substrate specificity (Beld et al., 1989) are boxed. Arrows (I to IV) show the positions of the primers used for cloning the gene by PCR.

mapping. The PCR-generated CHI clone, pCHI14, revealed a BclI polymorphism for the Landsberg and Columbia ecotypes of Arabidopsis. An EcoRI polymorphism was identified using the DFR PCR clone, pDFR2. The clones were used to probe blots containing genomic DNA representing 128 F_2 plants from a Landsberg/Columbia cross (Nam et al., 1989).

The CHI gene mapped to the short arm of chromosome 3 in the region of the *tt5* and *tt6* loci on the genetic map, as shown in Figure 3A. It was unlikely that CHI corresponded to the *tt6* locus. Thin-layer chromatographic analysis of pigments present in *tt6* plants indicates that this mutation affects one of the

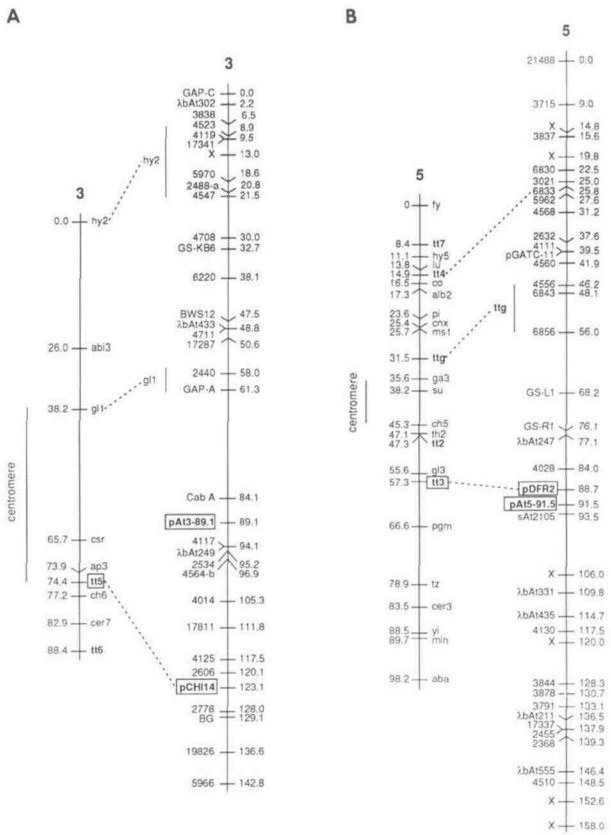

Figure 3. Correlation between CHI and DFR Sequences on the RFLP Map and the Locations of *tt* Loci on the Genetic Map.

The locations of pCHI14, pAt3-89.1, pDFR2, and pAt5-91.5 (boxed) were determined by RFLP mapping as described in the text. The locations of these clones on the RFLP map (Nam et al., 1989; S. Hanley and H. Goodman, unpublished results) are compared to the locations of *tt* mutants on the genetic maps (Koornneef, 1990a). The *tt* loci are in bold. Markers corresponding to unpublished clones are indicated by an X. The positions of three phenotypic markers—*hy2*, *gl1*, and *ttg*—that were used to integrate the genetic and RFLP maps are indicated by vertical bars (Nam et al., 1989). The contact points between the genetic and RFLP maps of the two chromosomes are indicated by dashed lines.

(A) Chromosome 3.

(B) Chromosome 5.

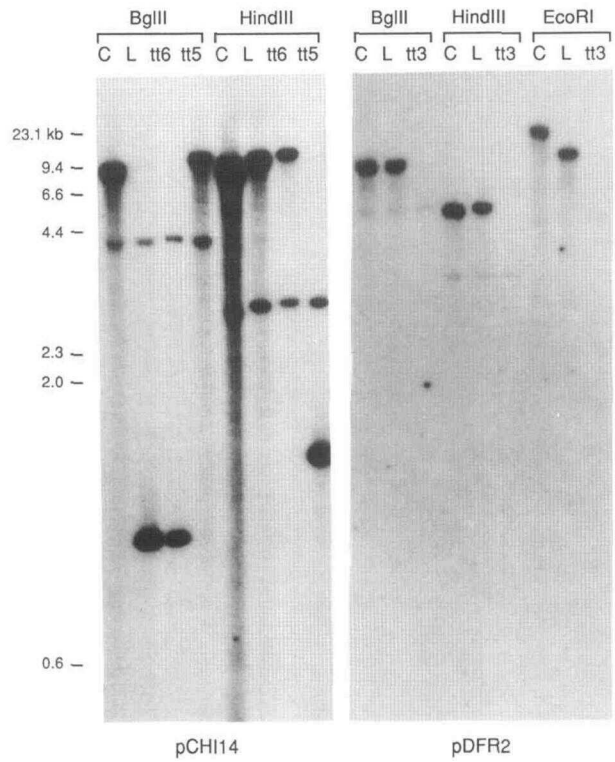

Figure 4. DNA Gel Blot Analysis of *tt3* and *tt5* Using PCR Clones.

Gel blots containing digests of genomic DNA (2 μg per lane) from Columbia (C), Landsberg (L), *tt5*, *tt6*, or *tt3* plants were hybridized with the PCR-generated clones pCHI14 or pDFR2.

intermediate enzymatic steps in flavonoid biosynthesis (B. Shirley, W. Kubasek, G. Stortz, M. Koornneef, F. Ausubel, and H. Goodman, unpublished results). In contrast, *tt5* plants appear to be totally devoid of flavonols and anthocyanidins (M. Koornneef, personal communication) and produce seeds that have a bright lemon-yellow color. This phenotype would be predicted for plants lacking CHI activity because this enzyme catalyzes an early step in flavonoid biosynthesis, the conversion of chalcones to flavanones. The DFR gene mapped to the long arm of chromosome 5, in the region of the *tt3* locus (Figure 3B). The phenotype of plants with mutations at this locus is consistent with a defect in the DFR gene. The seeds and vegetative tissues of *tt3* plants contain flavonols but no anthocyanidins, as would be expected in the absence of DFR enzyme activity (M. Koornneef, personal communication). These results suggested that *TT5* corresponded to the CHI gene, whereas *TT3* was the genetic locus for DFR.

Structural Changes in the CHI and DFR Genes in *tt5* and *tt3*

Ionizing radiation is known to cause chromosomal damage that in some cases includes large deletions or rearrangements detectable by DNA gel blot analysis (for a review, see

Sankaranarayanan, 1991). Radiation-induced alleles of *tt3* and *tt5* were isolated in studies on the mutagenic effects of ionizing radiation in plants (Dellaert, 1980; Koornneef, 1990b). Thus, the possibility that the CHI and DFR clones could identify structural changes at the *tt3* and *tt5* loci was examined.

The *tt5* mutation (allele 40.443) was isolated from a Landsberg population mutagenized with fast-neutron radiation (Koornneef, 1990b). A gel blot containing genomic DNA from this mutant, an ethyl methanesulfonate-generated allele of *tt6*, and wild-type plants was hybridized with pCHI14, as shown in Figure 4. The size of the bands in the digests of *tt5* DNA was different from those of the wild-type DNA. This provided strong evidence that *TT5* was the Arabidopsis CHI locus.

A similar analysis was performed for the DFR gene using an allele of *tt3*, M218, isolated after irradiating Arabidopsis seeds with x-rays (Koornneef, 1990b). A DNA gel blot containing wild-type and *tt3* (M218) DNA was hybridized with pDFR2. Figure 4 shows that sequences corresponding to this probe were completely missing in *tt3* plants. This showed that *tt3* (M218) was a DFR deletion mutant and indicated that *TT3* was the Arabidopsis DFR locus.

Structure of the *tt5* Allele

The lesions in *tt5* and *tt3* plants were examined in further detail to determine the extent and exact nature of the damage induced by ionizing radiation in these mutant plants. As a first step, maps were constructed of the wild-type and mutant CHI loci. A blot containing DNA from *tt5* and wild-type plants was hybridized with plasmid subclones covering the CHI transcription unit. Figure 5A shows that, as with the 314-bp PCR-generated clone (Figure 4), the three clones spanning the CHI coding region hybridized to HindIII and BglII fragments in *tt5* that were different in size than those in wild-type plants. Further analysis using Sau3AI digests of *tt5* and Landsberg DNA (data not shown) indicated that little, if any, of the CHI sequence was missing in the mutant. Instead, *tt5* plants appeared to contain a rearrangement of CHI gene sequences within the 1.5-kb region defined by the three adjacent BglII sites in the promoter and a BclI site in the third exon of the CHI gene (Figure 1). These findings, together with results described below, indicated that the structure of the *tt5* mutation was as illustrated in Figure 5B.

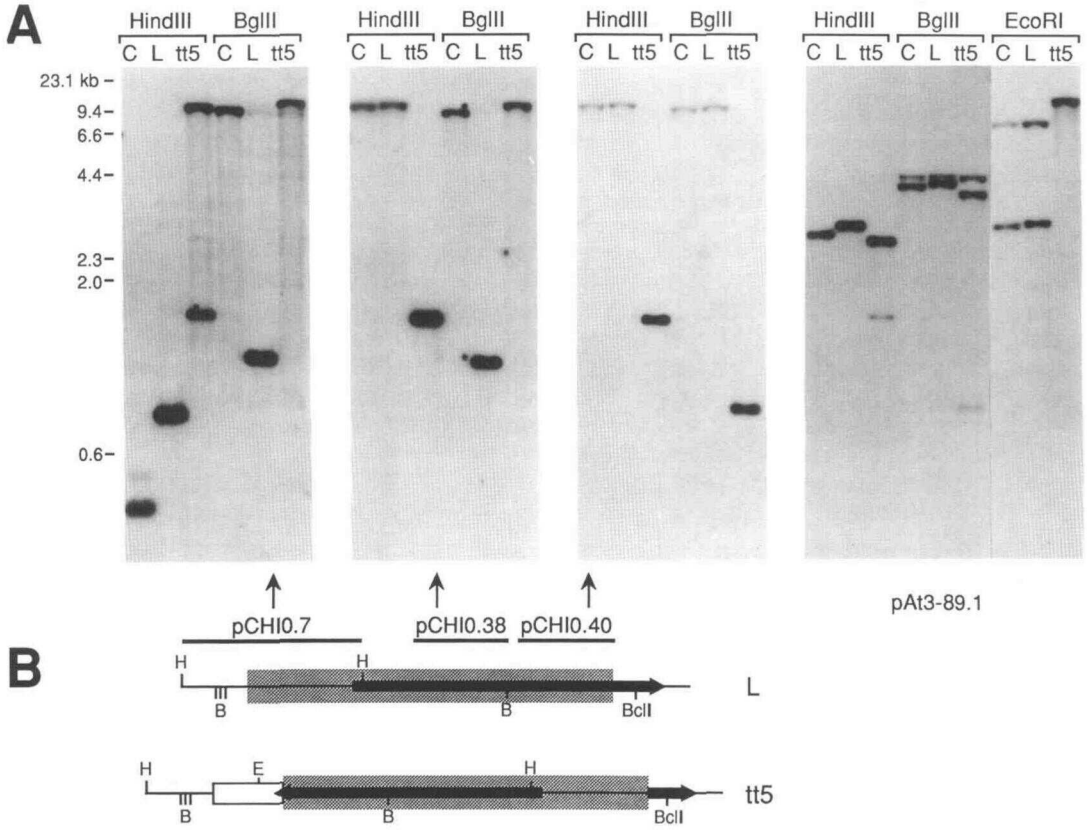

Figure 5. Structural Analysis of the CHI Gene in *tt5*.

(A) DNA gel blot containing EcoRI, HindIII, and BglII digests of DNA from wild-type (Columbia [C] and Landsberg [L]) and *tt5* plants probed sequentially with pCHI0.7, pCHI0.38, and pCHI0.40 (all described in Methods), and pAt3-89.1 (the wild-type clone from which the insertion was derived). **(B)** Structure of the CHI locus in Landsberg (L) and *tt5* plants. The shaded area indicates the region that is inverted in the mutant. The open box represents the insertion sequence. Restriction enzyme cleavage sites are as follows: BclI (not abbreviated); B, BglII; E, EcoRI; H, HindIII.

To isolate the regions surrounding the break points of the inversion, a phage library was constructed using genomic DNA from *tt5* plants. Five independent clones were isolated from this library by screening with a 4.8-kb BglII-EcoRI fragment containing the entire wild-type CHI gene. Subclones were made in a plasmid vector, and the structure of the CHI locus in *tt5* was determined by restriction mapping. The region between the three tandem BglII sites and the BclI site that flank the inversion was sequenced. The inversion break points are indicated by triangles in the wild-type sequence in Figure 1. Figure 6 illustrates the structure of the CHI locus in *tt5*. One break point was located 390 bp upstream of the AUG, thus leaving a substantial portion of the promoter attached to the coding region. The other break point was located in the fourth exon, 44 amino acids from the end of the predicted protein product. Sequencing showed that 4 bp were lost from this exon, as illustrated in Figure 7A. In addition, a 272-bp fragment, which had no homology to CHI gene sequences, was inserted at one end of the inversion. This insertion introduced two new amino acids and a stop codon in the middle of the fourth exon. No other sequence differences were found between *tt5* and Landsberg. These results indicate that the phenotype of *tt5* plants could arise because of a loss of CHI gene expression due to disruption of the structure of the promoter or the 3′ end of the gene or because the terminal 44 amino acids of the protein are essential for enzyme activity.

To determine the origin of the inserted DNA, wild-type sequences homologous to those inserted at the CHI locus in *tt5* were amplified by PCR and used to screen the Landsberg genomic library. From one phage clone, a 3.0-kb HindIII subclone (pAt3-89.1) was isolated that hybridized to the PCR product. A HindIII polymorphism between Landsberg and Columbia was used to determine the location of these sequences in wild-type plants. The clone mapped to a site 38 centimorgans (cM) proximal to the CHI gene on chromosome 3 (Figure 3A).

To determine whether the sequence inserted at the CHI locus in *tt5* had also been deleted at its original location, the wild-type clone, pAt3-89.1, was hybridized to the genomic DNA gel blot (Figure 5A). The clone hybridized to one HindIII band that was somewhat smaller in *tt5* than in Landsberg, suggesting that part of the region between these HindIII sites had been deleted in the mutant. The clone also hybridized weakly to bands from the CHI locus in *tt5* that contained the insertion and that also hybridized to pCHI0.7 and pCHI0.40. From sequence analysis, it was known that the 272-bp fragment inserted at the CHI locus contained an EcoRI site (Figure 5B). pAt3-89.1 also contained an EcoRI site. The clone hybridized to a single strong band in the *tt5* EcoRI digest and to two EcoRI fragments in wild-type DNA, consistent with the transfer of sequences flanking this site to the new location in *tt5*.

The sequences flanking the deletion were isolated from the *tt5* library by screening with pAt3-89.1. One phage clone was obtained from which a 2.7-kb HindIII fragment was subcloned. Comparison of the sequences adjacent to an XmnI site within this clone and the corresponding wild-type sequences in pAt3-89.1 showed that *tt5* contained a deletion at this location that corresponded exactly to the insertion at the CHI locus (Figure 7B). The 272-bp fragment appeared to have been transferred directly to the CHI locus. However, a 3-bp change in the *tt5* sequence had also occurred, which resulted in the in-frame stop codon at the border of the insertion in *tt5*.

The break points in *tt5* are compared to the corresponding wild-type sequences in Figure 7. Very little if any homology was present between the ends that were fused in the mutation. Thus, it was unlikely that the inversion and insertion were generated by mechanisms (e.g., template switching during replication repair) that act on homologous sequences. Instead,

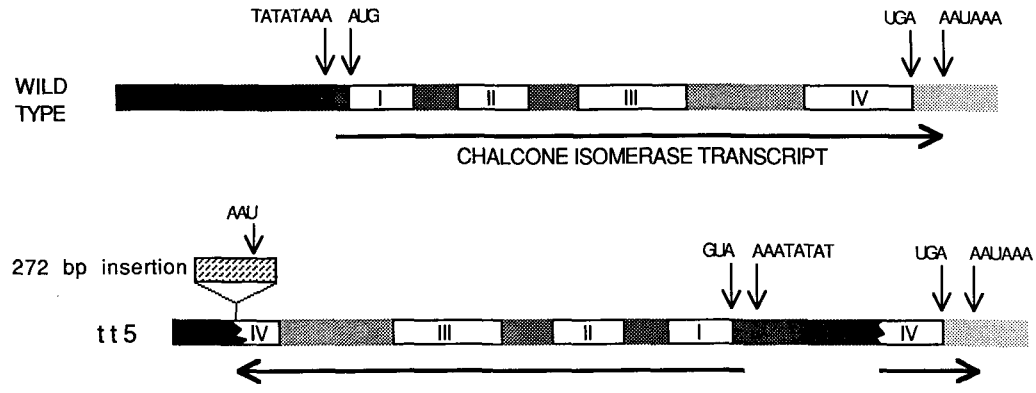

Figure 6. Structure of the *tt5* Locus.

Maps of the CHI gene in wild-type and mutant plants derived from restriction mapping and sequencing. Open boxes correspond to the four CHI exons (I to IV). Horizontal arrows indicate the locations of the CHI transcripts.

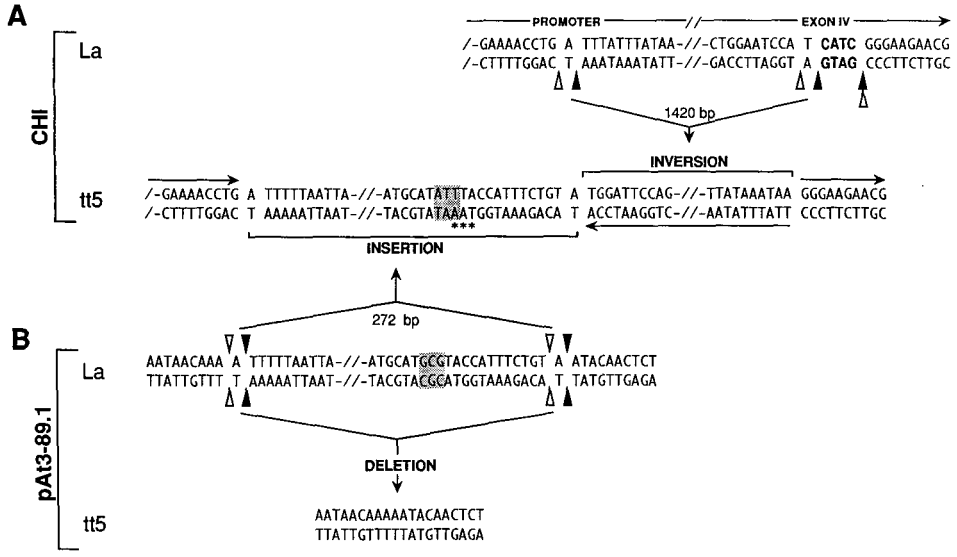

Figure 7. Sequences Surrounding the *tt5* Break Points.

Solid and open triangles indicate two possible junctions that cannot be distinguished by sequence analysis. Three base pairs of the 272 bp transferred to the CHI locus in *tt5* that are different in the wild type are shaded, the stop codon in the insertion is indicated by asterisks, and the 4 bp deleted from the fourth exon are in bold type. The direction of the wild-type CHI transcript is indicated by horizontal arrows next to the wild-type and mutant sequences.

(A) Sequence of the CHI gene in *tt5* and Landsberg.

(B) Sequence at the pAt3-89.1 locus in *tt5* and Landsberg.

it appears that breaks introduced by fast-neutron irradiation were aberrantly joined by way of mechanisms involving nonhomologous recombination.

Structure of the *tt3* Allele

The structure of the lesion induced by x-rays in the *tt3* (M218) allele was also examined in detail. To determine the extent of the deletion in *tt3*, a gel blot containing various digests of Columbia, Landsberg, and *tt3* genomic DNA was hybridized with a series of HindIII clones spanning an 11.8-kb region that included the DFR gene, as shown in Figure 8. Sequences contained in two of the clones, pDFR4.4 and pDFR2.3, were completely absent in *tt3*. Adjacent clones, pDFR2.1 and pDFR3.0, hybridized to HindIII bands that were longer in *tt3* than in Landsberg. Thus, the end points of the deletion in *tt3* were located within these two fragments. According to these results, ~8 kb of sequence had been deleted at the DFR locus (Figure 8B). In addition, pDFR2.1 and pDFR3.0 did not hybridize to the same HindIII band in *tt3*. This indicated that the break points at the DFR locus had not been rejoined to each other. New sequences had either been inserted between the two break points or the ends had been fused to other breaks in the Arabidopsis genome.

To distinguish between these possibilities, the sequences fused to the deletion end points were isolated. A genomic library was constructed for *tt3* and screened using pDFR2.1 and pDFR3.0 (Figure 8B). The five independent clones isolated from this library hybridized to either pDFR2.1 (two clones) or pDFR3.0 (three clones), but not to both probes. This indicated that these sequences might be separated by a significant distance in the mutant. Fragments corresponding to the 4.7- and 3.2-kb HindIII fragments in *tt3* (Figure 8, hybridizations with pDFR2.1 and pDFR3.0) were subcloned from two of the *tt3* genomic clones and designated ptt3-4.7 and ptt3-3.2, as illustrated in Figure 9. The 1.9-kb BglII-HindIII fragment and the 0.7-kb HindIII-BamHI fragment from these clones, which contained sequences not present in the wild-type DFR clones, were then isolated. These fragments hybridized to a single 3.6-kb HindIII fragment in Landsberg genomic DNA (data not shown, but see hybridization with pAt5-91.5, below). This showed that the ends of the DFR deletion were fused to the ends of a second break that had occurred at another site in the Arabidopsis genome. Thus, in addition to the DFR deletion, *tt3* plants contained either an inversion or a translocation.

To determine the location of the second break site, the BglII-HindIII and BamHI-HindIII fragments from ptt3-4.7 and ptt3-3.2 were used to isolate the 3.6-kb HindIII fragment from the Landsberg genomic library (Figure 9). This clone, designated

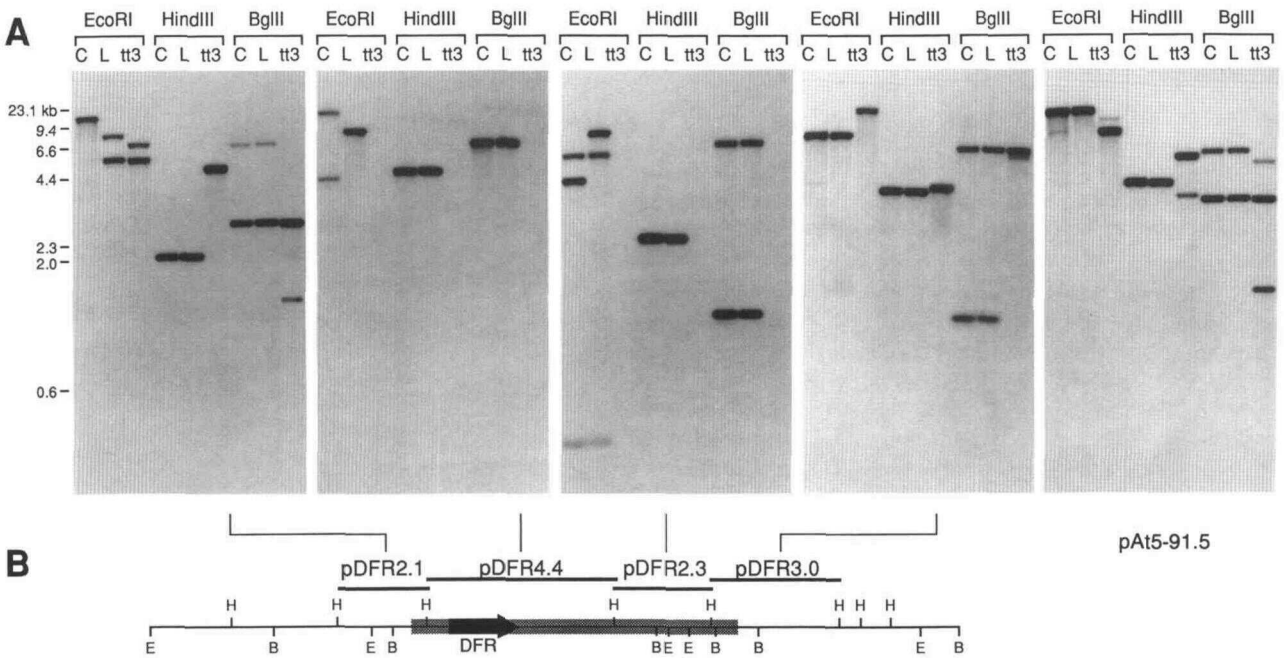

Figure 8. Structural Analysis of the DFR Locus in *tt3*.

(A) Gel blot containing DNA from Columbia (C), Landsberg (L), and *tt3* plants was probed sequentially with pDFR2.1, pDFR4.4 (which contains the DFR gene), pDFR2.3, pDFR3.0, and pAt5-91.5 (the wild-type clone containing the distal break point).
(B) Restriction fragment map of the DFR locus in Landsberg. The location of the DFR gene is indicated by the arrow. Bars above the map show the four HindIII fragments used as probes. The shaded area indicates the region deleted in *tt3*. B, BglII; E, EcoRI; H, HindIII.

pAt5-91.5 (see below), hybridized to 3.2- and 4.7-kb HindIII bands in *tt3* DNA (Figure 8). This was a composite of the hybridization pattern observed for pDFR2.1 and pDFR3.0 and thus confirmed that the deletion end points were contained on a single HindIII fragment in the wild-type genome.

pAt5-91.5 did not hybridize to any of the phage clones that contained the wild-type DFR sequences (data not shown) and hybridized to different yeast artificial chromosome clones (S. Hanley and H. Goodman, unpublished results), again suggesting that the second break had occurred a significant distance away from the DFR gene. To determine the location of this second break site, the position of pAt5-91.5 on the RFLP map was determined using a *Dra*I polymorphism. This showed that the second break had occurred 2.8 cM distal to the DFR gene on the same chromosome (Figure 3B). Based on information from the genetic and physical maps, this corresponds to ~560 kb in Arabidopsis (Nam et al., 1989; B. Hauge and H. Goodman, unpublished results).

The regions surrounding the break points in pDFR2.1, pDFR3.0, ptt3-4.7, ptt3-3.2, and pAt5-91.5 were sequenced. The results are presented schematically in Figure 10 and in detail in Figure 11. The deletion at the DFR locus encompassed 7.4 kb, starting 675 bp upstream of the AUG and extending 5.1 kb beyond the stop codon. In addition, 52 bp were deleted at the site of the second break point, and a 7-bp piece of filler DNA was present at one end of the inversion. Short imperfect direct repeats were present at the break points, but there was no homology between the regions joined in the mutant, indicating that, as in *tt5*, repair of radiation-induced DNA damage in *tt3* had involved nonhomologous recombination.

Analysis of Gene Expression in *tt3* and *tt5* Plants

To examine the effects of the *tt3* and *tt5* mutations on gene expression, 3-week-old plants were treated with high-intensity

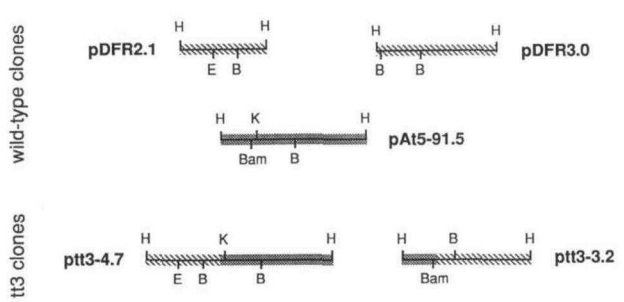

Figure 9. Break Point Clones from *tt3* and Landsberg.

Hatched areas correspond to sequences from the DFR locus; shaded areas identify sequences from the region of the distal break point. Bam, BamHI; B, BglII; E, EcoRI; H, HindIII; K, KpnI.

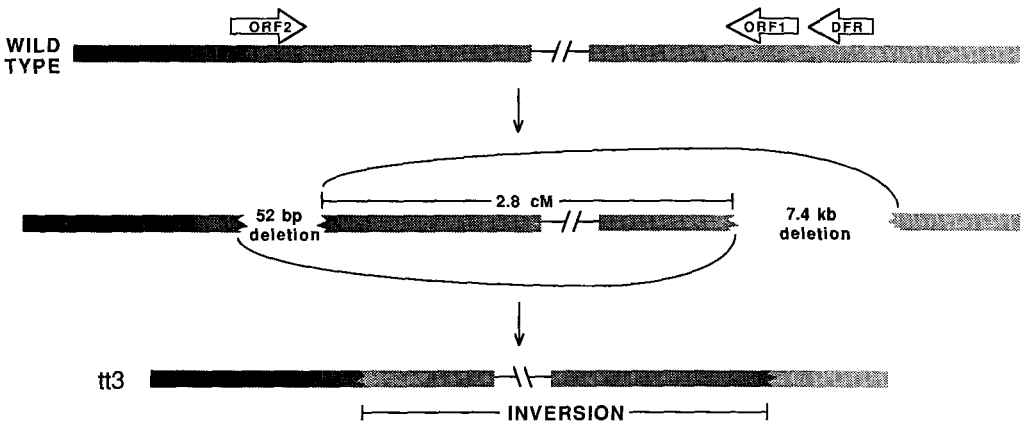

Figure 10. Structure of Chromosome Rearrangements in *tt3*.

The map of this region was derived from restriction mapping and sequencing clones from wild-type (Landsberg) and *tt3* genomic libraries and by RFLP mapping of the distal break point. The arrows indicate the locations of the three genes affected by the mutation. Only the direction of transcription of the three genes relative to each other was determined. It is not known whether transcription of ORF2 is toward the centromere (as shown) or toward the distal end of chromosome 5. ORF, open reading frame.

white light for 18 hr to induce expression of the flavonoid genes. Total RNA was isolated from these and control (untreated) plants and used to prepare a gel blot. The filter was hybridized with pCHI4.8 and then stripped and hybridized with pDFR4.4, clones that contained the entire CHI and DFR gene sequences, respectively. The results are shown in Figure 12. Like CHS (Feinbaum and Ausubel, 1988), CHI and DFR mRNA was induced to high levels in wild-type plants treated with high-intensity light. Low levels of CHI mRNA were also detectable in *tt5* plants following induction. This suggests that the 390-bp region upstream of the AUG might contain promoter sequences that control the response to high-intensity light. The reduced levels of CHI mRNA in *tt5* relative to Landsberg plants suggest that additional sequences necessary for full promoter activity lie upstream of the 5′ break point or that loss of 3′ sequences affected transcription or mRNA stability. This is similar to the observation of Feinbaum et al. (1991) that 186 bp of the Arabidopsis CHS gene promoter directed expression of a

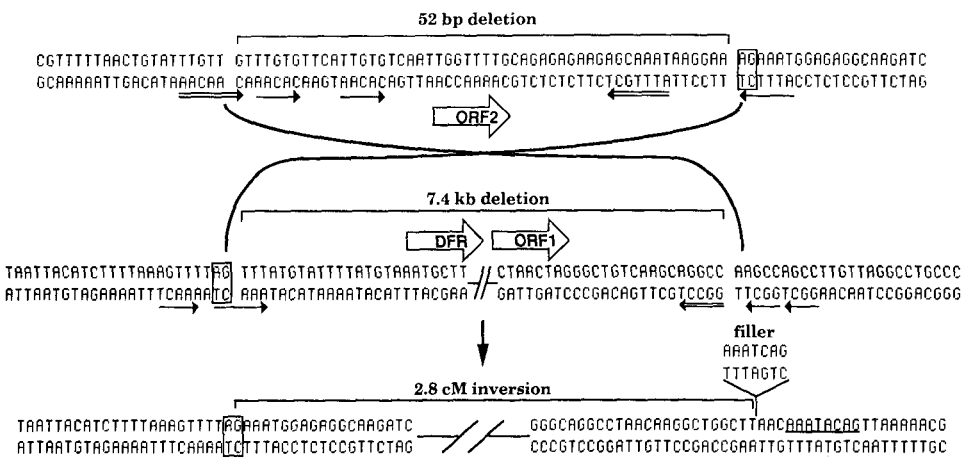

Figure 11. Sequences Surrounding the *tt3* Break Points.

Boxed sequences in the mutant were derived from either the DFR locus (7.4-kb deletion) or one end of the distal break point (52-bp deletion). Arrows under the sequence indicate small direct repeats at the borders of the break points, two of which are imperfect (double line). Sequences with imperfect homology to the filler sequence in *tt3* are underlined. ORF, open reading frame.

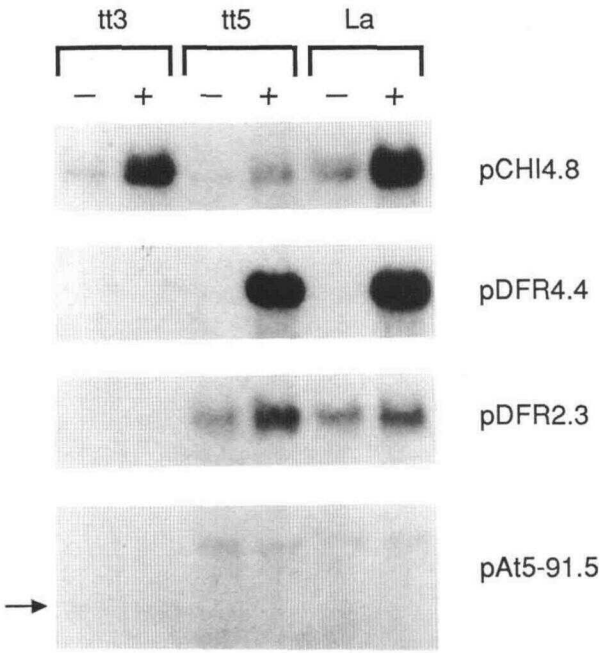

Figure 12. RNA Gel Blot Analysis of Gene Expression in La, *tt3*, and *tt5* Plants.

Three-week-old plants were exposed to high-intensity white light to induce expression of the flavonoid pathway. Total RNA was isolated from induced (+) and control (–) plants and analyzed on a gel blot (10 μg per lane). The filter was hybridized with pCHI4.8 (which contains part of the CHI coding region), pDFR4.4 (which contains the DFR gene), pDFR2.3 (fragment deleted in *tt3* located adjacent to DFR gene), and pAt5-91.5 (wild-type clone corresponding to the distal break point). The arrow indicates the position of the truncated transcript that hybridizes to pAt5-91.5 in *tt3*. La, Landsberg *erecta* ecotype.

reporter gene in response to high-intensity light and that higher overall levels of expression were observed using a 523-bp promoter fragment. No DFR mRNA was detected in the deletion mutant, *tt3*, as expected. Interestingly, the levels of CHI mRNA in the DFR mutant and DFR mRNA in the CHI mutant were similar to those found in the wild type. This indicates that changes in the concentrations of flavonoid intermediates may not affect the expression of the CHI and DFR genes.

The finding that the lesions in both *tt* mutants involved sites at other locations and that *tt3* contained a large deletion at the DFR locus indicated that genes other than CHI and DFR may have been disrupted by the mutations. No signal was detected when the RNA gel blot was hybridized with pAt3-89.1, which contains the wild-type sequences transferred to the CHI locus in *tt5*, and limited sequence analysis of this clone revealed no evidence of an open reading frame (data not shown). Thus, the effect of the *tt5* mutation on gene expression may be restricted to the CHI locus. However, when the pDFR2.3 fragment was used to probe the blot, a transcript was detected in *tt5* and Landsberg plants (Figure 12). The absence of this transcript in *tt3* plants is consistent with its being derived at least in part from the 2.3-kb HindIII fragment that is deleted in *tt3*. An open reading frame was present at one end of this clone (data not shown). Interestingly, the levels of this transcript were somewhat higher in both *tt5* and wild-type plants induced with high-intensity light. A small amount of hybridization was also detected when pAt5-91.5, which corresponds to the second break site in *tt3*, was used as a probe. The transcript was smaller in *tt3* than in Landsberg and *tt5* plants (indicated by the arrow in Figure 12). Sequencing revealed an open reading frame, the 3′ end of which would be lost as a result of the break and fusion to the DFR locus in *tt3* (data not shown). This is consistent with the finding that the BglII-HindIII fragment from this clone did not hybridize to the transcript in *tt3* (data not shown). The results of this analysis indicate that at least two other genes were disrupted in *tt3*. However, *tt3* plants do not exhibit any discernable phenotypes other than the absence of flavonoid pigments.

DISCUSSION

Deletion and inversion mutants have been used extensively to identify genes and study gene and chromosome structure in systems such as Drosophila, yeast, and some mammals. Deletions and rearrangements can also facilitate gene cloning by methods such as chromosome walking and genomic subtraction. However, the deletion of the DFR gene in *tt3* is, to our knowledge, only the fourth example of a specific locus for which a deletion mutant has been identified in Arabidopsis. The other loci for which deletion mutants have been found are *ga1*, a locus involved in giberrellin biosynthesis (Koornneef, 1979; Sun et al., 1992); *chl3*, which corresponds to one of the nitrate reductase genes (Wilkinson and Crawford, 1991); and *gl1*, which is involved in trichome development (Oppenheimer et al., 1991). These mutants were all isolated using ionizing radiation (fast-neutron, x-ray, or γ-ray), suggesting that a variety of radiation types may be effective in generating deletion mutants in Arabidopsis.

Although ionizing radiation–induced deletions have been reported in many organisms, the structures of these mutations have not been studied in detail. The mutations in the two flavonoid biosynthetic alleles examined here were much more complex than simple rejoining of adjacent break points. In the x-ray–induced *tt3* allele, breaks at sites located 2.8 cM apart were aberrantly rejoined in such a way that the intervening sequences, minus 52 bp and 7.4 kb at the ends, were inverted. These rearrangements affected the expression of at least two genes other than DFR. In contrast, only four nucleotides were lost in the rearrangements examined in *tt5*, which included inversion of a 1.5-kb fragment containing most of the CHI coding region and part of the promoter. A 272-bp fragment was transferred to this region from a site 38 cM away on the same chromosome, but this did not appear to disrupt additional

genes. Sequence analysis revealed little or no homology between the ends that were fused in *tt3* and *tt5*. In many organisms, fusion of broken ends of chromosomes occurs randomly and thus presumably by nonhomologous recombination (Roth and Wilson, 1988). Studies of end joining of foreign DNA in mammalian and amphibian cells have also shown that cells can ligate blunt or mismatched ends by mechanisms that require little or no homology (Roth and Wilson, 1986; Pfeiffer and Vielmetter, 1988). In addition, the 272-bp insertion at the CHI locus in *tt5* and the 7-bp insertion in *tt3* are reminiscent of the filler sequences found at illegitimate recombination junctions in mammalian cells (Roth and Wilson, 1988; Begley et al., 1989; Chen et al., 1990) and in T-DNA insertion sites in Arabidopsis and tobacco (Gheysen et al., 1991; Mayerhofer et al., 1991). Thus, the fusion of broken ends generated by ionizing radiation appears to occur by mechanisms involving nonhomologous recombination that are similar or identical to those that mediate the integration of foreign sequences into the genome.

There are two possible scenarios for the way in which the rearrangements in *tt3* and *tt5* arose. One possibility is that each mutation involved four double-strand breaks. In *tt5*, one fragment was inverted and another became inserted at a new site, leaving behind a 272-bp deletion. In *tt3*, aberrant rejoining of four ends resulted in an inversion and loss of two fragments. Alternatively, one or both of the deletions in *tt3* could have resulted from exonucleolytic digestion at the broken ends prior to rejoining. However, there is no evidence of extensive exonucleolytic digestion accompanying the rearrangements in *tt5*. In addition, little or no exonucleolytic activity is observed in the case of retroviral or transposon insertions in mammalian cells, which also involve free ends and illegitimate recombination (Roth and Wilson, 1988). It has been suggested that the ionizations from the track of a single charged particle could cause closely spaced double-strand breaks (Sankaranarayanan, 1991). In the case of the *tt5* lesion, two breaks apparently did occur close together, followed by inversion of the intervening CHI gene sequences. These findings argue that multiple breaks also occurred in *tt3* and that sequences were deleted as a consequence of the aberrant rejoining of the free ends.

The differences in the types of rearrangements present in *tt3* and *tt5* could be due to differences in the type of radiation used to generate these alleles. Early studies on mutagenesis of Arabidopsis and other plant species showed that different mutant spectra were obtained using radiation and chemical treatments. In general, a wider mutant spectrum was obtained using chemical treatments such as ethyl methanesulfonate than with radiation. However, some phenotypes were observed in Arabidopsis exclusively using fast neutrons (Dellaert, 1980), and differences in the mutant spectra after x-ray and fast-neutron irradiation have been observed in barley (Lundqvist et al., 1962; Persson and Hagberg, 1969). Although some of these differences could be due to secondary phenotypic effects of the mutations, experiments with dithiothreitol indicated that fast-neutron irradiation induced a relatively higher frequency of single- and double-stranded breaks in Arabidopsis, similar to the effects seen with fast neutrons and γ-rays in bacteriophage DNA (Hawkins, 1979). Although the structures of the other three deletion mutants identified in Arabidopsis have not been examined in detail, the lesions induced in *tt5* by fast neutrons were restricted to smaller regions than the lesions in *tt3*, which were induced by x-rays. It is possible that fast neutrons cause somewhat different lesions due to the higher linear energy transfer and densely ionized tracks of this type of radiation compared to the low linear energy transfer and sparse ionizations of x-rays and γ-rays (Hawkins, 1979). Although there is insufficient data to know whether this is a general effect of fast-neutron irradiation, the broader mutant spectrum observed for this mutagen (Dellaert, 1980) is consistent with the observation of a more restricted effect on the structure of *tt5* than *tt3*. It is also known that the conditions of irradiation, including dose, dose rate, and the oxygen and moisture content of the target tissue, can affect the outcome of mutagenesis in plants (Underbrink et al., 1970). However, there is as yet insufficient information on these factors to define conditions that would favor the production of deletions useful for gene cloning.

Differences in the types of rearrangements found in *tt3* and *tt5* could also reflect limitations on the types of mutations that can be sustained at some loci. It may not be possible to isolate deletion mutants for genes that must retain at least partial activity or are located in a region containing other essential genes. The latter may be an especially significant factor in plants such as Arabidopsis that have compact genomes and gene structures. This could also explain why efforts to isolate deletion mutants for the Arabidopsis alcohol dehydrogenase gene using γ radiation were unsuccessful, even though a number of point mutants were obtained in these experiments (B. Hauge, J. Giraudat, and H. Goodman, unpublished results; U. Hanfstingl and F. Ausubel, personal communication).

Curiously, in both *tt3* and *tt5* the rearrangements involved sequences from different sites on the same chromosome. It is possible that repair of DNA damage caused by radiation was influenced by the arrangement of these sequences in the nucleus. Chromosomal lesions in the dihydrofolate reductase gene in Chinese hamster ovary cells occurred adjacent to two regions attached to the nuclear scaffold (scaffold attachment regions [SARs]), which contain a consensus sequence for topoisomerase II cleavage (Käs and Chasin, 1987). Illegitimate recombination has also been linked to SARs and topoisomerase II sites in the mouse immunoglobulin κ-chain gene (Sperry et al., 1989). It is possible that the *tt3* and *tt5* alleles arose by illegitimate recombination events mediated by neighboring SARs that brought broken ends from different chromosomal locations into proximity and resulted in aberrant rejoining events. Sequences adjacent to the break points in both *tt3* and *tt5* are AT rich, a characteristic of mammalian SARs. Unfortunately, consensus sequences for plant SARs and topoisomerase II cleavage sites have not yet been reported.

The characterization of the *tt3* and *tt5* alleles raises a number of points regarding the use of ionizing radiation–induced alleles in molecular genetic experiments. This work has shown that structural mutants can provide direct correlations between

cloned gene sequences and genetic loci using DNA gel blot analysis. In Arabidopsis, these correlations provide important new contact points between the genetic and RFLP maps. Chromosome rearrangements can also provide useful information about the functions of gene sequences. For example, the break in the CHI promoter in *tt5* delimits promoter sequences that mediate the response to high-intensity light. Furthermore, although high-intensity light did induce some expression of the mutant gene, no anthocyanins were detected in these plants (data not shown). It thus appears that the C terminus of the protein is essential for the activity of the enzyme or the stability of the protein, even though this region is highly divergent in the proteins from Arabidopsis, snapdragon, petunia, and bean. Although the deletion in *tt3* gives no information about the structure of the DFR gene, it does provide a null background. This can be particularly useful, for example, for complementation experiments that can be subject to cosuppression effects. This type of gene silencing, which results from some as-yet-unknown interaction between a transformed gene and the corresponding endogenous gene(s), has been reported for a number of plant genes, including CHS and phenylalanine ammonia-lyase genes (Liang et al., 1989; Napoli et al., 1990; van der Krol et al., 1990). In addition, analysis of gene expression showed that CHI mRNA levels were similar to wild-type levels in the DFR null mutant *tt3*, which would be expected to accumulate flavonols. Similarly, wild-type levels of DFR mRNA were found in the apparent absence of CHI activity and thus the absence of DFR substrates in *tt5*. This suggests that CHI and DFR gene expression may not be affected by changes in the concentrations of flavonoid biosynthetic intermediates. On the other hand, the 2.8-cM inversion in *tt3* could complicate genetic experiments involving crosses to wild-type plants, for example, by causing additional chromosome rearrangements or distorting genetic distances by suppressing recombination. It should also be noted that because two genes are covered by a single large deletion in *tt3* and the sequences between this deletion and the middle of a third gene are inverted, the three genes disrupted in *tt3* cannot be separated by standard genetic techniques such as back-crossing. This mutation could therefore complicate efforts to assign phenotypes to specific loci.

The complexity of the rearrangements found in *tt3* and *tt5* also has implications for the use of ionizing radiation–induced alleles for gene cloning. The lesion induced in *tt5* by fast-neutron irradiation was essentially localized within the CHI gene, except for the 272-bp deletion at the 38-cM distal site. This type of rearrangement is ideally suited for chromosome walking experiments because the end point of the walk could be determined by DNA gel blot analysis, which would simultaneously provide unambiguous identification of the gene of interest. This *tt5* allele could not have been used for subtraction cloning of the CHI gene, however. Conversely, the 2.8-cM inversion in *tt3* would preclude chromosome walks from the distal side of the DFR locus. However, the 7.8-kb deletion in *tt3* is ideal for cloning by genomic subtraction. In the case of a novel gene, analysis of additional alleles or complementation of the mutant phenotype would then be used to positively identify the gene of interest. Because it is not possible to know beforehand the structure of radiation-induced mutations, the results of the analysis of *tt3* and *tt5* suggest the use of several approaches and multiple alleles in these types of gene cloning efforts.

In summary, it is evident that ionizing radiation can generate lesions that will be useful for gene cloning experiments in plants. However, the structures of the *tt3* and *tt5* alleles demonstrate that radiation can induce gross chromosome rearrangements that may involve multiple loci and that may not always be accompanied by localized deletions or inversions. Further analysis of the effects of mutagenic agents and the cellular mechanisms involved in the repair of DNA damage may lead to improved methods for generating specific types of mutations at high efficiency.

METHODS

Plants and RNA and DNA Isolation

Arabidopsis thaliana, ecotype Columbia (Col-0), was obtained from F. Ausubel (Harvard Medical School, Boston, MA). The Landsberg *erecta* ecotype and *tt3*, *tt5*, and *tt6* lines were obtained from M. Koornneef (Agricultural University, Wageningen, The Netherlands). Plants were grown in flats under standard greenhouse conditions using a 16-hr light/8-hr dark cycle. High-intensity light treatments were as described by Feinbaum et al. (1991). Tissue for DNA and RNA isolation was harvested just before the bolting stage (~3 weeks after planting) into liquid nitrogen and stored at −70°C. Genomic DNA was isolated using the method of Watson and Thompson (1986). Total RNA was isolated using the method of Ausubel et al. (1989).

Polymerase Chain Reaction Cloning of the CHI and DFR Genes

DNA fragments corresponding to the Arabidopsis chalcone flavanone isomerase (CHI) and dihydroflavanol 4-reductase (DFR) genes were synthesized by polymerase chain reaction (PCR) according to the method of Gould et al. (1989). Genomic DNA was isolated from 3-week-old Arabidopsis plants, ecotypes Landsberg and Columbia. Degenerate synthetic oligonucleotides were designed based on the amino acid sequences of the two CHI proteins from petunia and the cDNA for the gene from kidney bean (van Tunen et al., 1989; Blyden et al., 1991). The DFR primers were designed based on the deduced protein sequences for the maize, petunia, and snapdragon genes (Schwarz-Sommer et al., 1987; Beld et al., 1989). Primers were synthesized on a DNA synthesizer (model No. 8700; MilliGen/Biosearch, San Rafael, CA). The sense primers were synthesized with EcoRI linkers and the antisense primers with HindIII linkers to further ensure against cloning artifacts (i.e., products synthesized using the same primer on both strands). The positions of the primers in the CHI and DFR gene sequences are indicated in Figures 1 and 2. The primers had the following

sequences (linker sequences are given in lowercase letters, degenerate sites indicated in brackets or with an N for positions in which all four nucleotides were present):

CHI I (sense):
5′-ccggaattcAA[G,A]TT[C,T]ACNG[C,T]NAT[C,T,A]GGNGT-3′

CHI II (sense):
5′-ccggaattcAT[C,T,A]GGNGTNTA[C,T][C,T]TNGA[G,A]GA-3′

CHI III (antisense):
5′-cccaagctTNA[G,A][C,T]TT[C,T]TC[G,A]AANGGNC-3′

CHI IV (antisense):
5′-cccaagctTGNGCNAC[G,A]CA[G,A]TT[C,T]TC-3′

DFR I (sense):
5′-ccggaattcACNGTN[T,C][T,G]NGTNACNGGNGC-3′

DFR II (sense):
5′-ccggaattcGGNTT[T,C][G,A]TNGGN[A,T][C,G]NTGG[T,C]T-3′

DFR III (antisense):
5′-cccaagcttGCNGT[A,T,G]ATNA[A,G]N[G,C][T,A]NGGNGG-3′

DFR IV (antisense):
5′-cccaagctTC[A,G]CANA[A,G][A,G]TC[A,G]TCNA[A,G][A,G]TG-3′

PCR reactions were performed using *Taq* DNA polymerase (Cetus, Norwalk, CT) as described by Gould et al. (1989). The products obtained with the outside primers (I and IV) were reamplified with the set of internal primers (II and III) to ensure that the desired sequences were obtained. Annealing temperatures were based on the average of the estimated T_m of the primers (Suggs et al., 1981): 46°C for CHI I+IV, 48°C for CHI II+III, 48°C for DFR I+IV, and 52°C for DFR II+III. Hybridization of a gel blot of the PCR products with a petunia CHI cDNA clone (van Tunen et al., 1988) confirmed that CHI sequences of the predicted size had been amplified.

Library Construction and Screening

Genomic clones for the CHI and DFR genes were isolated from a λ FIX library of total Landsberg genomic DNA (Voytas et al., 1990). Genomic libraries for *tt3* and *tt5* were constructed in λ FIX II vectors (Stratagene, La Jolla, CA) and packaged using Gigapack II Plus packaging extracts (Stratagene). The Landsberg library was plated on ER1458; the *tt3* and *tt5* libraries were plated on P2392. Plaque lifts were made using Hybond filters (Amersham Corp., Arlington Heights, IL), which were then autoclaved for 10 min, UV-irradiated using a Stratalinker (Stratagene), and baked for 1 hr at 80°C. Filters were hybridized with probes as described for RNA and DNA gel blots (below).

DNA Subcloning and Sequencing

The PCR products were digested with EcoRI and HindIII and isolated from 0.8% agarose Tris-acetate-EDTA mini-gels using DEAE paper (Yang et al., 1979). Phage DNA was prepared from ER1458 lysates according to the mini-prep method of Grossberger (1987). DNA fragments were subcloned into pBluescript KS+ vectors (Stratagene) and used to transform JM109. Plasmid clones were as follows: pCHI14 (clone of the 314-bp PCR product obtained using CHI primers I and IV); pCHI4.8 (4.8-kb SalI-EcoRI subclone from λCHI-1); pCHI0.7 (0.7-kb HindIII fragment containing the CHI promoter and 58 bp of coding region); pCHI0.38 (375-bp Sau3AI fragment internal to the CHI coding region); pCHI0.40 (400-bp BglII-HinfI fragment containing sequences from the 3′ end of the CHI gene); pDFR2 (clone of the 841-bp PCR product obtained using DFR primers II and III); and pDFR4.4, pDFR3.0, pDFR2.3, and pDFR2.1 (HindIII subclones from λDFR-1 and λDFR-3; pDFR4.4 contains the DFR gene).

Double-stranded DNA was isolated from plasmid clones using the miniprep method of Birnboim and Doly (1979). Sequencing was performed using α-^{35}S-dATP and Sequenase (United States Biochemical Corp., Cleveland, OH) according to the manufacturer's protocol for double-stranded DNA sequencing, except that the DNA was denatured by boiling for 2 min and then quick-chilled in dry ice/ethanol. Sequence analysis was performed using the Sequence Analysis Software Package (Devereux et al., 1984) by the Genetics Computer Group, Inc. (Madison, WI) and the BLAST network service of the National Center for Biotechnology Information (Bethesda, MD). The nucleotide sequence data for the Arabidopsis CHI gene has been submitted to GenBank as accession number M86358. Data for the DFR gene has been submitted to GenBank as accession number M86359.

RNA and DNA Gel Blot Analysis

DNA gels were run in Tris-borate-EDTA buffer and transferred to Biotrans filters (International Chemical and Nuclear Corp., Irvine, CA) in 10 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate; Maniatis et al., 1982). RNA samples were electrophoresed in formaldehyde gels (Maniatis et al., 1982) and transferred to Biotrans filters in 25 mM sodium phosphate, pH 6.5. Filters were UV-crosslinked using a Stratalinker (Stratagene) and baked for 1 hr at 80°C. Inserts that were 1 kb or shorter were labeled by PCR using T3 and T7 oligonucleotide primers in a 20-μL reaction volume. Longer inserts were purified from phage or plasmid clones in low-melting agarose and labeled by the random-primer method (Feinberg and Vogelstein, 1983, 1984). Probes were separated from unincorporated nucleotides using a 1-mL Sephadex G-50 spin column and denatured in a microwave (Stroop and Schaefer, 1989). Prehybridizations (1 hr) and hybridizations (overnight) were at 65°C in the hybridization buffer of Church and Gilbert (1984). Filters were washed two times for 30 min in 500 mL of 40 mM $NaHPO_4$, pH 7.2, 1 mM EDTA, 1% SDS at 65°C (Church and Gilbert, 1984). The damp filters were autoradiographed at −80°C using intensifying screens. Filters were stripped in 2 mM Tris, pH 8.0, 2 mM EDTA at 70°C for 15 min prior to reprobing (Church and Gilbert, 1984).

ACKNOWLEDGMENTS

We thank Arjen van Tunen for the petunia CHI cDNA clone and Dan Voytas and Fred Ausubel for the Landsberg genomic library. We would also like to acknowledge Inhwan Hwang's assistance in dissecting the structure of the *tt5* allele and thank all of the members of the Goodman and Ausubel laboratories for advice and support. We thank two anonymous reviewers for careful reading of the manuscript and helpful suggestions. Finally, we thank Tom Gerats and Arjen van Tunen for inspiring our interest in the flavonoid pathway and Maarten Koornneef for making this study of Arabidopsis flavonoid mutants possible. This work was supported by a grant from Hoechst AG (Frankfurt, Germany).

Received November 15, 1991; accepted January 20, 1992.

REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1989). Current Protocols in Molecular Biology. (New York: Green Publishing Associates Wiley Interscience).

Bednar, R.A., Fried, W.B., Lock, Y.W., and Pramanik, B. (1989). Chemical modification of chalcone isomerase by mercurials and tetrathionate. J. Biol. Chem. **264,** 14272–14276.

Begley, C.G., Aplan, P.D., Denning, S.M., Haynes, B.F., Waldmann, T.A., and Kirsch, I.R. (1989). The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. Proc. Natl. Acad. Sci. USA **86,** 10128–10132.

Beld, M., Martin, C., Huits, H., Stuitje, A.R., and Gerats, A.G.M. (1989). Flavonoid synthesis in *Petunia*: Partial characterization of *dihydroflavonol 4-reductase genes.* Plant Mol. Biol. **13,** 491–502.

Bender, W., Spierer, P., and Hogness, D.S. (1983). Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the bithorax complex in *Drosophila melanogaster.* J. Mol. Biol. **168,** 17–33.

Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids Res. **7,** 1513–1523.

Blyden, E.R., Doerner, P.W., Lamb, C.J., and Dixon, R.A. (1991). Sequence analysis of a chalcone isomerase cDNA of *Phaseolus vulgaris* L. Plant Mol. Biol. **16,** 167–169.

Chen, Q., Cheng, J.-T., Tsai, L.-H., Schneider, N., Buchanan, G., Carroll, A., Crist, W., Ozanne, B., Siciliano, M.J., and Baer, R. (1990). The *tal* gene undergoes chromosome translocation in T cell leukemia and potentially encodes a helix-loop-helix protein. EMBO J. **9,** 415–424.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA. **81,** 1991–1995.

Coen, E.S., Romero, J.M., Doyle, S., Elliott, R., Murphy, G., and Carpenter, R. (1990). *floricaula*: A homeotic gene required for flower development in *Antirrhinum majus.* Cell **63,** 1311–1322.

Dellaert, L.M.W. (1980). The effect of dithiothreitol on radiation-induced genetic damage in *Arabidopsis thaliana* (L.) Heynh. Mutation Res. **71,** 109–126.

Devereux, J., Haeberli, P., and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. Nucl. Acids Res. **12,** 387–395.

Feinbaum, R.L., and Ausubel, R.M. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. Cell. Biol. **8,** 1985–1992.

Feinbaum, R.L., Storz, G., and Ausubel, F.M. (1991). High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. Mol. Gen. Genet. **226,** 449–456.

Feinberg, A., and Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132,** 6–13.

Feinberg, A., and Vogelstein, B. (1984). A technique for radiolabeling DNA restriction fragments to high specific activity. Anal. Biochem. **137,** 266–267.

Gheysen, G., Villarroel, R., and Van Montagu, M. (1991). Illegitimate recombination in plants: A model for T-DNA integration. Genes Dev. **5,** 287–297.

Goff, S.A., Klein, T.M., Roth, B.A., Fromm, M.E., Cone, K.C., Radicella, J.P., and Chandler, V.L. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulating genes into maize tissues. EMBO J. **9,** 2517–2522.

Gould, S.J., Subramini, S., and Scheffler, I.E. (1989). Use of the DNA polymerase chain reaction for homology probing: Isolation of partial cDNA or genomic clones encoding the iron-sulfur protein of succinate dehydrogenase from several species. Proc. Natl. Acad. Sci. USA **86,** 1934–1938.

Grossberger, D. (1987). Minipreps of DNA from bacteriophage lambda. Nucl. Acids Res. **15,** 6737.

Guiltinan, M.J., Marcotte, W.R., Jr., and Quatrano, R.S. (1990). A plant leucine zipper protein that recognizes an abscisic acid response element. Science **250,** 267–271.

Harrison, B.J., and Stickland, R.G. (1974). Precursors and genetic control of pigmentation. 2. Genotype analysis of pigment controlling genes in acyanic phenotypes in *Antirrhinum majus.* Heredity **33,** 112–115.

Hawkins, R.B. (1979). Double strand-breaks and DNA-to-protein cross-links induced by fast neutrons in bacteriophage DNA. Int. J. Radiat. Biol. **35,** 1–13.

Herman, P.L., and Marks, M.D. (1989). Trichome development in *Arabidopsis thaliana.* II. Isolation and complementation of the *GLABROUS1* gene. Plant Cell **1,** 1051–1055.

Jordan, B.R. (1988). Megabase methods: A quantum jump in recombinant DNA techniques. BioEssays **8,** 140–145.

Käs, E., and Chasin, L.A. (1987). Anchorage of the Chinese hamster dihydrofolate reductase gene to the nuclear scaffold occurs in an intragenic region. J. Mol. Biol. **198,** 677–692.

Konz, C., Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Reiss, B., Redei, G.P., and Schell, J. (1990). Isolation of a gene encoding a novel chloroplast protein by T-DNA tagging in *Arabidopsis thaliana.* EMBO J. **9,** 1337–1346.

Koornneef, M. (1979). Intragenic recombination within the GA-1 locus of *Arabidopsis thaliana.* Arabid. Inf. Serv. **16,** 41–46.

Koornneef, M. (1990a). Linkage map of *Arabidopsis thaliana.* In Genetic Maps, S.J. O'Brien, ed (New York: Cold Spring Harbor Laboratory Press), pp 94–97.

Koornneef, M. (1990b). Mutations affecting the testa color in *Arabidopsis.* Arabid. Inf. Serv. **27,** 1–4.

Liang, X., Dron, M., Schmid, J., Dixon, R.A., and Lamb, C.J. (1989). Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco plants. Proc. Natl. Acad. Sci. USA **86,** 9284–9288.

Lundqvist, U., von Wettstein-Knowles, P., and von Wettstein, D. (1962). Induction of *eceriferum* mutants in barley by ionizing radiation and chemical mutagens II. Hereditas **59,** 473–504.

Luo, D., Coen, E.S., Doyle, S., and Carpenter, R. (1991). Pigmentation mutants produced by transposon mutagenesis in *Antirrhinum majux.* Plant J. **1,** 59–69.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Martin, C., Prescott, A., Mackay, S., Bartlett, J., and Vrijlandt, E. (1991). Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus.* Plant J. **1,** 37–49.

Mayerhofer, R., Koncz-Kalman, Z., Nawrath, C., Bakkeren, G., Crameri, A., Angelis, K., Redei, G.P., Schell, J., Hohn, B., and Koncz, C. (1991). T-DNA integration: A mode of illegitimate recombination in plants. EMBO J. **10,** 697–704.

McClintock, B. (1984). The significance of responses of the genome to challenge. Science **226,** 792–801.

Meyer, P., Heidmann, I., Forkmann, G., and Saedler, H. (1987). A new petunia flower color generated by transformation of a mutant with a maize gene. Nature **330,** 677–678.

Meyerowitz, E.M., Bowman, J.L., Chang, C., and Kempin, S. (1990). RFLP map of *Arabidopsis thaliana.* In Genetic Maps, S.J. O'Brien, ed (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp 98–99.

Nam, H.-G., Giraudat, J., den Boer, B., Moonan, F., Loos, W.D.B., Hauge, B.M., and Goodman, H.M. (1989). Restriction fragment length polymorphism linkage map of *Arabidopsis thaliana.* Plant Cell **1,** 699–705.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes *in trans.* Plant Cell **2,** 279–289.

Oppenheimer, D., Herman, P., Sivakumaran, S., Esch, J., and Marks, M.D. (1991). A *myb* gene required for trichome differentiation in *Arabidopsis* is expressed in stipules. Cell **67,** 483–493.

O'Reilly, C., Shepherd, N.S., Pereira, A., Schwarz-Sommer, Z., Bertram, I., Robertson, D.S., Peterson, P.A., and Saedler, H. (1985). Molecular cloning of the *a1* locus of *Zea mays* using the transposable elements *En* and *Mu1.* EMBO J. **4,** 877–882.

Persson, G., and Hagberg, A. (1969). Induced variation in a quantitative character in barley. Morphology and cytogenetics of *erectoides* mutants. Hereditas **61,** 115–178.

Pfeiffer, P., and Vielmetter, W. (1988). Joining of nonhomologous DNA double strand breaks *in vitro.* Nucl. Acids. Res. **16,** 907–924.

Roth, D.B., and Wilson, J.H. (1986). Relative rates of homologous and nonhomologous recombination in mammalian cells. Proc. Natl. Acad. Sci. USA **82,** 3355–3359.

Roth, D., and Wilson, J. (1988). Illegitimate recombination in mammalian cells. In Genetic Recombination, R. Kucherlapati and G.R. Smith, eds (Washington, DC: American Society for Microbiology), pp. 621–653.

Sankaranarayanan, K. (1991). Ionizing radiation and genetic risks. III. Nature of spontaneous and radiation-induced mutations in mammalian in vitro systems and mechanisms of induction of mutations by radiation. Mutation Res. **258,** 75–97.

Schulze-Lefert, P., Dangl, J.L., Becker-André, M., Hahlbrock, K., and Schulz, W. (1989). Inducible *in vivo* DNA footprints define sequences necessary for UV light activation of the parsley chalcone synthase gene. EMBO J. **8,** 651–656.

Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P.A., and Saedler, H. (1987). Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays.* EMBO J. **6,** 287–294.

Sperry, A.O., Blasquez, V.C., and Garrard, W.T. (1989). Dysfunction of chromosomal loop attachment sites: Illegitimate recombination linked to matrix association regions and topoisomerase II. Proc. Natl. Acad. Sci. USA. **86,** 5497–5501.

Stafford, H.A. (1990). Flavonoid Metabolism. (Boca Raton, FL: CRC Press).

Staiger, D., Kaulen, H., and Schell, J. (1989). A CACGTG motif of the *Antirrhinum majus* chalcone synthase promoter is recognized by an evolutionarily conserved nuclear protein. Proc. Natl. Acad. Sci. USA **86,** 6930–6934.

Straus, D., and Ausubel, F.M. (1990). Genomic subtraction for cloning DNA corresponding to deletion mutations. Proc. Natl. Acad. Sci. USA **87,** 1889–1893.

Stroop, W.G., and Schaefer, D.C. (1989). Comparative effect of microwaves and boiling on the denaturation of DNA. Anal. Biochem. **182,** 222–225.

Suggs, S.V., Hirose, T., Miyake, T., Kawashima, E.H., Johnson, M.J., Itakura, K., and Wallace, R.B. (1981). Use of synthetic oligodeoxyribonucleotides for the isolation of specific cloned DNA sequences. ICN-UCLA Symp. Mol. Cell. Biol. **23,** 683–693.

Sun, T.-P., Goodman, H.M., and Ausubel, F.M. (1992). Cloning the Arabidopsis *GA1* locus by genomic subtraction. Plant Cell **4,** 119–128.

Underbrink, A.G., Sparrow, R.C., Sparrow, A.H., and Rossi, H.H. (1970). Relative biological effectiveness of X-rays and 0.43-MeV monoenergetic neutrons on somatic mutations and loss of reproductive integrity in *Trandescantia stamen* hairs. Radiat. Res. **44,** 187–203.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M., and Stuitje, A.R. (1990). Flavonoid genes in petunia: Addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell **2,** 291–299.

van Tunen, A.J., Koes, R.E., Spelt, C.E., van der Krol, A.R., Stuitje, A.R., and Mol, J.N.M. (1988). Cloning of the two chalcone flavanone isomerase genes from *Petunia hybrida*: Coordinate, light-regulated and differential expression of flavonoid genes. EMBO J. **7,** 1257–1263.

van Tunen, A.J., Hartman, S.A., Mur, L.A., and Mol, J.N.M. (1989). Regulation of chalcone flavanone isomerase (CHI) gene expression in *Petunia hybrida*: The use of alternative promoters in corolla, anthers and pollen. Plant Mol. Biol. **12,** 539–551.

Voytas, D.F., Konieczny, A., Cummings, M.P., and Ausubel, F.A. (1990). The structure, distribution and evolution of the *Ta1* retrotransposable element family of *Arabidopsis thaliana.* Genetics **126,** 713–721.

Watson, J.C., and Thompson, W.F. (1986). Purification and restriction endonuclease analysis of plant nuclear DNA. Methods Enzymol. **118,** 57–75.

Wienand, U., Sommer, H., Schwarz, Z., Shephard, N., Saedler, H., Kreuzaler, F., Ragg, H., Hahlbrock, K., Harrison, B., and Peterson, P.A. (1982). A general method to identify plant structural genes among genomic DNA clones using transposable element induced mutations. Mol. Gen. Genet. **187,** 195–201.

Wilkinson, J.Q., and Crawford, N.M. (1991). Identification of the *Arabidopsis CHL3* gene as the nitrate reductase structural gene *NIA2.* Plant Cell **3,** 461–471.

Yang, R., Lis, J., and Wu, R. (1979). Elution of DNA from agarose gels after electrophoresis. Methods Enzymol. **68,** 176–182.