Chapter I

LITERATURE REVIEW

I.1 Relevance to Agriculture

Disease in agriculture has a profound impact on the global economy. It is estimated that every year the total crop production lost to disease is nearly 30% (Thomas, 1999). For example, in the United States, crop losses in 1999 were estimated to be $9.1 billion per year and worldwide losses total more than $175 billion annually (Thomas, 1999). Current control strategies for pests and plant disease rely upon pesticides, genetic engineering and innate resistance in the plant. Chemical controls can be costly to the grower and harmful to the environment and are often beyond the means of farmers in developing nations. Therefore, innovative strategies that lead to more effective resistance to pathogens are necessary. Genetic approaches to improve innate defense responses in plants have the potential to provide low input, environmentally friendly resistance to disease. However, host resistance is often overcome by pathogens that have evolved mechanisms to defeat plant defenses. To design new genetic strategies that remain durable against continuously co-evolving pathogens, a broader understanding of the coevolutionary "arms race" between plants and pathogens is necessary.

I.1.2 Overview of the Plant-Pathogen Arms Race

Plants are attacked by a wide range of disease-causing organisms, including bacteria, viruses, nematodes, fungi, and oomycetes. To block invasion, plants maintain preformed physical and chemical barriers. Physical barriers include a waxy surface coat and cell walls. Plants also produce anti-microbial compounds that are often stored in the vesicles or cell walls, to be released upon pathogen attack (Hammond-Kosack and Jones, 2000). These preformed barriers constitute the first line of defense against pathogen ingress.

Plants can also activate a second line of inducible defense responses against pathogens that have overcome preformed defenses. These defenses may include antibiotic production and tissue strengthening at the infection site. In addition, plant cells adjacent to the infection site can commit suicide by programmed cell death (referred to as the hypersensitive response or HR). This response can directly kill the pathogen and/or inhibit access to nutrients (Hammond-Kosack and Jones, 1996).

These inducible defense responses are highly effective, but can carry a high metabolic cost and cause considerable collateral damage to the plant (McDowell and Woffenden, 2003). For this reason, defenses are only employed if the plant detects that it is being attacked. Thus, successful recognition of the invading pathogen is of key importance for efficient self-defense.

Plants have evolved multiple surveillance mechanisms to detect the presence of disease-causing organisms. To begin with, plants can recognize pathogen-associated molecular patterns (PAMPs) that are conserved across distantly related pathogen species (Martin et al., 2003; Greenberg and Vinatzer, 2003; Nurnberger et al., 2004). This is referred to as PAMP-triggered immunity (PTI). Plant defenses can be activated by a number of PAMPs, which include flagellin and lipo-polysaccharide from bacteria, and chitin and egosterol from fungi or oömycetes (Zipfel et al., 2005). Receptors for several of these PAMPs have now been cloned. For example, flagellin is perceived in *Arabidopsis* by FLS2, which is an extracellular surface receptor in *Arabidopsis*. This receptor recognizes the conserved 22 amino acid peptide of bacterial flagellin (flg22) (Felix et al., 1999). FLS2 encodes a receptor-like protein kinase (RLK) consisting of extracellular leucine-rich repeats (LRRs, described in detail below), a transmembrane domain, and a cytoplasmic serine/threonine kinase domain. FLS2 recognition of flg22 triggers defenses that restrict bacterial growth (Zipfel et al., 2004). PTI contributes to basal resistance in host plants but is also important for non-host resistance (Nurnberger et al., 2002, 2005; Dangl and Jones, 2001). Emerging evidence suggests that PTI can be very potent and may represent a major determinant of pathogen host range (Hann and Rathjen, 2007; Ham et al., 2007).

Although PTI can be a very effective weapon for the plant, certain pathogens can interfere with the downstream signaling events that lead to activation of basal defense mechanisms (Abramovitch and Martin, 2004; Kim et al., 2005). This suppression can be carried out by so-called "effectors", which are proteins encoded by the pathogen that are secreted into the plant cell cytosol. Bacteria use a type III secretion system (TTSS), to deliver the effectors into plant cells (Abramovitch and Martin, 2004). Once inside, these effectors target specific plant regulators of defense (referred as effector-triggered susceptibility or ETS). For example, the effector AvrPtoB from *Pseudomonas syrinage*

promotes bacterial growth *in planta* and suppresses programmed cell death (Abramovitch et al., 2003). This effector functions as a molecular mimic of eukaryotic E3 ubiquitin ligases and promotes disease in tomato by degrading a plant surveillance protein (Abramovitch et al., 2006, 2006). Additionally, AvrPtoB was shown to suppress papillae formation, which is a common defense response used by plants to fortify the cell wall (DebRoy et al., 2004; Hauck et al., 2003).

In response to defense-suppressive effectors, plants have evolved a molecular surveillance system based on dozens of constitutively expressed resistance (R) genes that are capable of recognizing pathogen effectors as signals of invasion. Effector-triggered immunity (ETI) is characterized by a plant resistance gene that perceives the product of a corresponding effector gene from the pathogen. In this interaction the pathogen effector gene is re-classified as an "avirulence" (Avr) gene because it triggers the plant resistance responses that render the pathogen avirulent (non-disease causing). This secondary plant surveillance system activates robust and rapid defense responses that can override effectors' defense-suppressive ability. ETI often involves a rapidly induced hypersensitive response that halts pathogen growth.

Pathogens have evolved strategies to cope with ETI by avoiding recognition. For example, pathogens can mask the avirulence factors from detection by changing the structure of the surface that interacts with the R protein. Additionally, certain effectors can be completely deleted and through functional redundancy the deletion can be compensated (Hulbert et al., 2001; Abramovitch and Martin, 2004; Chang et al., 2004). These avoidance mechanisms can successfully render the R protein-dependent recognition useless and place pressure on the plant to evolve new surveillance genes.

In sum, the selective pressure upon both plants and pathogens creates a continuous cycle of detection and mutation (Michelmore and Meyers, 1998). Jones and Dangl (2006) have likened the alternating pattern of resistance and susceptibility in the plant immune system to a zigzag schematic (Fig. I.1**)** that consists of four phases: In phase 1, PTI acts as an early warning system for the presence of a potential pathogen. In phase 2, effector proteins are evolved by pathogens to suppress host defenses. The evolution of effectors in pathogens consequently led to the evolution of ETI, which is phase 3 and is considered to be a stronger activation of PTI-associated defenses. In phase

4 the strength of selection leads to the formidable tactics, described above, utilized by pathogens to avoid detection.

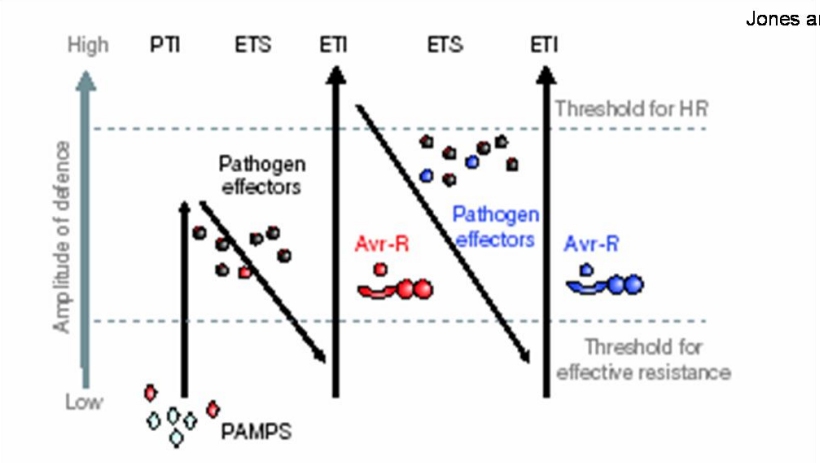


Fig. I.1 Zigzag model: In phase I, PAMP recognition triggers PAMP-triggered immunity (PTI). In phase 2, successful pathogens deploy effectors that can interfere with PTI by altering resistance signaling and interfering with the resistance response while promoting pathogen virulence. This is referred as effector-triggered susceptibility (ETS). In phase 3, direct or indirect recognition of an effector by an R protein activates effector triggered immunity (ETI). In phase 4, natural selection drives pathogens to avoid recognition using various strategies (refer to text) then in turn selection favors new plant R alleles that can recognize new or altered pathogen variants, resulting again in ETI.

Ultimately, the strong evolutionary pressure to maintain surveillance and keep pace with the rapidly evolving pathogen populations presents a considerable challenge to the plant.

I.1.3 Molecular Mechanisms of R Protein Surveillance

The classic gene-for-gene model described by Flor (1971) proposes that resistance is triggered by a specific interaction between a plant that expresses a dominant *R* gene and a pathogen that expresses a complimentary dominant *Avr* gene. The molecular basis of this genetic model was described with the elicitor-receptor model, which proposes that *R* gene-encoded receptors recognize and directly bind the pathogen Avr protein or its enzymatic product (elicitor) (Gabriel and Rolfe, 1990). In other words, this model predicts that *R* genes encode receptors that bind *Avr* gene products. There is genetic specificity between R-Avr interactions, as well as an array of allelic diversity within the plant and pathogen immune system that is comparable to the specificity and diversity observed within the vertebrate immune system. Similar to the receptor-ligand

relationship in vertebrate innate immunity, the genetic specificity of the R-Avr interaction is most easily explained as the effector molecule binding to a site on the target R protein.

In support of the elicitor-receptor model, direct interactions have been demonstrated between certain combinations of R and Avr proteins. For example, the tomato resistance gene *Pto*, for bacterial speck resistance, and the AvrPto protein of *Pseudomonas syringae pv. tomato* was shown to interact through yeast two-hybrid analysis (Scofield et al., 1996; Tang et al., 1996). *AVR-Pita*, which is expressed by the rice blast fungus *Magnaporthe grisea*, was shown to function as the elicitor molecule and directly bind the Rice Pi-ta resistance protein *in vitro* (Jia et al, 2000). A type III effector, Avr protein PopP2, was shown through yeast two-hybrid analysis to interact with the *Arabidopsis thaliana RRS1-R* resistance gene, which confers resistance to several strains of *Ralsonia solanacearum*, the causal agent of bacterial wilt (Deslandes et al., 2003). In the flax system, a direct interaction between the flax rust resistance protein L5 and AvrL567 variants A and B was demonstrated through yeast two-hybrid analysis as well as a direct interaction between L6 and AvrL567 variants A, B, and D (Dodds et al., 2006).

The direct interactions described above provide strong support for the elicitor-receptor model. However, many other attempts to demonstrate direct interactions between various R and Avr proteins have failed. To explain these negative results, Van der Biezen and Jones (1998) proposed the "guard model". This model predicts that R proteins continuously monitor specific plant proteins (termed "guardees") that are targeted and modified by effectors. Resistance is triggered when the R protein detects the attempted attack on its guardee. In contrast to the elicitor-receptor model, a direct interaction between the R protein and the cognate Avr/effector protein is not assumed. Experimental support for this model has been demonstrated with the *Arabidopsis R* genes *RPM1* and *RPS2*. Both of these genes are activated in response to the effector-dependent modification or degradation of the guardee, *RIN4*. RIN4 is a membrane-associated protein that physically co-localizes with RPM1 and RPS2 and is targeted by *Pseudomonas syringae* virulence effectors. Considering the diversity of potential Avr/effector proteins, "guarding" is a good strategy because a single R protein could provide for recognition of multiple effectors that induce the same "danger" signal in the

plant. Additional data collectively suggest a significant proportion of R proteins conform to the guard model (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003; Shao et al., 2003; Kim et al., 2005). In sum direct *R-Avr* interactions and R protein guarding occur in nature.

I.1.4 *R* Gene Structure and Specificity

Conserved structural motifs of R proteins have provided clues to understanding their molecular function. The largest class of R proteins contain a consensus nucleotide-binding site (NBS), followed by tandem arrays of leucine-rich repeats (LRRs). The LRR motif contains leucines or other hydrophobic residues that form a structural scaffold, which is thought to facilitate protein interactions or ligand binding (Hammond-Kosack and Jones, 1996). The NBS motif, also found in many ATP and GTP binding proteins, is predicted to be involved in signaling (Hammond-Kosack and Jones, 1996). The exact role of the NBS is unknown, but the tomato R proteins I-2 and Mi-1 have been shown to be capable of binding and hydrolyzing ATP (Tameling et al., 2002).

The NBS-LRR gene superfamily can be subdivided based upon domains found at the amino (N) terminus. Some R proteins possess a domain denoted the Toll/Interleukin-1/receptor (TIR) domain because of its similarity to the cytoplasmic signaling domain of the Toll and Interleukin transmembrane receptors (TIR-NBS-LRR: TNL). Other NBS-LRR R proteins (including the RPP8 proteins utilized in this project) contain a coiled coil (CC) motif at the N terminus (CC-NBS-LRR: CNL). This is a common motif found in other proteins to facilitate protein-protein interactions. However, the exact roles of the TIR and CC domains also remain to be established.

NBS-LRR genes have been identified in every plant species examined to date. NBS-LRR genes recognize pathogens as diverse as bacteria, fungi, oomycetes, and nematodes, but individual NBS-LRR genes typically have very narrow recognition capabilities. The molecular basis for the differences in recognition specificity is not yet known, but the LRR region is a likely determinant for *R* protein specificity. LRRs have previously been shown to form a flexible solvent-exposed, beta-strand/beta-turn motif that can provide a ligand-binding surface that is both versatile and highly evolvable (Kobe and Deisenhofer, 1994; Kobe and Kajava, 2001) (Fig. I.2).

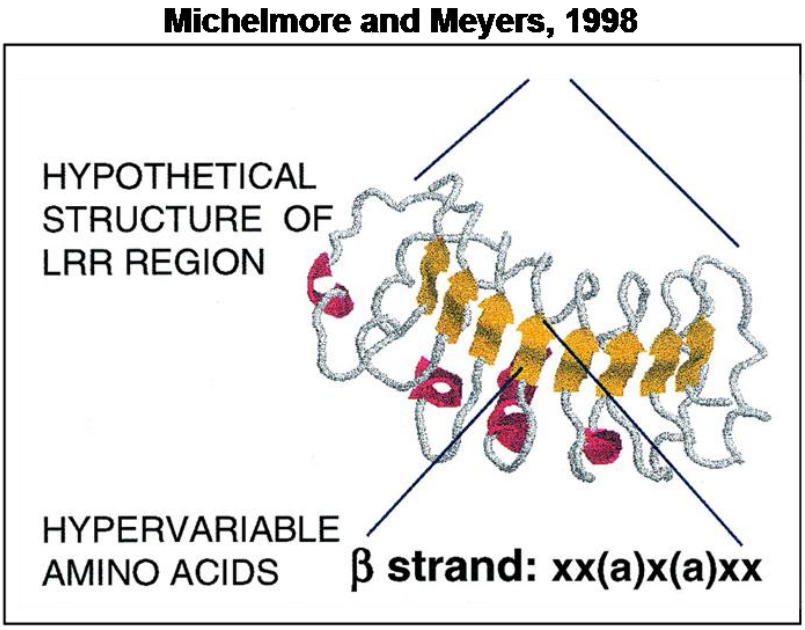


Fig. I.2 LRR Motif: the LRR forms barrel-like structures (α helices) with a parallel sheet (β sheets) that occupies the inner concave surface. The α helices and β sheets are connected by loops or turns that creates a curved and elongated shape.

Evolutionary studies indicate that the LRR region is under selection for amino acid diversification, which could give rise to altered binding specificities as potential ligand binding sites for Avr proteins are generated (Ellis et al., 2000). This hypothesis has received experimental support from domain swapping experiments, which showed that resistance specificity was determined by the LRR region of the gene (Ellis et al., 1999). Changes within the LRR organization and structure are therefore thought to contribute to the evolution of new ligand-binding surfaces (Ellis et al., 2000).

The molecular events that lead to R protein recognition of the pathogen and activation of disease resistance signaling pathways are not clearly defined. However, there is evidence that suggests resistance signaling is initiated by conformational changes within the NBS-LRR R protein domains. These changes are described by the "jack-knife model" (Fig. I.3), which proposes that intramolecular interactions between R protein

domains can regulate NBS-LRR signaling. The domains of the NBS-LRR protein serve as a platform for the regulatory factors/interactors to assemble. The amino-terminal and carboxy-terminal domain within the LRR accommodate different interaction partners. The C-terminal LRR serves as a platform for upstream activators that appear to facilitate recognition. The N-terminal LRR serves as a platform for signaling partners that modulate activation. The NBS is responsible for hydrolysis of ATP and release of the signal that interacts with the downstream signaling partners that assemble at the CC or TIR domain. Moffett et al (2002) demonstrated *in vivo* that interactions between NBS-LRR domains create a signaling-competent conformation that can be positively or negatively regulated upon assembly of various putative regulatory factors necessary for controlled signaling. Notably, this supporting data does not preclude the effect of intermolecular interactions with *trans* participants. The events described above represent some of the earliest events associated with activation of disease resistance by R proteins.

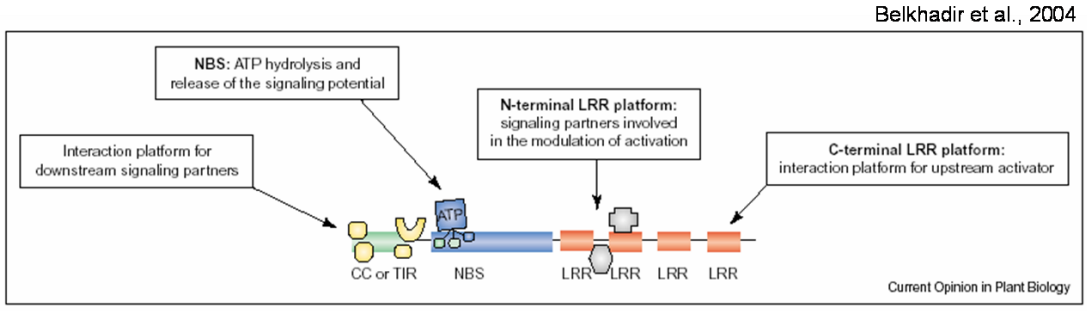


Fig. I.3 Jack-knife model: The domains of the NBS-LRR protein serve as a platform for the regulatory factors/interactors to assemble. The C-terminal LRR serves as a platform for upstream activators that appear to facilitate bacterial recognition. The N-terminal LRR serves as a platform for signaling partners that modulate activation. The NBS is responsible for hydrolysis of ATP and release of the signal that interacts with the downstream signaling partners that assemble at the CC or TIR domain.

I.1.5 *R* Gene Evolution and Diversity

As described above, pathogens can deploy vast and variable arrays of effectors. R protein-dependent recognition is consequently heavily burdened to keep pace with the rapidly evolving pathogen populations. Maintenance of surveillance therefore presumably necessitates mechanisms that can accelerate change and genetic diversity in the surveillance system.

The mechanisms that drive the evolution of new *R* genes are poorly understood. However, studies into the genomic organization of *R* gene loci have provided some clues about how plants can produce new *R* genes. Most plants contain dozens or even hundreds of NBS-LRR genes in their genomes. Imporantly, many are organized as tightly linked, contiguous clusters. For example, 109 of the 149 *Arabidopsis* NBS-LRR genes reside in 40 clusters ranging in size from two to eight genes (Richly et al., 2002; Meyers et al., 2003). Other plants, like rice and tomato, also maintain large NBS-LRR gene families (Pan et al., 2000; Monosi et al., 2004). Clusters can contain genes with different pathogen recognition specificities (Botella et al., 1998; Ellis et al., 1999; Hulbert et al., 2001; Parniske et al., 1997). This clustered distribution is thought to have significant evolutionary importance, because it could accelerate *R* gene diversification through several mechanisms. To begin with, multigene clusters provide for functional redundancy. If one gene of a cluster is mutated, silenced or inactivated, then another gene of that cluster may be able to compensate for the lost function. Functional redundancy may allow for one gene to evolve a new function (e.g. recognition of a different pathogen effector) while the other gene would maintain its preexisting function. Another source of new *R* genes is through duplication, which can copy whole genes or blocks of genes and transfer that segment so it is contiguous with the original one or transfer to another site, creating more diversity (Leister et al., 2004). Furthermore, the physical proximity of genes within clusters increases the likelihood of new *R* genes "assembled" by recombination. In this respect, a multigene cluster could serve as a reservoir for sequence diversity (Meyers et al., 1998). For genes that are not active, function could be restored by recombination between active and inactive genes.

I.1.6 General Mechanism of Recombination

The process of recombination involves the exchange or transfer of genetic information between DNA molecules (Holliday, 1964). Several models for the mechanism of recombination have been proposed. In the Holliday model, which was the first widely-accepted model, homologous recombination by chromatid pairing, single-strand DNA breakage, and strand invasion result in a dynamic structure called a "Holliday junction" (Holliday, 1964). Following strand exchange, the Holliday junction

can slide along the length of the paired DNA strands. This is referred to as Branch Migration. Symmetrical heteroduplex DNA is created as the Holliday junction migrates (in either direction) along the paired chromosomes. The resolution of the Holliday junction is characterized by final cutting and religation of DNA to yield a crossover event (Holliday, 1964). Depending upon which pair of opposing strands are cut, resolution of the Holliday junction may result in either a non-crossover event or a cross-over that includes the exchange of flanking DNA markers. Additionally, gene conversion can occur from the repair of the mismatched region of heteroduplex DNA that is formed. Gene conversions are frequently associated with crossovers, which are conservative but reciprocal exchanges of genetic information from one homolog to another homolog (Holliday, 1964). However, a gene conversion is a non-reciprocal exchange of information from one homolog to another homolog, which results in the loss of genetic information (Szostak et al., 1983). In contrast to the Holliday model, which suggests a symmetrical exchange of genetic information, the Meselson-Radding model suggests that exchange of DNA occurs asymmetrically (Meselson and Radding, 1975). In both of these models recombination is initiated by a single-strand nick. However, in the model proposed by Szostak et al. (1983), recombination is initiated by a double-strand break, hence the name Double-Strand-Break repair model. Studies in yeast have shown recombination can be stimulated by double-strand breaks (Szostak et al., 1983). Currently the Double-Strand-Break repair model is the most widely accepted model for meiotic recombination.

Recombination can occur in somatic or meiotic cells. Recombination in somatic cells can occur at any stage of development. Recombination is best studied during meiosis because the resulting recombinant alleles are heritable and these gene products may be evolutionarily important for genetic diversity. This is in contrast to recombination that occurs in somatic cells, which the resulting traits are heritable only if they occur in cell lineages that give rise to gametes.

There are also differences in the mechanics of recombination. Intrachromosomal recombination involves interactions between two homologous sequences within a single chromosome (or chromatid) during meiosis. This may result in the looping out (loss) or inversion of DNA sequences. For example, a recombination substrate arranged as direct

repeats generates a short lived extrachromosomal circle and the sequence between the two repeats is deleted (Fig. I.4A). A recombination substrate arranged in the opposing orientation inverts the intervening sequence (Fig. I.4B). Interchromosomal recombination involves interactions between homologous chromosomes. An unequal crossover event is characterized by chromosomes that have misaligned and paired. At a complex locus, unequal crossovers may occur in the region between the genes (intergenic) or within the gene (intragenic). Early evidence of unequal crossing-over was observed at the *bar* locus in Drosophila. Reversion of the mutant *bar* gene, characterized by mutant narrow eyes, to the normal round eye phenotype was shown to be the result of unequal crossing-over of three loci (*forked*, *bar*, and *fused*), all on the X chromosome (Sturtevant, 1925). Crossing-over on the left portion of *bar* (*forked*) on one chromosome and on the right portion (*fused*) on the homologous chromosome was found to cause mutations in the *bar* gene that change the characteristics of the Drosophila eyes (Sturtevant, 1925). Another early example of unequal crossing-over by sister chromatid exchange (i.e. interactions between sequences located on separate but identical sister chromatids) was demonstrated with segregation analysis of an inserted *LEU2* gene within an rRNA cluster in yeast (Petes, 1980).

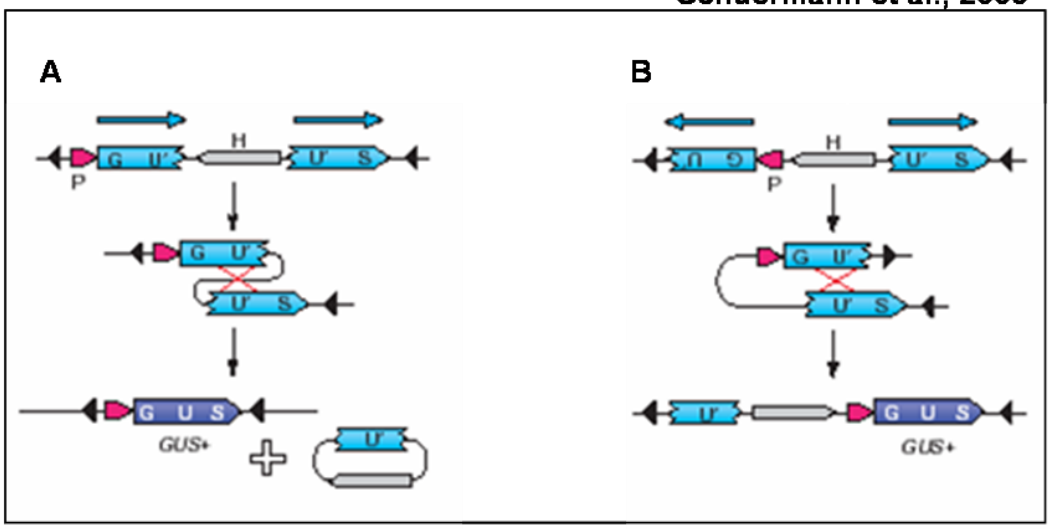


Fig. I.4 Intrachromosomal Recombination with direct and indirect repeats: A. Direct repeats: a recombination substrate arranged in the direct orientation generates a short lived, extrachromosomal circle as the sequence between the two repeats is deleted. B. Indirect repeats: A recombination substrate arranged in the indirect orientation inverts and retains the intervening sequence

A very important aspect of the process of unequal crossing-over is that it leads to the concomitant production of four altered gene products. At a genetic locus organized as a gene cluster, homologous chromosomes may misalign due to the high sequence similarity among the paralogous genes. The misalignment followed by an unequal crossover between paralogous genes will give rise to a deletion, duplication, and two reciprocal recombinant genes (Fig. I.5). Evidence of this process has been observed in human amylase genes, mammalian Major Histocompatibility Complex (*MHC*), plant ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*RBCS*), and plant resistance (*R*) genes (Gumucio et al., 1988; Hughes, A.L. and M. Yeager, 1997; Jelesko et al., 1999, 2004; Hulbert et al., 2001).

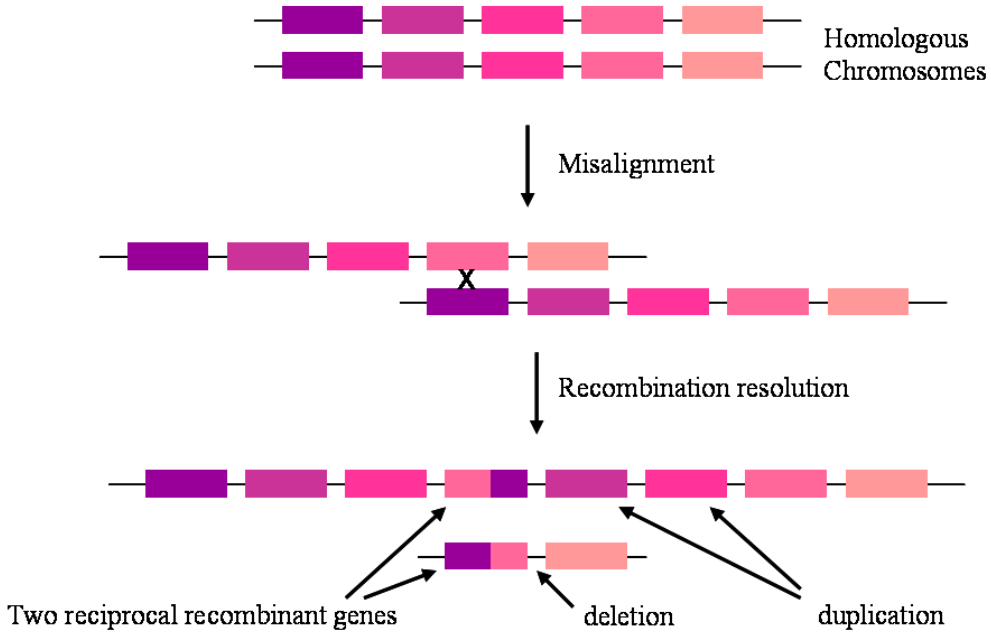


Fig. I.5 Recombination within gene clusters: The misalignment followed by an unequal crossover between paralogous genes will give rise to a deletion, duplication, and two reciprocal recombinant genes.

The predominant model for studies of meiotic recombination in eukaryotes is yeast (*Saccharomyces cerevisiae*). While much of the information from these studies has provided important insights into the frequency and molecular biology of meiotic recombination, many aspects of this important process that have been determined in yeast are not the same in multicellular organisms. It was observed that meiotic recombination in yeast is about 10,000 times greater than that of plants and mammals (Jackson and Fink, 1985). Also, with the exception of the rDNA genes, the yeast genome contains virtually no gene clusters larger than simple gene duplications. This is in marked contrast to multicellular eukaryotes where large gene clusters are prevalent. Considering that recombination may play an important role in *R* gene evolution, a more precise account of the frequency of meiotic recombination in a multicellular organism is necessary to expand our understanding of how meiotic recombination leads to rapid genomic change.

I.1.7 *R* Gene Evolution and Recombination

Gene Duplication

An important source for new *R* genes is tandem duplication. Tandem duplication creates adjacent chromosome segments where the duplicated segment is contiguous with the original duplication. As mentioned above the physical clustering of many NBS-LRR genes is thought to facilitate this process. As previously mentioned, there are a large number of NBS-LRR gene clusters in *Arabidopsis*. An equivalent proportion of clustered genes are also found in the rice genome (Bai et al., 2002; Monosi et al., 2004; Zhou et al., 2004). Additional support for tandem duplication was demonstrated with phylogenetic analysis which showed that genes within a cluster often occupy the same phylogenetic lineage (Richly et al., 2002; Meyers et al., 2003). Furthermore, at the *Rp1* gene cluster in maize haplotypes, the gene copy number ranges from 1-52 (Smith et al., 2004). In addition, the genomic sequence analysis of three maize bacterial artificial clones (BACs) from the *Rp1* region revealed duplicated segments of *Rp1* homologs, which supports tandem duplication (Ramakrishna et al., 2002**)**.

It is important to note that some NBS-LRR gene clusters are actually comprised of a mix of evolutionarily distant NBS-LRR genes. In *Arabidopsis* there are at least 10 clusters that contain interspersed CNLs and TNLs (defined previously in I.1.4) (Richly et al., 2002; Meyers et al., 2003). It is unlikely that a mixed cluster could have originated from a single progenitor gene and tandem duplication. The proliferation of closely related *R* genes that are physically dispersed in the genome is another observation that does not comply with simple tandem duplication. Two models have been proposed to describe the above mentioned NBS-LRR distribution patterns. In the rapid rearrangement model, a single gene or small groups of genes are transposed to distal locations (ectopic duplication). The lack of large-scale synteny in sequences that flank related NBS-LRR genes in cereals and Arabidopsis have provided support for ectopic duplications (Leister et al., 1998; Richly et al., 2002; Meyers et al., 2003). In the second model, the conserved synteny model, a large-scale duplication would take place followed by local rearrangement. This was illustrated with statistical approaches using phylogenetics and phylogeography that defined chromosomal regions as geographical populations, from which migration patterns could be evaluated (Baumgarten et al., 2003).

The impact that NBS-LRR gene duplication has had on the proliferation and diversity of plant *R* genes has been substantial. Sequence exchange by mispairing and unequal crossing over between linked NBS-LRR genes (intragenic recombination) (see below) also is thought to contribute to NBS-LRR gene cluster diversity as described in the following section.

Sequence Exchange in *R* Gene Evolution

Sequence exchange has been shown with genetic and molecular evidence to be a significant factor in *R* gene evolution (Ellis et al., 2000; Hulbert et al., 2001). The *Maize Rp1* resistance gene cluster provides an informative example of *R* gene diversification by unequal crossing over. The *Rp1* complex contains 14 genes (*Rp1-A* to *Rp1-N*) that confer resistance to different races of *Puccinia sorghi* (Hulbert, 1997). To investigate recombination, test-cross families were created to identify unusual *Rp1* variants. Individuals that were homozygous or heterozygous for the dominant *Rp1* genes were crossed to susceptible individuals that were homozygous or heterozygous for the recessive *rp1* allele. Using RFLP markers that flanked the *Rp1* locus, numerous *Rp1* variants associated with the exchange of flanking markers were identified. These variants were inoculated with 11 *P. sorghi* biotypes, which were found to induce different virulence phenotypes, and their resistance specificities were evaluated (Hulbert, 1997). Strikingly, *Rp1* variants that showed resistance to a different spectrum of rust biotypes than either parent (i.e. non-parental race specificities) were identified. Flanking marker analysis showed that recombination was associated with the non-parental race specificities. This suggests recombination by unequal crossing-over was the source of *R* gene diversification at the *Rp1* locus.

Genetic diversification at the *Rp1* locus was also shown with deletion mutants from the *Rp1-D* haplotype (consists of nine homologous genes) that were created by a transposon-tagging approach using *Activator* (*Ac*) or *Dissociation* (*Ds*) insertion mutants (Collins et al., 1999). DNA gel blot analysis of the mutants showed insertion/excision of *Rp1-D* gene family members. It was suggested that most of the insertions/deletions arose from recombination or other rearrangements among the *Rp1-D* gene family members. The transposon insertion correlated with either a loss or gain of *Rp1-D* resistance that was

determined by testing the mutants for their ability to revert in the presence of *Ac*. However, some of the mutations did not revert in the presence of *Ac*, suggesting that an event other than transposon insertion created the mutation (Collins et al., 1999). Nevertheless, recombination was associated with the different mutants of the *Rp1-D* haplotype.

An example of a naturally occurring recombination event that lead to new recognition specificities was observed at the flax rust resistance locus *L* (Luck et al., 2000). The TIR-encoding region of the *L6* allele was replaced with the corresponding regions from *L2* or *LH* by recombination. The resistance specificity in the recombinant allele changed from *L6* to *L7*. In addition, the replacement of the TIR and most of the NBS-encoding region of *L10* by *in vivo* recombination with the equivalent region from *L2* or *L9* produced recombinant alleles that have a novel specificity.

R Gene Turnover

Although the above examples indicate that sequence exchanges are important for *R* gene evolution, the importance of sequence exchange appears to vary between different *R* loci as well as between genes within an individual cluster. There are *R* gene lineages that appear to be evolving in relative isolation with little or no sequence exchange (Michelmore and Meyers., 1998; Ellis et al., 2000). Conversely, there are lineages that do recombine frequently and/or different genes within the cluster appear to exchange sequences at very different rates. Dodds et al (2001) described heterogeneous evolution with the *N* cluster for flax rust resistance. *N* locus genes from different haplotypes could be divided into three subclasses based on sequence identity. Genes within a subclass that contains two paralogous genes that share a high degree of sequence identity frequently recombine. However, the sequence exchange is limited to genes within subclasses as there is no sequence exchange between subclasses.

Another example of heterogeneous evolution was demonstrated with the *RGC2* cluster in cultivated lettuce and wild relatives. At the *RGC2* cluster, there are between 12-32 gene copies. Sequence comparisons involving a large number of *RGC2* haplotypes revealed that there are two types of *RGC2* genes and that they differ by their rate of sequence exchange (Kuang et al., 2004). Type I genes evolve rapidly and are

characterized by chimeric structures which suggest frequent sequence exchange whereas Type II genes appear to recombine infrequently and are relatively conserved. Kuang et al subsequently showed with the potato *R1* cluster that three subclusters of Type I genes frequently exchange sequence but not between subclusters. By many accounts there is evidence that supports the importance of sequence exchange in the evolution of *R* gene clusters. However, the different evolutionary modes and tempos of sequence exchange raises questions regarding the impact of frequent or infrequent exchange within or between gene clusters and the influence it has on the diversification and/or homogenization of gene clusters. Due to the uncertainty about the relevance and impact of recombination, more research on the frequency and functional consequences of recombination within *R* gene clusters is sorely needed.

I.1.8 Recombination Assays

Assays based on reporter genes (phenotypic/molecular markers, antibiotic resistance, histochemical/bioluminescence) have been useful for measuring and visualizing recombination in plants. Phenotypic and molecular markers are informative for meiotic recombination frequencies because recombination is assayed in the recombinant progeny (Hulbert, 1997). However, genotyping these plants can be a time consuming task. The recombination-dependent activation of stably integrated, overlapping, inactive fragments of an antibiotic resistance gene led to frequent antibiotic resistant cells (Tovar and Lichtenstein, 1992; Swoboda et al., 1994). One of the first such systems utilized a resistance marker transgene that contained a functional hygromycin phosphotransferase (*hyg*) gene flanked by a pair of defective neomycin phosphotransferase (*neo*) genes in the inverted orientation (Tovar and Lichtenstein, 1992). This construct made it possible to distinguish between reconstitution of an active *neo* gene by either gene conversion or reciprocal crossover events. The defective *neo* genes were inactivated by non-allelic mutations in the coding region. Detection of recombination relied upon selection for kanamycin drug resistance resulting from either crossing over or gene conversion. DNA analysis of the recombinant tissue distinguished cross-over events from gene conversion based upon the identification of transgenic plants containing the *hyg* gene in the inverted orientation (i.e. crossovers) and those that did not

contain changes in the overall structure of the recombination substrate but did contain a *neo* gene corrected by a gene conversion event.

Although assays that use the drug resistance reporter genes are effective, these assays are difficult to use because they require cell culture of plant tissue. Assays that allow the visualization of the recombination event at the whole plant level have many advantages. For example, reporter gene analysis of recombination has been studied with the β-glucuronidase (*GUS*) gene (Swoboda et al., 1994; Lucht et al., 2002). Lucht et al. (2002) utilized inactive segments of a *GUS* reporter gene construct arranged in either direct or inverted orientation (Fig I.4). Somatic recombination events were identified as blue sectors observed by histochemical staining for GUS activity. The number of blue sectors was representative of the frequency of recombination events at that reporter gene locus. Another study utilized *Nicotiana tabacum* plants transgenic for two non-functional, overlapping copies of the luciferase (*LUC*) gene. Homologous recombination within the region of homology results in the reconstitution of an intact luciferase transgene (Kovalchuk et al., 2003) and recombination events were observed as bioluminescent spots on leaf tissue.

Different orientations of the truncated reporter gene constructs enable analysis of intramolecular or intermolecular recombination events. Additionally, through the use of molecular markers flanking the repeats of the truncated reporter gene, recombination between sister chromatids and between homologous chromosomes can be distinguished. Homologous recombination can occur between genetically linked homologous sequences (intramolecular recombination) or between homologous sequences located on different homologous chromosomes (intermolecular recombination). Molinier et al (2004) compared the intermolecular recombination repair frequencies between sister chromatids and between homologous chromosomes using plants that were either hemizygous or homozygous for the truncated reporter gene construct in a specialized arrangement that can undergo intermolecular recombination. The somatic recombination frequency in homozygous plants was slightly more than two times the recombination frequency in hemizygous plants (Molinier et al., 2004). This analysis (2004) did not determine whether specific recombination events took place between sister chromatids or homologous chromosomes. Molinier et al (2004) assumed that the increased somatic

recombination frequency in homozygous plants was due to recombination between homologous chromosomes. It remains to be determined if the increased recombination frequency was due to more genes or recombination between homologous chromosomes.

To avoid unwanted gene rearrangements, recombination must be tightly controlled and regulated. Consistent with this assertion, the frequency of recombination is low. In tobacco protoplasts, the frequency of somatic recombination occurred at frequencies of ~3 x 10^{-5} to 1 x 10^{-6} (Tovar and Lichtenstein, 1992). This recombination frequency is consistent with the results obtained by others using genetic screens for recombinants (Peterhans et al., 1990). The recombination frequency in *Arabidopsis* was also very similar to the observed frequency in tobacco at >10^{-6} for somatic recombination events and <2 x 10^{-5} and 1 to 3 x 10^{-6} for meiotic recombination events (Assaad and Signer, 1992; Jelesko et al., 1999).

The data based on flanking marker and reporter gene analysis have proven to be effective methods to identify recombination events but they do not illuminate the more intricate details of recombination, such as the effect of recombination on gene structure and evolution. Another factor that remains poorly understood is the influence that genomic changes have on successive generations. Notably, the meiotic recombination events are the evolutionarily important events because they are passed to progeny in somatic recombination, whereas they generally are not passed on to the next generation. The majority of the reporter-gene based screens that assay homologous recombination are performed in somatic tissue and are not examined in the next generation. Therefore somatic recombination frequencies may not be representative of the frequency of heritable mutations. Additionally, somatic screens are generally not able to assess the adaptive value of the genetic changes. To accurately evaluate the role of recombination in creating genetic diversity, it will require detailed measures of the frequency of meiotic recombination events and information about the sequence characteristics of meiotic recombination products.

I.1.9 Synthetic *R* Gene Cluster Technology

Estimations of recombination frequency rely on the ability to identify the recombination events. If the detection strategy employed is biased towards certain

recombination products, then the estimations of the frequency of recombination may not be truly representative. Most forward genetic screens of gene clusters have been based on a loss-of-function phenotype and therefore are biased towards identifying the deletion products of recombination. Identification of gene duplication and/or chimeric genes is more difficult because the investigator may not know what phenotype to look for. Even if a phenotype is predictable and observable, one may not see any phenotypic alteration in the organism due to redundant functions.

Some of these problems have been addressed in plants with synthetic gene cluster technology that couples chimeric gene formation to the activation of the firefly luciferase (*LUC)* gene, which imparts a bioluminescent phenotype that can be assayed non-invasively in intact plants (Jelesko et al., 1999). Synthetic gene cluster technology provides many advantages over forward genetic screens used to identify recombinants. The screen is not biased towards identifying deletion products since it identifies recombinants independent of an altered phenotype of the recombinant gene. This screen allows the rapid screening of large populations to identify recombinant alleles that display a luc^+ phenotype. Approximately 7,500 five day-old seedlings can be screened every ten minutes with over 1.2 million seedlings screened over 24 hours of imaging time (Jelesko et al., 1999). The sensitivity of the video-imaging equipment allows extremely low levels of recombinant chimeric gene expression to be detected. As little as 23 photons per twenty minutes of imaging time can identify a luc^+ seedling. In comparison to genetic screens that rely on DNA markers to identify a recombinant, this screen is less labor intensive because it eliminates the repetitive genotyping of the non-recombinant parental genotype.

The first study describing this approach utilized a synthetic *RBCSB* gene cluster (*synthRBCSB*) that was composed of a silent *ΔRBCS1B::LUC* gene fusion lacking all 5' transcription and translation signals, followed by wildtype *RBCS2B* and *RBCS3B* genomic segments (Jelesko et al., 1999). A homologous recombination event between *ΔRBCS1B::LUC* and *RBCS3B* results in a novel recombinant *RBCS3B/1B::LUC* chimeric gene whose expression was driven by *RBCS3B* transcription and translation signals. This screen can identify rare meiotic recombination events forming chimeric *RBCS3B/1B::LUC* genes associated with *RBCS2B* gene duplications. The frequency of

recombinants identified ranged from 1-3 x 10^{-6} (Jelesko et al., 2004). Chimeric gene formation was verified by sequencing. Recombination resolution sites mapped to three distinct regions. The region containing the most recombinants was the longest interval of DNA sequence identity. This fits the idea that homologous recombination requires regions of DNA sequence identity and that longer intervals show more crossover events than shorter regions of identity (Lyznik et al., 1991; Puchta and Hohn, 1991). Half of the recombinants obtained from this screen fit this model. However, the other two regions of recombination resolution sites/crossovers did not follow this trend, but rather mapped to areas where there was an abrupt transition from a region of high sequence similarity to a region of low sequence similarity. The majority of the recombinants were simple crossovers without associated gene conversion tracts, although a few of the latter were also identified. The non-uniform distribution of the crossovers may be an indication that recombination within a gene cluster of paralogous genes can facilitate the diversification of the gene cluster. Potentially, this could produce functionally distinct genes. Each recombinant obtained from the *synthRBCSB* cluster contained polymorphic DNA sequence that was derived from the two parental genotypes, thus providing evidence that the cluster is being diversified and not homogenized by this process (Jelesko et al., 2004).

I.1.10 Stress and Elevated Recombination Frequency

There is a long-standing hypothesis that the frequency of recombination could be elevated by biotic or abiotic stress, thereby facilitating the evolution of new traits (McClintock, 1984). This hypothesis has received experimental support from several studies. For example, plant somatic recombination frequencies are enhanced by abiotic stresses such as ionizing radiation, mitomycin C, or heat. Lebel et al (1993) observed ionizing radiation doubled the frequency of recombination in plant somatic cells in comparison to untreated controls. Similarly, treatment with mitomycin C increased the frequency by 9-fold and heat shock increased somatic recombination by 6.5-fold.

The frequency of recombination can also be enhanced by pathogen infection or activation of pathogen defense responses by treatment with a synthetic inducer such as BTH (a structural analog of the defense signal salicylic acid). Kovalchuk et al (2003) tested the response of a reporter gene to tobacco mosaic virus (TMV) by utilizing the

reporter system based on the recombination-dependent activation of the duplicated inactive luciferase genes. Infection with TMV induced a three to four-fold increase in recombination. In another study, the oomycete pathogen *Hyaloperonospora parasitica* was found to stimulate a 1.8-fold increase in somatic recombination in *Arabidopsis* (Lucht et al., 2002). BTH stimulated a 2.5-fold and 4.6-fold increase in recombination, respectively, depending upon whether the recombination substrate was arranged as inverted or direct repeats. The stress-induced elevations in somatic recombination rates can be passed on to progeny, which could be a potent source of genetic variation. However the meiotic mutations produced by recombination ensure these traits will be passed on to successive generations. Moreover, an increase in somatic recombination due to the stress-induced stimulus suggests that meiotic recombination may also be affected by stress.

Following exposure to DNA damaging agents, DNA repair pathways are activated (Tuteja et al., 2001). DNA double strand breaks (DSBs) can be repaired by homologous recombination. Notably, deletions and filler DNA insertions may accompany DSBs by a process called non-homologous end joining (NHEJ) that does not require a region of homology. Therefore DSB repair by NHEJ can be mutagenic. As previously mentioned plants exposed to stress conditions exhibit an increased rate of somatic recombination. Arabidopsis plants challenged with UV-C, xylanase, and bleomycin lead to increased rates of recombination (Molinier et al 2005). UV-C irradiation and xylanase, a fungal elicitor that induces an oxidative burst and defense responses, damages DNA possibly by the production of reactive oxygen species (Molinier et al., 2005). Bleomycin chelates Fe^{3+} and reduces it to Fe^{2+} while releasing oxidative degradation products (Norskov-Lauritsen et al., 1990). Bleomycin, UV-C, and xylanase have all been shown to cause DNA DSBs (Norskov-Lauritsen et al., 1990; Menke et al., 2001). Microarray analysis showed these treatments differentially regulated the expression of genes associated with the defense and stress response, signaling, and transcription factors (Molinier et al 2005). The expression data along with the increased recombination frequency suggests the possibility of shared components in the DNA repair pathway and the defense/stress response pathway. Another example of a mechanistic link between the defense response and recombination was described by

Durrant et al (2007). Using mutants for *SNI1*, a repressor that regulates transcription of *PR* genes, and *RAD51D*, which plays a role in *PR* gene expression and defense, Durrant et al demonstrated reduced somatic recombination after treatment with BTH, INA, or pathogen challenge. *SNI1* and *RAD51D* appear to have a dual role in that they can regulate both defense gene expression and DNA recombination. It will be interesting to determine the influence of abiotic stress on the frequency of recombination with endogenous multigene clusters and whether a similar correlation between DNA repair pathway and defense/stress response pathway components can be made.

I.1.11 Objectives of Research

There is considerable speculation on the role of recombination in the evolution of resistance gene clusters in plants. A more comprehensive understanding of the molecular biology of meiotic unequal crossing over is necessary. The objectives of this research were to develop a genetic screen that models meiotic unequal crossing over at a plant resistance gene cluster in *Arabidopsis* and to assess the effect of abiotic stress on recombination within the synthetic *RBCSB* gene cluster (*synthRBCSB*). The specific aims of this research were to (1) mutagenize the promoter of *RPP8* to create a transcriptionally inactive *synthRPP8* gene cluster; (2) generate *synthRPP8* gene cluster constructs that can be utilized for recombination studies; (3) identify the optimal conditions to perform the recombination assay (4) and develop a UV-C irradiation assay that can demonstrate the influence of abiotic stress on meiotic recombination with a *synthRBCSB* gene cluster.

Chapter II

Construction and Optimization of a *synthRPP8* Resistance Gene Cluster to Model Meiotic Unequal Crossing Over in *Arabidopsis thaliana*

II.2 Introduction

There is increasing evidence that recombination plays an important role in the evolution of *R* genes (reviewed in Ellis et al., 2000; Hulbert et al., 2001). Sequence exchange between genes in the same or different clusters influence *R* gene cluster evolution (Dodds et al., 2001; Kuang et al., 2004, 2005; Bakker et al., 2006). For example, at the flax rust resistance locus *L*, naturally occurring interallelic recombination was shown to create chimeric genes encoding novel proteins with race-specificities that differ from both parental alleles (Ellis et al., 1999; Luck et al., 2000). .

Additional evidence supporting the relevance of recombination to R gene evolution has been provided by previously published studies of different alleles of the *Arabidopsis RPP8* locus. The *RPP8* locus in the Landsberg erecta (Ler) accession (*RPP8*-Ler) contains 2 NBS-LRR genes: *RPP8* (confers resistance to the Emco5 isolate of *Hyaloperonospora parasitica*) and *RPH8A* (unknown function) (Fig. II.1A). *RPP8* and *RPH8A* share a high degree of DNA sequence similarity (~91% identity) (McDowell et al., 1998). In contrast, the *RPP8* locus in the Columbia accession of *Arabidopsis (RPP8*-Col) contains a single chimeric gene, which resembles the 5' half of *RPP8* and the 3' half of *RPH8A* (McDowell et al., 1998) (Fig. II.1B). It was suggested that the chimeric structure of *RPP8*-Col was likely derived from recombination within a Ler-like ancestral cluster (McDowell et al., 1998). *RPP8*-Col does not provide resistance to Emco5 and its function is currently unknown.

Functional divergence at the *RPP8* locus was conclusively demonstrated by identification of the *HRT* gene from the Dijon-17 accession of *Arabidopsis*, and the *RCY1* gene of the C24 accession. Both of these genes are allelic to *RPP8*. However, both alleles recognize completely unrelated pathogens: *HRT* recognizes Turnip Crinkle Virus (TCV), and *RCY1* recognizes the yellow strain of Cucumber Mosaic Virus (CMV-Y) (Takahashi et al., 2002). The coat protein of TCV acts as the elicitor of the resistance response in TCV (Zhao et al., 2000) and it was postulated by Ren et al (2000) that *HRT* might recognize a protein complex composed of the TCV coat protein and a TCV coat protein-interacting protein (TIP). The coat protein of CMV-Y acts as the elicitor of the resistance response in C24 (Takahashi et al., 2001). The coat proteins of these two pathogens share very little sequence similarity. Interestingly, *HRT* and *RCY1* have the

chimeric structure of *RPP8*-Col (Fig. II.1C and D). The different recognition specificities suggest recombination has played a role in the evolution of functionally divergent *RPP8* alleles that provide resistance to diverse pathogens through recognition of sequence-unrelated elicitors. However, the *RPP8-Col*, *HRT,* and *RCY1* alleles are distinguished from each other by a large number of nucleotide substitutions that change the sequence of the encoded protein. Therefore, the recognition specificities of these genes may be due to either the chimerism alone and/or the base pair changes that occurred after recombination.

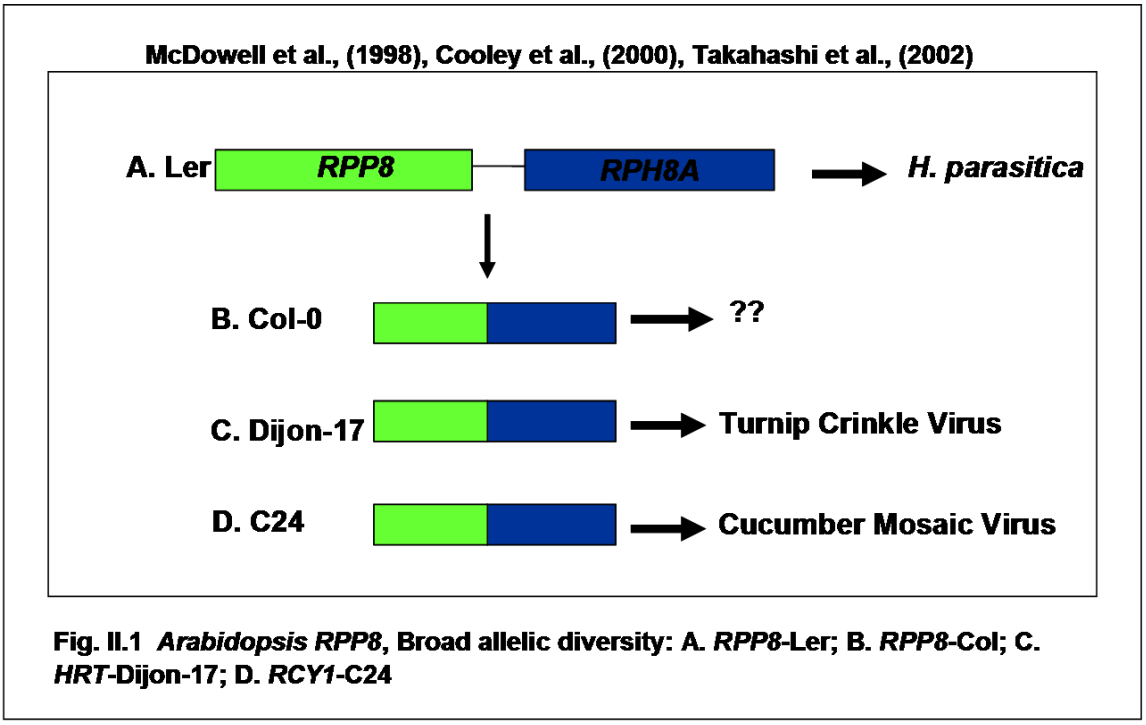


Fig. II.1 *Arabidopsis RPP8*, Broad allelic diversity: A. *RPP8*-Ler; B. *RPP8*-Col; C. *HRT*-Dijon-17; D. *RCY1*-C24

Although these and other studies support the relevance of recombination to *R* gene evolution (reviewed in McDowell and Simon, 2006), there are relatively few estimates of the rates of specific types of recombination between *R* gene clusters (Parniske et al., 1997; Molinier et al., 2004; Yandeau-Nelson et al., 2006). More studies that provide quantitative measures of recombination with statistical significance are necessary. Additionally, an assay that allows higher resolution mapping of recombination sites is needed to effectively evaluate the sequence characteristics of recombination and the influence of recombination on creating genetic diversity. The

immediate functional consequences of recombination are of particular interest because we are uncertain if novel recognition specificities can arise from one round of recombination.

In this chapter, I report the development of a genetic screening system that utilizes synthetic gene cluster technology to investigate meiotic unequal crossing-over at a synthetic *RPP8* locus (*synthRPP8*) in *Arabidopsis thaliana*. Two *synthRPP8* clusters (*synthRPP8-1* and *synthRPP8-2*) were constructed. Transgenic *synthRPP8* lines were created in *Arabidopsis* and lines with a single-copy insertion of the transgene were isolated (*synthRPP8-1.21* and *synthRPP8-2.37*). Numerous optimization steps were performed to fine-tune the bioluminescence assay and the screening conditions so that large population sizes could be screened with maximal throughput and high sensitivity.

An initial screen of ~1 million *synthRPP8* transgenic plants was performed and plants that expressed the luc$^+$ phenotype were isolated and analyzed. Unexpectedly, background bioluminescence was found to interfere with the identification of bona fide luc$^+$ *synthRPP8* recombinants. An abiotic stress response assay was performed and the data suggests activation of a putative stress response element in the promoter of *RPP8* is responsible for background levels *in vivo* luciferase activity. Therefore, attempts to reduce the background of bioluminescence were made. The background bioluminescence could not be sufficiently reduced. Therefore, two *synthRPP8* constructs, *synthRPP8-3* and *synthRPP8-4*, were constructed with a large portion (535 bp and 679 bp respectively) of the *RPP8* promoter removed, to lessen the chances of activation of the *RPP8* promoter. Transgenic *synthRPP8-3* and *synthRPP8-4* lines were created and selection for single-copy transgene insertions have identified one line, *synthRPP8-4.64*, that may be used to investigate meiotic unequal crossing-over at a synthetic *RPP8* locus (*synthRPP8*) in *Arabidopsis thaliana*.

II.2.2 Materials and Methods

Plasmids and plant lines utilized in this study: Construction of all recombination substrates is described in detail below. Table I lists the plasmids and plant lines utilized. Please note: *synthRPP8* is used as a generic name to reference the synthetic *RPP8* locus.

Table 1: Recombination Substrates: Plasmid and Plant Lines

Plasmids and Plant Lines			
Plasmid	**Genotype**	**Locus**	**Transgenic Plant Designation**
pJGJ184	*LUC-NOS*	N/A	N/A
pJGJ329	*RPP8*	N/A	N/A
pCG8	*RPP8-Lox-LUC-NOS*	N/A	N/A
pSS5	$^{C\Delta T}$*RPP8*	N/A	N/A
pBW44	*RPP8::Lox::LUC-Lox*	N/A	N/A
pSS6	$^{C\Delta T}$*RPP8::Lox::LUC-Lox*	N/A	N/A
pBW56	*Lox::LUC-Lox-NOS*	N/A	N/A
pBW65	*UTR*	N/A	N/A
pSS8	*Lox::LUC-Lox-UTR*	N/A	N/A
pBW57	*Intergenic*	N/A	N/A
pSS9	*Lox::LUC-Lox-UTR-Intergenic*	N/A	N/A
pSS10	$^{C\Delta T}$*RPP8::Lox::LUC-Lox-UTR-Intergenic*	N/A	N/A
pBW63	*RPH8A*	N/A	N/A
pSS11	$^{C\Delta T}$*RPP8::Lox::LUC-Lox-UTR-Intergenic-RPH8A*	N/A	N/A
pSS12	$^{C\Delta T}$*RPP8::Lox::LUC-Lox-NOS-Intergenic-RPH8A*	N/A	N/A
pSS13	*pCambia 3300 with restriction enzyme cassette*	N/A	N/A
pSS15	$^{C\Delta T}$*RPP8::Lox::LUC-Lox-UTR-Intergenic-RPH8A*	synthRPP8-1	synthRPP8-1.21
pSS16	$^{C\Delta T}$*RPP8::Lox::LUC-Lox-NOS-Intergenic-RPH8A*	synthRPP8-2	synthRPP8-2.37
pSS35	$^{535\Delta}$*RPP8::Lox::LUC-Lox-NOS-Intergenic-RPH8A*	synthRPP8-3	N/A
pSS36	$^{679\Delta}$*RPP8::Lox::LUC-Lox-NOS-Intergenic-RPH8A*	synthRPP8-4	synthRPP8-4.64

Table I: Symbols and Abbreviations

- In-frame gene fusion= ::
- Linked genes= -
- *RPP8*= *RPP8* gene
- *RPH8A*= *RPH8A* gene
- *LUC*= luciferase gene
- *Lox*= 32 bp Cre recombinase recognition sequence
- *NOS*= nopaline synthase gene
- *UTR*= untranslated region
- *Intergenic*= intervening sequence between *RPP8* and *RPH8A*
- *CΔT*= C to T transition
- *535*Δ= 535 bp deletion
- *679*Δ= 679 bp deletion

Site-directed mutagenesis of a putative cis-element in the *RPP8* promoter

The point mutation in the *RPP8* promoter was created with the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), following the manufacturer's protocol. The following oligonucleotide primers were designed to incorporate the mutation: oSAS3 (5'-CTATCTAGCTACCAATCTCTTGACCGAGGATTCTCTAC-3') and oSAS4 (5'-GTAGAGAATCCTCGGTCAAGAGATTGGTAGCTAGATAG-3'). Plasmid pJGJ329 was the substrate used for mutagenesis of the *RPP8* promoter to create pSS5 ($^{C\Delta T}$*RPP8*). Mutagenesis was confirmed by DNA sequencing. The XmaI/BsmBI mutagenized fragment from pSS5 ($^{C\Delta T}$*RPP8*) was subcloned into XmaI and BsmBI sites in pBW44 (*RPP8*::Lox::*LUC*-Lox fusion in pBluescript II SK+) to create pSS6.

RPP8::Lox::LUC-Lox-UTR-RPH8A gene fusion and *RPP8::Lox::LUC-Lox-NOS-RPH8A* gene fusion

Prior to fusing $^{C\Delta T}$*RPP8* to *LUC*, the 3' UTR of *RPP8* and part of the intergenic region that separates *RPP8* from *RPH8A* was subcloned into pBW56 (*Lox-LUC-Lox-NOS* plasmid construct; pBluescript II SK$^+$ backbone vector). The base plasmid for construction of pBW56 was pJGJ184 (Jelesko et al., 1999), which consists of the coding region of luciferase and the nopaline synthase gene (NOS) in pBluescript II SK$^+$. pBW56 was constructed in 3 parts (fragment A, B, and C) with three PCR products designed to be inserted into pJGJ184. Fragment A (210 bp) was amplified from template pCG8

(*RPP8-Lox-LUC-NOS*) using primers oBJW6 (5'-CCATCTTGTTGGCATGGCGGCCGCTAGCCCTCAGCCTGAGCGATCGCGCATCCGCGGAGCTCCATGGAGATAACTTCGTATAATGTATGC-3') and bwLucAS1 (5'-CAGGGCGTATCTCTTCATAG-3'). Fragment B (102 bp) was amplified from template pJGJ184 using primers oBJW7 (5'-CAGAGAGATCCTCATAAAGGC-3') and oBJW8 (5'-CTGCAGTCAGCTACTTACAATAACTTCGTATAGCATACATTATACGAAGTTATTTACAATTTGGACTTTCCGCC-3'). Fragment C (301 bp) was amplified from template pJGJ184 using primers oBJW9 (5'-CTGCAGGATCGTTCAAACATTTGGCAA-3') and oBJW10 (5'-GGTACCTCAGGGCCGGCCAGTCGGCGCGCCGTCAGCCCGGGCGATCTAGTAACATAGATGACA-3'). Fragments A, B, and C were created with the proofreading polymerase Elongase (Elongase® Enzyme Mix, Invitrogen Corporation, Carlsbad, California) and the PCR parameters used to make these fragments followed the Manufacturer's protocol with the exception of using only Buffer B and no Buffer A, 1ng of template and the following amplification procedure. Amplification consisted of one cycle of 3 min at 94°C; 25 cycles of 30 sec at 94°C, 30 sec at 42°C, 30 sec at 68°C; followed by a 10 min extension at 68°C. The PCR fragments were cloned into Invitrogen vector pCR2.1 using the TA cloning kit pCR2.1 TOPO, according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, California). PCR fragment A replaced the region of pJGJ184 between the *Bst*XI site in the 5' polylinker and a primer (bwLucAS1) binding site just downstream of the *Nar*I site in luciferase. Fragment A contained restriction enzyme sites for *Bst*XI, *Not*I, *Bbb*CI, *Sgf*I, *Sac*II, and *Nco*I. The *Nco*I site was designed in-frame with the 36 bp Lox site (5'-ATAACTTCGTATAATGTATGCTATACGAAGTTATTG-3'). The 5' end of the luciferase coding region through the binding site for the primer bwLucAS1 followed and completed fragment A. PCR fragment B was inserted into sites *Eco*NI-*Kpn*I, and PCR fragment C was inserted into sites *Pst*I-*Kpn*I. The final construction of pBW56 created a cassette consisting of a recognition site for the Cre recombinase in frame with the coding region for firefly Luciferase (*LUC*) through the codon just prior to the stop codon,

followed by a second Lox site in the same orientation as the first (Lox sites are in direct repeat orientation), followed by stop codons in all three reading frames, followed by the 3' untranslated region from the nopaline synthase (*NOS*) gene for efficient translation to create (*Lox::LUC::Lox::NOS*).

Next, the 3' UTR was subcloned from pBW65 as a *Pst*I-*Xma*I fragment into pBW56 (creating pSS8). The intergenic region (pBW57) was inserted as an *Age*I-*Asc*I fragment into the *Xma*I-*Asc*I digested pSS8 (pSS9). *Age*I and *Xma*I produce compatible overhangs. Ligation of these two ends destroys the *Xma*I site. The mutagenized *RPP8* fragment of pSS6 was then subcloned into pSS9 as a *Not*I-*Nar*I fragment (pSS10). The assembly order described above allowed the firefly luciferase gene (*LUC*) to be fused, in-frame, to the 3' end of *RPP8*. Subsequently, the *Sac*II-*Xho*I *RPH8A* fragment from pBW63 was subcloned into pSS10 to create pSS11 (*RPP8::Lox::LUC-Lox-UTR-RPH8A*).

All of the above steps, except for the first step that replaced the *NOS* terminator for the 3' UTR, were followed to create the second version, pSS12 (*RPP8::Lox::LUC-Lox-NOS-RPH8A*), of the synthetic *RPP8* cluster. To facilitate the insertion of pSS11 and pSS12 into the binary vector pCambia 3300, a PCR amplified cassette containing the restriction sites *Xma*I-*Bst*XI-*Not*I-*Bbv*CI-*Sgf*I-*Sac*II-*Nco*I-*Asc*I-*Fse*I-*Kpn*I-*Sac*I was inserted into pCambia 3300 using restriction sites *Xma*I and *Sac*I (pSS13). This 81 base pair cassette was constructed using oligonucleotide primers oSAS9 (5'-CCCGGGCCATCTTGTTGGGCGGCCGCCCTCAGCGCGATCGCCCGCGGCCAT-3') and oSAS10 (5'-GAGCTCGGTACCGGCCGGCCGGCGCGCCCCATGGCCGCGGGCGATCGCGCT-3'). Recombinant plasmids were identified by restriction digest of miniprep DNA with *Asc*I (not present in pCAMBIA 3300 but is present within 81 base pair cassette) and confirmed by sequencing. In preparation for transformation of *A. thaliana*, pSS11 and pSS12 was subcloned from pBluescript into the binary vector pSS13 to create pSS15 (*$^{C\Delta T}$RPP8::Lox::LUC-Lox-UTR-RPH8A*; *synthRPP8-1*) and pSS16 (*$^{C\Delta T}$RPP8::Lox::LUC-Lox-NOS-RPH8A*; *synthRPP8-2*)

Plant Transformation, Growth Conditions, and Selection

Plant transformation

Binary plasmid clones were transformed into *Agrobacterium tumefaciens* strain GV3101 using electroporation as described in Ausubel et al., 1996. Transformed cells were selected by plating onto medium containing gentamycin, rifampicin and kanamycin. The cells that contained *synthRPP8-1* and *synthRPP8-2* were introduced into the Col-0 and/or CW84 plant lines (derived from a cross between Wassilewskija (WS-0) (Botella et al., 1998) and Columbia (Col-0) of *A. thaliana* using the floral dip method (Bechtold et al., 1993). Transgenic T_1 plants were identified through BASTA selection: ~30,000 seeds were sown onto soil in a 20" length x 10" width x 2.5" height tray and stratified under a dome at 4°C for two days. On day three, seedlings were transferred to growth racks and remained covered under the dome for 3 days. On day 4 the dome was propped open. On day 5 the dome was removed and spraying with the herbicide BASTA commenced. A 1:10,000 dilution (100ul/L) of Liberty "BASTA" herbicide plus 0.005% Silwet (50ul/L) was used. The plants were sprayed heavily once with BASTA for 3 continuous days. A second round of BASTA application was performed 7 days after completion of the first application.

Growth conditions

Plants sown on soil (Sunshine Mix 1, Sun Gro Horticulture, Bellevue, WA) were grown under fluorescent lights (Philips Universal/H-Vision, F32T8/TL741) on a 16-hr light/8-hr dark cycle at 23°C.

Selection for plants with single copy inserts that had not undergone transgene rearrangements during transformation

Transgenes with single copy inserts were selected based on segregation of BASTA resistance as a single genetic trait (~3:1 for resistant to susceptible) in the T_2 generation and Southern blot analysis. For Southern blot analysis, 10 µg of genomic DNA was digested with 10 units of *Bsr*GI for ~12 hours at 37°C. Digested genomic DNA was probed with a ~1300 bp luciferase fragment that was PCR amplified off of

~100 ng of pSS16 with oligonucleotide primers oSAS18 (5'-GATAACTTCGTATAATGTATGCTATACGA-3') and oSAS19 (5'-CTCCAGAATGTAGCCATCCATCCTTGTCA-3'). The luciferase probe was labeled with digoxigenin (DIG)-dUTP (alkali-labile) according to the manufacturer's instructions (Roche Applied Science; Cat. No. 1 573 152). Hybridization of the membrane with the probe was performed at 42°C overnight (~16hrs). Two low stringency (2X SSC with 0.1% SDS) washes followed by two high stringency (0.5X SSC with 0.1% SDS) washes was carried out at 37°C for five minutes and 65°C for fifteen minutes, respectively (procedure outlined in the DIG Application Manual for Filter Hybridization, Roche Molecular Biochemicals). Probe-target (*LUC* gene) hybrids were detected with the alkaline- phosphatase-conjugated antibody, Anti-Digoxigenin-Alkaline Phosphatase (Roche Applied Science; Cat. No. 11 093 274 910), using the CDP-Star substrate (Roche Applied Science; Cat. No. 1 685 627). The alkaline phosphatase conjugate of the anti-digoxigenin antibody binds to the hybridized probe. The antibody-probe hybrid was visualized with the chemiluminescent alkaline phosphatase substrate, CDP-Star. CDP-Star generates a luminescent signal that is recorded with X-ray film.

Southern blot analysis was used to determine whether the entire *synthRPP8* construct into genomic DNA was incorporated into transgenic plants. Rearrangement in the structural organization of the transgene was assessed by digesting 10 µg of genomic DNA with *Sph*I, which cuts once 5' of *synthRPP8*, once 3' of *synthRPP8*, and once within the luciferase gene. The *Sph*I digested genomic DNA was probed as described above with the same 1300 bp luciferase fragment labeled with DIG-dUTP (alkali-labile). Hybridization and detection of DNA blots were performed as described above.

Assay for Bioluminescence

Growth and imaging conditions for LUC assay

Approximately 10^6 T_4 seeds obtained from homozygous *synthRPP8-1* (*$^{C\Delta T}$RPP8::Lox::LUC-Lox-UTR-RPH8A*) and *synthRPP8-2* (*$^{C\Delta T}$RPP8::Lox::LUC-Lox-NOS-RPH8A*)) were collected into 10 independent lots. Each lot contains seed collected from five to six 3 ½ in. pots with approximately nine plants per pot. Approximately 7500 T_4 seedlings from a single independent seed lot were germinated on 20 cm X 20 cm

Whatman 3MM chromatography paper moistened with 1X Hoagland's solution (plant nutrient solution). The seedlings were stratified for 2 nights at 4°C and then placed under fluorescent lights (Philips Universal/H-Vision, F32T8/TL741) on a 16-hr light/8-hr dark cycle for 7 days at 23°C. T_4 seedlings were then assayed for *in vivo* luciferase activity as follows: 20 min prior to imaging, seedlings were sprayed with 1 mM synthetic D-luciferin (Biosynth, Basel), 0.01% Triton X-100 solution. The tray was imaged for 20 minutes in photon-counting mode using an intensifier charge-coupled device video camera (model C2400 47), an Image Intensifier Controller (model M4314), an Image Processor (Argus 50), and an imaging chamber (model A417) containing a Xenon CM 120 lens (Schneider, Bad Kreuznach, Germany). Unless otherwise stated the imaging equipment are products of Hamamatsu Photonics, Hamamatsu City, Japan (refer to Jelesko et al., 1999 for more detailed information on imaging equipment). If a putative luc^+ plant appeared, the tray was imaged for an additional 10 minutes. The approximate region on the tray where the luc^+ signal appeared was estimated and all of the seedlings in this area were transferred to a water-agar (Difco™ Agar, Becton, Dickinson and Company, Sparks, MD) plate and re-imaged to identify the individual luc^+ seedling. Luc^+ seedlings were then transferred to soil and grown under standard conditions as described above.

Diagnostic restriction marker assay to distinguish the wildtype *RPP8* promoter from the mutated promoter

Primers oJM7 (5'-GATCCCATGGAACAACTAGTCGTCGAGAAT-3') and oSAS8 (5'-GGTTCCGACAACTAAGCAAACTGC-3') were used to amplify the *RPP8* promoter fragment from ~100 ng of genomic DNA from the luc^+ plants. Amplification consisted of one cycle of 3 min at 94°C; 35 cycles of 30 sec at 94°C, 30 sec at 53°C, 45 sec at 72°C; followed by a 10 min extension at 72°C. 0.5 units of restriction enzyme *Hae*III was incubated with 10 ul of the PCR product for ~ 4 hours at 37°C to cleave PCR products containing the wildtype promoter.

***synthRPP8* and Abiotic Stress Assay**

Abiotic stress regime

Water deprivation/drought: plants were germinated on soil and grown as previously described for seven days. The plants were watered Monday, Wednesday, and Friday until day seven. Watering ceased on day seven and the water deprivation continued until the plants were 3 weeks of age.

Constant light: plants that were subjected to water deprivation were placed under fluorescent lights (Philips Universal/H-Vision, F32T8/TL741) on a 24-hr light cycle at 23°C. The constant light regime began on day seven and continued until the plants were 3 weeks of age.

Assembly of modified *synthRPP8*

Deletion of the promoter of *synthRPP8* to create *synthRPP8-3* and *synthRPP8-4*

Two modified *synthRPP8* (*synthRPP8-3* and *synthRPP8-4*) gene clusters were created. The first is pSS35 ($^{535\Delta}$*RPP8::Lox::LUC-Lox-NOS-RPH8A*; *synthRPP8-3*), which has ~535 bp deleted from the *RPP8* promoter in the original construct pSS16. The *RPP8* sequence in pSS35 begins 151 bp 5' to the ATG start site in *RPP8*. The second, is pSS36 ($^{679\Delta}$*RPP8::Lox::LUC-Lox-NOS-RPH8A*; *synthRPP8-4*), from which 679 bp was deleted from the *RPP8* promoter in the original construct pSS16. The *RPP8* sequence in pSS36 begins 7 bp 5' to the ATG start site in *RPP8*.

pSS35 was constructed by using restriction sites to remove a large portion of the *RPP8* 5' flanking region: pSS16 was digested with enzyme *Ahd*I. This created a 3'-1 bp overhang that was removed using T4 DNA polymerase. The pSS16/*Ahd*I/T4 polymerase treated DNA was then digested with *Sma*I, which cuts 5' to the ATG start site in *RPP8* and creates a blunt end. In the final step, the blunt ends created by *Ahd*I and *Sma*I were ligated with T4 DNA ligase to circularize the deletion-containing pSS16.

pSS36 was constructed using exonuclease digestion to remove a large portion of the *RPP8* 5' flanking region. First, pSS16 was digested with enzyme *Ahd*I, creating a 3'-1 bp overhang. The 3' overhang was removed using T4 DNA polymerase. Following

the removal of the 3'-1bp overhang, the Erase-a-Base® System (Promega Corporation, Madison, WI) was applied to the pSS16/*Ahd*I/T4 polymerase treated DNA to generate nested deletions. The Erase-a-Base® System created nested deletions using the following steps. First, the DNA was treated with exonuclease III, which digests DNA from the blunt-end restriction site. Second, the samples of the Exonuclease III digestion were removed at timed intervals and added to tubes containing S1 nuclease. S1 nuclease removed the remaining single-stranded tails. Third, the S1 nuclease was neutralized and heat inactivated. Fourth, Klenow DNA polymerase was added to fill in the ends, which were then ligated with T4 DNA ligase to circularize the deletion-containing pSS16.

II.2.3 Results

Overview of Experimental Design

The *synthRPP8* cluster was derived from the *RPP8* gene cluster found in the Ler ecotype of *Arabidopsis thaliana* (Fig. II.1A). This cluster contains two genes: *RPP8* (confers resistance to *Hyaloperonospora parasitica*) and a closely-linked homolog, *RPH8A* (unknown function). *SynthRPP8* is composed of a transcriptionally-inactive *RPP8::Lox::LUC-Lox-NOS/UTR* gene fusion followed by the *RPH8A* gene (Fig. II.2).

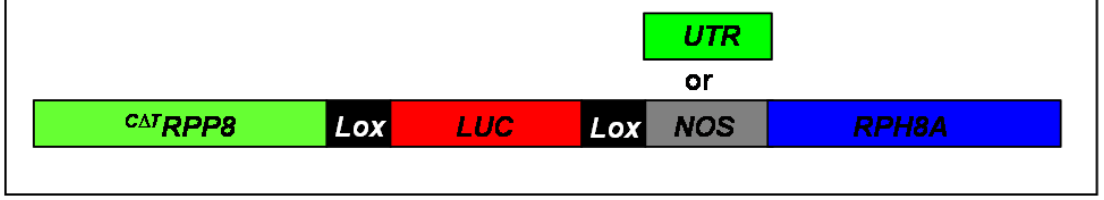


Fig. II.2 *synthRPP8*

In order to model meiotic recombination of the endogenous *RPP8* cluster as accurately as possible, our experimental design incorporated the following objectives. First, the *RPP8* promoter should be completely inactivated, because the assay for identifying recombinant chimeric gene formation couples chimeric gene formation to the activation of the firefly luciferase gene (*LUC*). Any residual luciferase activity would interfere with accurate identification of luc$^+$ seedlings that contain recombinant genes.

Second, the luciferase gene should be removable. One of our long-term objectives is to test the function of chimeric genes by pathogen challenge assays. It is necessary to remove the luc gene prior to pathogen challenge because the *LUC* fusion could interfere with the resistance function of the chimeric protein. Therefore, the *LUC* gene in *synthRPP8* is flanked by two direct repeats of the 32 base pair recognition sequence for Cre recombinase (*Lox*) (Hoess and Abremski., 1985). Cre recombinase is a Type I topoisomerase from bacteriophage P1 that catalyzes the site-specific recombination of DNA between Lox sites. The *Lox* sites enable excision of the luciferase gene by site-specific recombination between the directly repeated *Lox* sites. To remove the luciferase gene, transgenic lines containing chimeric genes will be crossed to a

transgenic line expressing Cre recombinase. The *LUC* gene located between the directly repeated Lox sites will be excised as an aberrant extrachromosomal circle that lacks an origin of replication.

Third, previous evidence from our lab suggests that the *NOS* terminator fused to the *LUC* gene may interfere with the resistance function of the construct (Woffenden, unpublished). We made two constructs in which the *RPP8::Lox::LUC-Lox* gene fusion is followed by either the native *RPP8* 3' untranslated (UTR) region or the 3' untranslated region from the nopaline synthase (*NOS*) gene.

Fourth, to recapitulate recombination at the *RPP8* locus more accurately, the configuration of *synthRPP8* should reflect the endogenous *RPP8* cluster structure as closely as possible. In particular, the spacing between *RPP8* and *RPH8A* should mimic the endogenous *RPP8* locus. This is important because the frequency of recombination is influenced by the physical distance between linked genes i.e. the probability of crossing over decreases with physical distance. The *RPP8::Lox::LUC-Lox-NOS/UTR* gene is followed by the intergenic region that separates *RPP8* and *RPH8A*. This region was incorporated into both versions of the *synthRPP8* cluster because it is the intervening sequence in the native Ler*RPP8* cluster. The length of the intergenic region does not correspond to the endogenous intergenic sequence between *RPP8* and *RPH8A*. However, the spacing between the two genes was preserved such that the length of *synthRPP8* reflects the length of the endogenous *RPP8* locus.

Fifth, the *synthRPP8* cluster was introduced into the CW84 plant line, which is a hybrid of a cross between the Columbia and Wassilewskija ecotypes of *Arabidopsis*. One of the major long-term objectives of this project is to characterize the resistance function of the *RPP8/RPH8A* chimeric genes. This can be achieved by challenging with pathogens that define the parental gene recognition specificities as well as a panel of virulent pathogens to determine whether recombination has given rise to new resistance specificities. CW84 was chosen as the plant line to introduce the *synthRPP8* transgene into because several *RPP* genes have been bred out of this hybrid (Botella et al., 1998). Thus, CW84 is susceptible to a wide variety of *Hyaloperonospora parasitica* isolates that can be used to probe for chimeric genes that have a gain-of-function (new resistance) phenotype.

Finally, the recombination assay will be performed with plants that contain single copy transgene inserts, because multicopy insertions would not recapitulate the structure of the endogenous *RPP8* cluster, which contains only two genes.

Below, we describe the assembly process used to create the *synthRPP8* gene cluster, as well as the steps taken to implement the synthetic gene cluster technology and the bioluminescence assay with *synthRPP8*.

Mutation of the *RPP8* Promoter

My first attempt to render *RPP8::Lox::LUC-Lox* transcriptionally inactive was to introduce a point mutation into a putative cis-element upstream of the *RPP8* coding sequence (Holt et al., 2002). Utilization of a single point mutation versus deletion of the entire promoter was preferred because we wanted to leave the 5' region of *RPP8* as intact as possible so that we could detect recombinants that resolved in the 5' flanking region. The first strategy for inactivating the gene was to engineer a point mutation in a putative cis element that was previously identified through EMS mutagenesis of the *RPP8* gene (Holt et al., 2002). This mutation replaced a cytosine with thymine, 189 base pairs upstream from the Ler*RPP8* translational start codon. This mutation lies within a putative cis-regulatory element that is conserved among *RPP8* gene family members (Holt et al., 2002). As a result of the point mutation the accumulation of *RPP8* mRNA is reduced to levels undetectable by an RT-PCR assay (Holt et al., 2002). In addition, this mutation completely disables the pathogen resistance provided by *RPP8* (Holt et al., 2002).

Site-directed mutagenesis was used to replace the cytosine with thymine within the Ler*RPP8* sequence of pJGJ329 (Material and Methods). The effect of the mutation was initially assessed by assaying luciferase activity in transgenic T_1 plants containing the $^{C\Delta T}$*RPP8::Lox::LUC-Lox-NOS* fusion, compared to the wildtype *RPP8::Lox::LUC-Lox-NOS* control lines. Fifty transgenic T_1 plants from $^{C\Delta T}$*RPP8::Lox::LUC-Lox-NOS* (Fig. II.3A) and the wildtype control *RPP8::Lox::LUC- Lox-NOS* (Fig. II.3B) were assayed. As expected, every plant containing $^{C\Delta T}$*RPP8::Lox::LUC-Lox-NOS* exhibited a luc$^-$ phenotype. This result suggests that the point mutation was sufficient to disable the *RPP8* promoter to a point at which luciferase activity was undetectable.

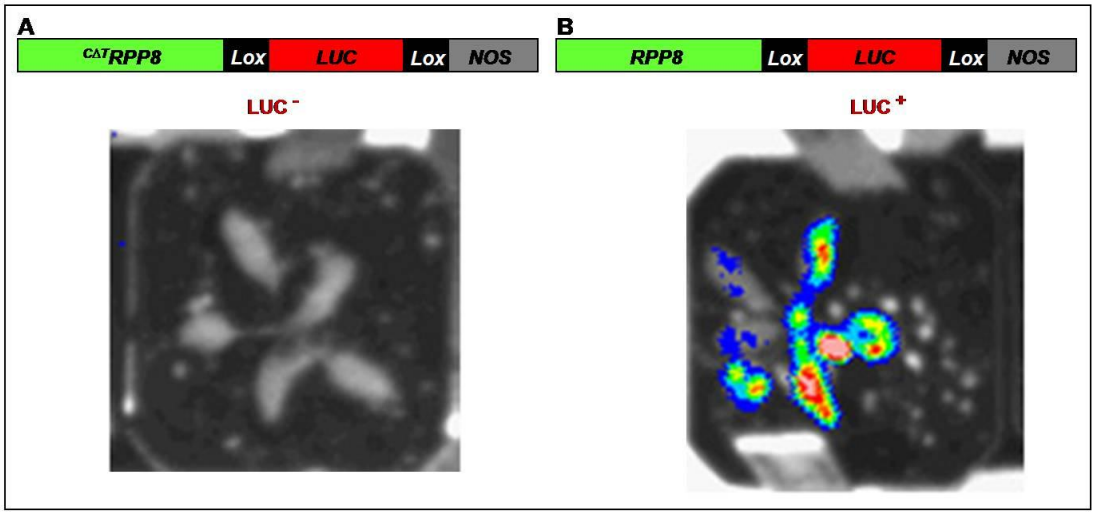


Fig. II.3 A. *CΔTRPP8::Lox::LUC-Lox-NOS* fusion produces LUC⁻ phenotype vs B. *RPP8:: Lox::LUC-Lox-NOS* fusion that produces LUC⁺ phenotype

Assembly of the *synthRPP8* Constructs

Multiple intermediate cloning steps were performed for the assembly of the full length *synthRPP8* cluster. The Material and Methods section outlines the assembly steps used to create the two versions (*synthRPP8-1* and *synthRPP8-2*) of the *synthRPP8* cluster described above. The Materials and Methods section and Table 1 provide a detailed description of all plasmids and plant lines utilized in this dissertation.

Creating Transgenic *synthRPP8* Lines

Transgenic *Arabidopsis* CW84 lines carrying *synthRPP8-1* (*CΔTRPP8::Lox::LUC-Lox-UTR-RPH8A)* or *synthRPP8-2* (*CΔTRPP8::Lox::LUC-Lox-NOS-RPH8A)* were generated as described in the Materials and Methods. Our strategy to identify lines with single copy inserts was to select lines with a single transgene locus based on segregation of BASTA resistance (expected ratio of ~3 resistant to 1 susceptible in the T_2 generation) (Tables 2 and 3) and follow up with Southern blot analysis to assess transgene copy number.

Table 2: Segregation of BASTA Resistance in *synthRPP8-1* T_2 Plant Lines

Segregation of BASTA Resistance				
Plant Line	**Resistant**	**Susceptible**	**R:S**	**R:S ratio**
AtSS15.1	**4**	**16**	**4:16**	**1:4**
AtSS15.6	**2**	**10**	**2:10**	**1:5**
AtSS15.15	**10**	**8**	**10:8**	**1:1**
AtSS15.19	**1**	**8**	**1:8**	**1:8**
AtSS15.20	**10**	**4**	**10:4**	**3:1**
AtSS15.21	**22**	**22**	**22:22**	**1:1**
Duplicate 15.21	**14**	**23**	**14:23**	**1:2**
AtSS15.29	**8**	**6**	**8:6**	**1:1**
AtSS15.32	**9**	**18**	**9:18**	**1:2**
AtSS15.33	**1**	**3**	**1:3**	**1:3**
AtSS15.34	**15**	**10**	**15:10**	**2:1**
AtSS15.37	**2**	**4**	**2:4**	**1:2**
AtSS15.38	**21**	**7**	**21:7**	**3:1**
AtSS15.44	**3**	**4**	**3:4**	**1:1**
AtSS15.45	**19**	**4**	**19:4**	**5:1**

Table 3: Segregation of BASTA Resistance in *synthRPP8-2* T_2Plant Lines

Segregation of BASTA Resistance				
Plant Line	Resistant	Susceptible	R:S	R:S ratio
AtSS16.2	5	17	5:17	1:3
AtSS16.4	18	9	18:9	2:1
AtSS16.7	56	44	56:44	1:1
AtSS16.9	27	18	27:18	2:1
AtSS16.12	26	16	26:16	2:1
AtSS16.15	40	36	40:36	1:1
AtSS16.16	17	10	17:10	2:1
AtSS16.17	7	6	7:6	1:1
AtSS16.22	4	49	4:49	1:12
AtSS16.24	25	55	25:55	1:2
AtSS16.25	38	26	38:26	1:1
AtSS16.27	11	8	11:8	1:1
AtSS16.28	41	18	39:18	2:1
AtSS16.29	18	14	18:14	1:1
AtSS16.32	4	1	4:1	4:1
AtSS16.35	30	26	30:26	1:1
AtSS16.37	19	5	19:5	4:1
Duplicate 16.37	25	11	25:11	2:1
AtSS16.40	27	10	27:10	3:1
AtSS16.41	20	14	20:14	1:1
AtSS16.42	3	0	3:0	N/A
AtSS16.43	18	7	18:7	3:1
AtSS16.44	6	0	6:0	N/A
AtSS16.45	7	46	7:46	1:7

As shown in Tables 2 and 3, most of the lines tested had non-mendelian rates of segregation. Transgenic lines exhibiting segregation ratios with a higher susceptible value compared to the resistant value i.e. 1 resistant to 3 susceptible were possibly the result of silencing of the BASTA resistance trait. Since the segregation data provided inconclusive evidence for genotypes with only single copy inserts of *synthRPP8-1* and *synthRPP8 -2*, we focused on transgenic lines that did not appear to exhibit silencing of the BASTA resistance trait. These lines were analyzed by Southern blot analysis.

For Southern blot analysis, genomic DNA from *synthRPP8-1* and *synthRPP8-2* plant lines was digested with *Bsr*GI, which cuts twice at the 3'end of *RPH8A* and once within the luciferase gene**,** and probed with a fragment derived from the *LUC* gene (Fig. II.4).

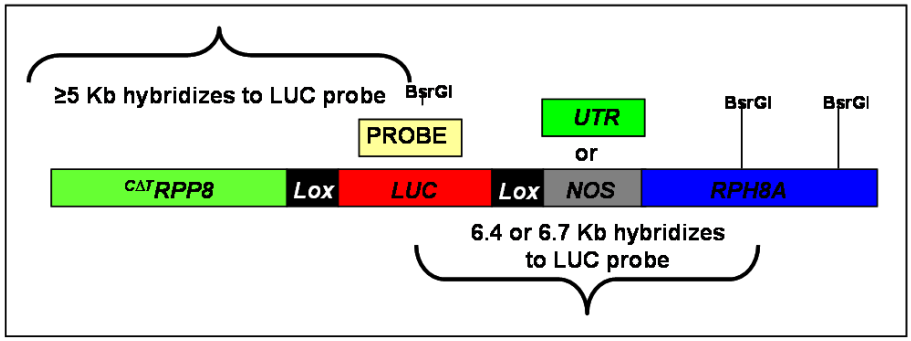


Fig. II.4 Southern Blot with LUC probe: single copy inserts from *synthRPP8-1* produce 6.7 Kb and ≥5 Kb; single copy inserts from *synthRPP8-2* produce 6.4 Kb and ≥5 Kb.

Transgenic lines with single copy insertions of *synthRPP8-1* produce two hybridizing bands; one predicted to be 6.7 Kb and another band ≥5 Kb (the size of this band depends on the location of the next *Bsr*GI site in the *Arabidopsis* genome). Transgenic lines with a single copy insertion of *synthRPP8-2* should produce two hybridizing bands; a predicted 6.4 Kb band and another band ≥5 Kb (again depending on the location of the next *Bsr*GI site that is in the *Arabidopsis* genome). Each of the two hybridizing bands represents a fragment within the *synthRPP8* gene cluster. If more than one transgene copy was present, then three or more hybridizing bands would be obtained. Single copy lines were necessary because multiple transgene insertions may trigger gene

silencing and the endogenous *Arabidopsis RPP8* locus is single copy. Approximately 50 transgenic lines for both *synthRPP8-1* and *synthRPP8-2* were screened by Southern blotting. One transgenic line containing a single copy insertion of *synthRPP8-1* was identified (*synthRPP8-1.21*) and one transgenic line containing a single copy insertion of *synthRPP8-2* was identified (*synthRPP8-2.37*) (Tables 2 and 3 and Fig. II.5).

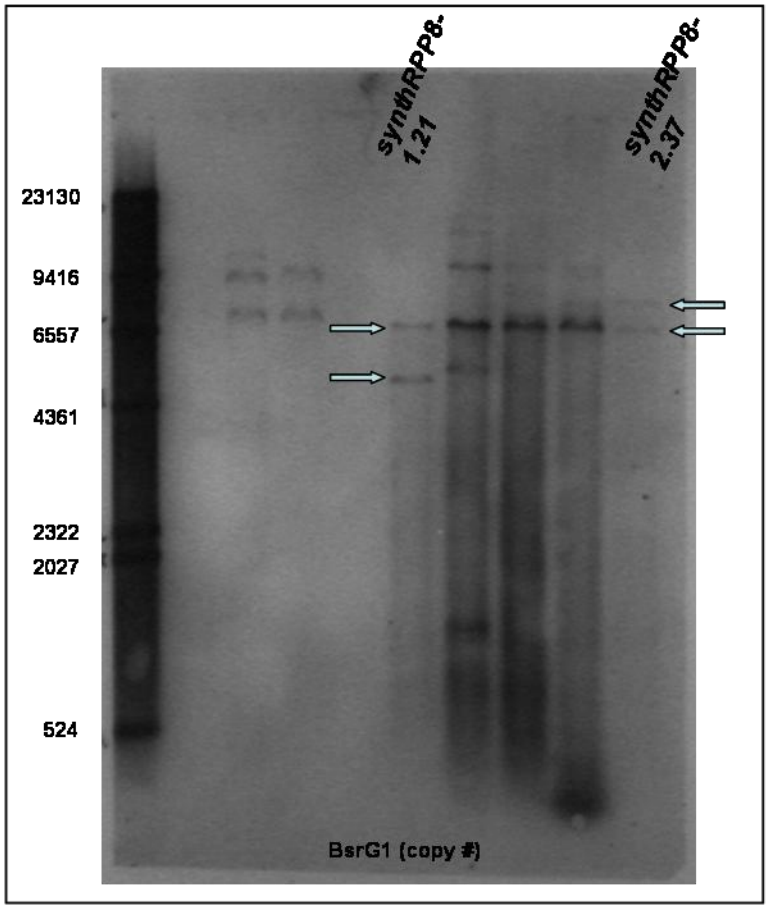


Fig. II.5 Southern Blot with LUC probe: A single copy insertion of *synthRPP8-1* was identified (*synthRPP8-1.21*) and a single copy insertion of *synthRPP8 -2* was identified (*synthRPP8-2.37*)

Because the *synthRPP8* transgene is large at ~14 Kb, Southern blot analysis was used to determine if the entire *synthRPP8* transgene was incorporated into the *Arabidopsis thaliana* genome, without rearrangements. Genomic DNA from *synthRPP8-*

1.21 and *synthRPP8-2.37* plant lines was digested with *Sph*I, which cuts once 5' of *RPP8*, once 3' of *RPH8A*, and once within the luciferase gene, and probed with a fragment derived from the *LUC* gene (Fig. II.6). Both *synthRPP8-1.21* and *synthRPP8 - 2.37* produced the two expected hybridizing bands of 4.9 Kb and 9.3Kb and 4.9 Kb and 8.9 Kb, respectively (Fig. II.7), demonstrating that the entire synthRPP8 was present in the genome of these lines.

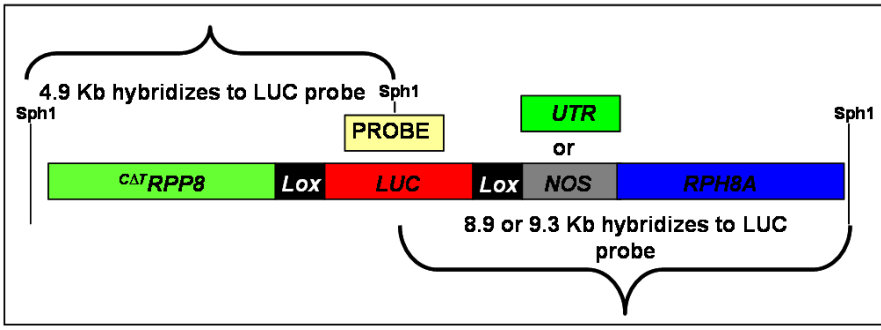


Fig. II.6 Transgene Rearrangement Southern Blot with LUC probe: intact *synthRPP8-1* produces 4.9 Kb and 9.3Kb; intact *synthRPP8-2* produces 4.9 Kb and 8.9 Kb.

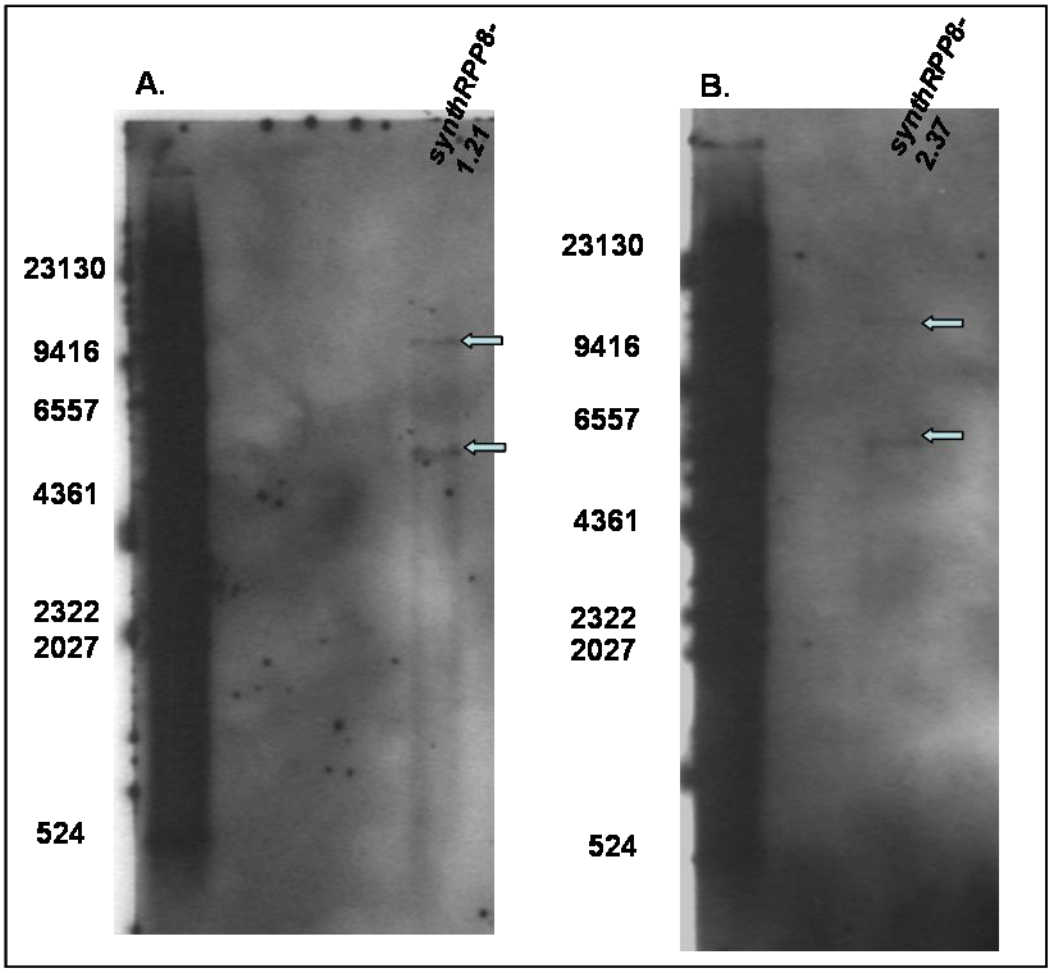


Fig. II.7 Transgene Rearrangement Southern Blot with LUC probe: Both *synthRPP8-1.21* and *synthRPP8-2.37* were intact without any rearrangements in gene structure

Another important measure for selecting transgenic lines to be used in the *synthRPP8* recombination assay was to verify that there was no residual luciferase activity produced by these plants. Plants exhibiting residual luciferase activity could be the result of the transgene inserting proximal to an endogenous transcriptional regulatory element. In the T_2 and T_3 generation, approximately 50 lines from *synthRPP8-1.21* and *synthRPP8 -2.37* were assayed on soil for the expression of luciferase activity at ~2 weeks of age and confirmed to be luc^- (data not shown).

Optimizing the Luciferase Bioluminescence Assay

Based on previous results with *synthRBCSB* gene clusters, as well as other previously established measures of somatic and meiotic recombination, we predict these recombination events to be rare (~10^{-6} to 10^{-5}) (Assaad and Signer, 1992; Jelesko et al., 1999, 2004). Therefore the screening conditions used should be quick and efficient so we can screen large population sizes in a relatively short time frame. Furthermore, we must be able to detect recombinant alleles that display a luc^+ phenotype at high sensitivity, otherwise we will underestimate the frequency of recombination.

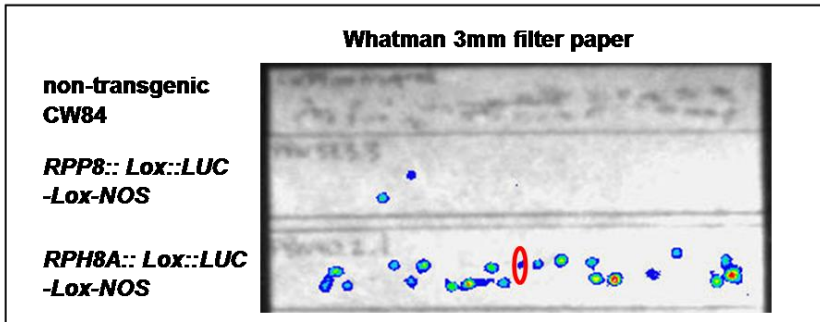
The bioluminescence assay can be performed at almost any age/developmental stage of plant growth. However, young plants are preferred because this lessens the wait time to perform the assay and most importantly, many-fold more plants can be screened per unit of time. Additionally, young plants tend to take up the luciferin substrate more readily, which will affect the distribution of bioluminescence and the sensitivity of the screening process.

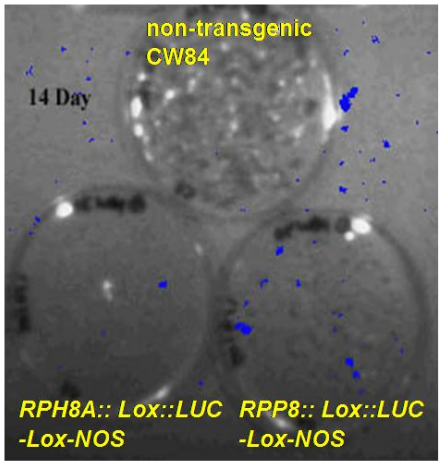
With these considerations in mind, we assessed the appropriate age to assay for *in vivo* luciferase activity (photon emission) by comparing light emission from control seedlings (*RPP8::Lox::LUC-Lox-NOS*) that were 5, 7, 10 and 14 days of age. At 5 days, seedlings were small and provided very low levels of luciferase activity. Even though luc^+ seedlings could be detected at 5 days, *R* gene promoters are often weak and we were concerned that a recombinant chimeric gene may display a lower level of luciferase activity compared to control seedlings. Therefore, it was necessary to screen with conditions that permit higher levels of luciferase activity. The leaves of plants older than 10 days did not bioluminesce evenly. This may be due to uneven uptake of luciferin into the leaves. Seedlings at 7 days of age were the best time to screen because they tend to exhibit higher and more even bioluminescence compared to 5 day-old plants. More importantly though, beyond 10 days fungal contamination became a problem when using the Whatman 3MM filter paper. Therefore 7 days was the preferred age.

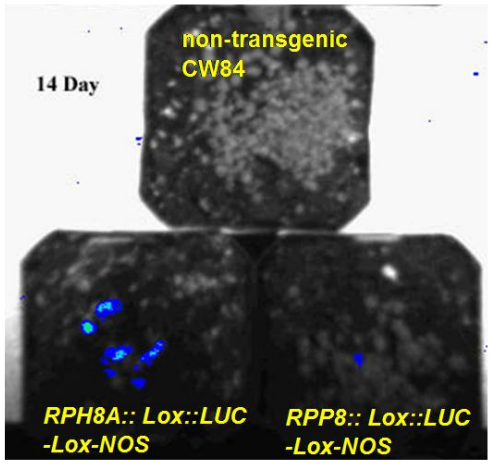
Luc^+ plants obtained from this screen can be either hemizygous or homozygous for the recombinant chimeric transgene. Plants hemizygous for the transgene may show reduced levels of luciferase activity relative to plants homozygous for the transgene. Therefore, the effect of transgene zygosity was assessed. To determine whether meiotic

unequal crossover events that yield a recombinant hemizygous or homozygous gene could be detected, we screened segregating and non-segregating populations of control seedlings that contain either the *RPP8* promoter fused to luciferase or the *RPH8A* promoter fused to luciferase. Luc^+ plants were detected in the segregating population at a ratio of ~3 luc^+ to 1 luc^-, which suggests that plants hemizygous for the transgene can be effectively detected. In addition, luc^+ plants were also detected in the homozygous population of control seedlings.

In order to optimize the conditions for luciferase imaging and screening of *synthRPP8* lines, I compared different plant growth media for assaying for *in vivo* luciferase activity. Seedlings from control lines (*RPP8::Lox::LUC-Lox-NOS* and *RPH8A::Lox::LUC-Lox-NOS*) and non-transgenic CW84 (used as a negative control) were imaged on soil, agar-based growth media, and on Whatman 3MM filter paper moistened with 1X Hoagland's solution (Fig. II.8). All conditions assayed showed photon emission levels equivalent to those observed with an empty imaging chamber (zero photon emission to very low levels (depicted as blue spots of photon emission)). Plants imaged on soil consistently provided the least amount of background activity. The agar based growth media provided the highest amount of background activity and the Whatman 3MM filter paper moistened with 1X Hoagland's solution provided very low levels of background photon emission but sometimes showed luciferase activity where no plant was present. Notably, the level of background photon emission with the Whatman 3MM filter matrix was not so high that it would impede optimal luciferase imaging. There was not a substantial difference in our ability to detect luc^+ plants in any of the growth conditions. However, in contrast to performing the screen on soil, the Whatman 3MM filter paper moistened with 1X Hoagland's solution allows seedlings that did not germinate to be accounted for because one can see directly on the filter paper if a seedling germinated. All seedlings must be accounted for to accurately assess the rate of recombination. Another reason for choosing to perform the screen on the Whatman 3MM filter paper moistened with 1X Hoagland's solution was that the preparation involved in using this method is less time consuming compared to the agar-based growth media.







Agar-based growth media

Soil

Fig.II.8 Seedlings from control lines (*RPP8::Lox::LUC-Lox-NOS* and *RPH8A::Lox::LUC-Lox-NOS*) and non-transgenic CW84 (used as a negative control) were imaged on soil, agar-based growth media, and on Whatman 3MM filter paper moistened with 1X Hoagland's solution

Another parameter was to determine the minimal amount of imaging time required for the initial detection of a luc$^+$ seedling. Seven day old seedlings were imaged for 10, 20, 30, 40, and 60 minutes. Sufficient imaging time was considered to be the length of time necessary to detect luc$^+$ seedlings with either a *RPP8* or *RPH8A* functional promoter. Twenty minutes of photon counting was sufficient for the detection of a luc$^+$ seedling with either a *RPP8* or *RPH8A* functional promoter. Ten minutes was insufficient.

The maximum number of seedlings that could be screened within twenty minutes without compromising our ability to detect a luc$^+$ seedling was investigated. This was accomplished by placing ~10 luc$^+$ control seedlings (*RPP8::Lox::LUC-Lox-NOS* and *RPH8A::Lox::LUC-Lox-NOS*) in marked positions on the tray, then adding non-transformed CW84 seedlings at densities of 1000, 4000, and 7500 per tray. Three lines

of *synthRPP8* seedlings were sown on each tray. The luc$^+$ control seedlings were placed in marked locations so that the control seedlings could be distinguished from luc$^+$ seedlings that may have come from the *synthRPP8* seedlings. *In vivo* luciferase assays at a seed density of ~7,500 seedlings per imaging tray permitted the detection of luc$^+$ seedlings (Fig. II.9).

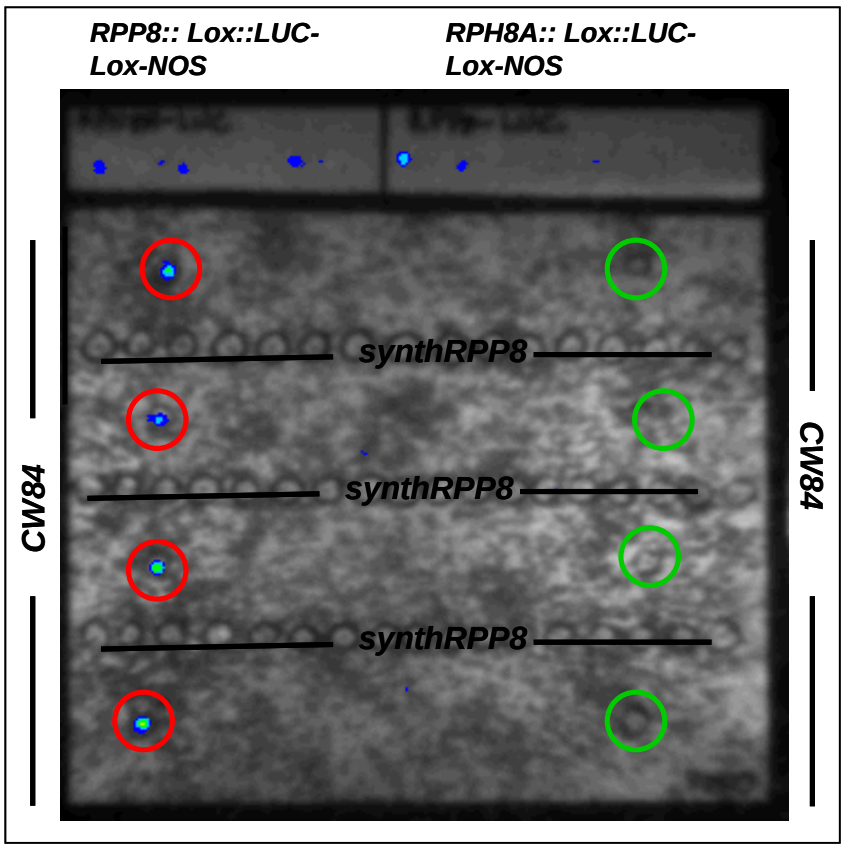


Fig. II.9 Detection of control luc$^+$ seedlings in seed dense population of ~7500: red circle marks location of *RPP8:: Lox::LUC-Lox-NOS* seedling; green circle marks location of *RPH8A:: Lox::LUC-Lox-NOS* seedling. NOTE: not all control seedlings germinated therefore no luciferase activity was detected

In summary, the optimal conditions for luciferase imaging were with 7 day-old seedlings on Whatman 3MM filter paper moistened with 1X Hoagland's solution. Considering the assay could be performed in 20 minutes with ~7,500 seedlings, the

screen should be fast and efficient, enabling the screening of very large populations of seedlings.

Bioluminescence Assay to Identify *synthRPP8* Recombinants

The *synthRPP8* recombination assay couples chimeric gene formation to the activation of the firefly luciferase gene. In the event that chromosomes with *synthRPP8* alleles misalign during meiosis, homologous genes *RPP8* and *RPH8A* could pair and undergo unequal crossing over between the inactive $^{C\Delta T}$*RPP8:: Lox::LUC* gene and the active *RPH8A* gene. As illustrated in Fig. II.10, recombination between misaligned $^{C\Delta T}$*RPP8:: Lox::LUC* and *RPH8A* genes would create a recombinant chimeric *RPH8A/RPP8::Lox::LUC* gene and a duplicated *LUC* gene. The chimeric gene imparts a luc^{+} phenotype to the plant because it contains a functional *RPH8A* promoter whose 5' transcription signals are responsible for the expression of luciferase from the chimeric *RPH8A/RPP8::Lox::LUC.*

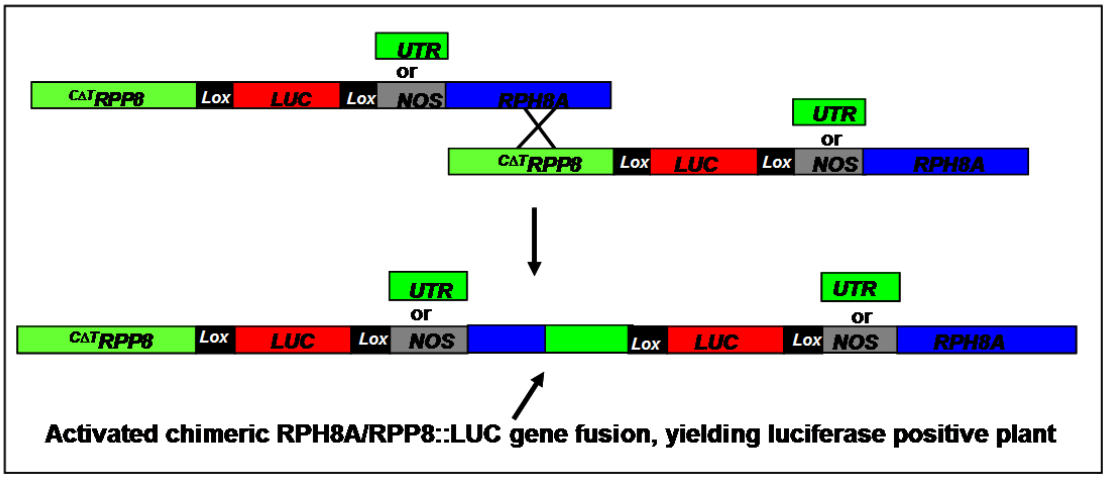


Fig. II.10 Recombination between misaligned $^{C\Delta T}$*RPP8* and *RPH8A* genes creates a recombinant chimeric *RPH8A/RPP8::Lox::LUC* gene and a duplicated *LUC* gene. The chimeric gene imparts a luc+ phenotype due to the functional *RPH8A* promoter whose 5' transcription signals are responsible for the expression of luciferase from the chimeric *RPH8A/RPP8::Lox::LUC*.

Approximately 10^6 homozygous T_5 seedlings (combined total includes seedlings from both independent transformed lines, *synthRPP8-1.21* and *synthRPP8 -2.37*) were

screened for *in vivo* luciferase activity under the optimized experimental conditions described in the previous section. The seedlings used in the screen were obtained from independent T_4 seed lots to ensure that the luc^+ plants isolated would truly represent independent recombination events as opposed to a somatic event in one T_4 plant that subsequently produced many T_5 progeny with the same recombinant allele. An uneven distribution of luc^+ seedlings in a particular seed lot would suggest a somatic recombination event. Somatic recombination in the inflorescence meristem gives rise to flowers/gametes with mitotic recombinant alleles (Kovalchuk et al., 2003). Moreover, recombinants obtained from a somatic event would all have the same recombination resolution site. Unexpectedly, a large number of luc^+ plants were observed in multiple trays from at least six of the ten representative lots isolated (Note: the plants were collected into ten independent lots). Luc^+ plants were isolated at a rate of ~1×10^{-3}. The luc^+ plants exhibited photon emission levels that were comparable to the photon emission levels produced by the control seedlings (*RPP8::Lox::LUC-Lox-NOS* or *RPH8A:: Lox::LUC-Lox-NOS*) used throughout the experiments (Fig. II.11). The number of luc^+ plants observed suggested recombinants created by meiotic unequal crossing over were produced at a rate much higher than any measure of recombination that had been previously reported in *Arabidopsis* i.e. $<2 \times 10^{-5}$ and 1 to 3×10^{-6} (Assaad and Signer, 1992; Jelesko et al., 1999, 2004) and all lots screened for luciferase showed this pattern.

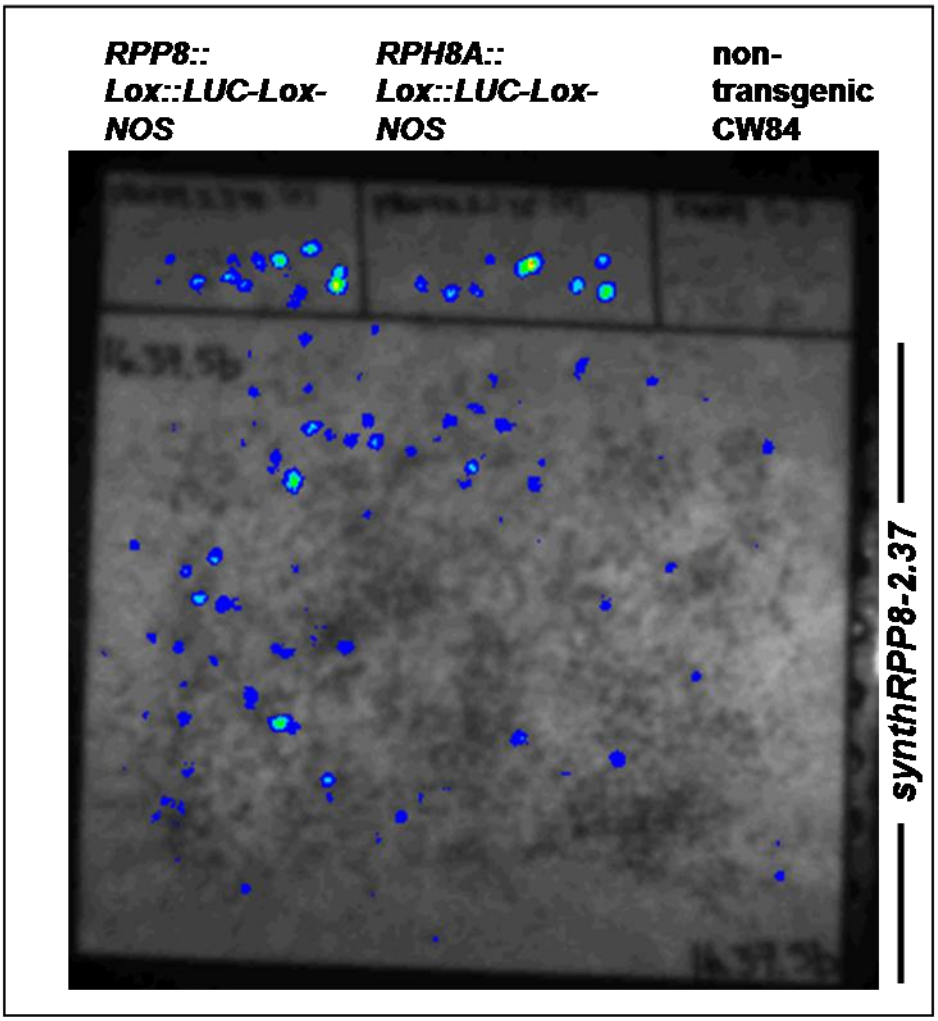


Fig. II.11 Bioluminescence Assay to Identify *synthRPP8* Recombinants: Seedlings from non-transgenic CW84 (- control), *RPP8:: Lox::LUC-Lox-NOS* (+ control), *RPH8A:: Lox::LUC-Lox-NOS* (+ control), *and synthRPP8-2.37* were screened for *in vivo* luciferase activity.

Molecular Analysis of Luc$^+$ Plants

The unexpectedly high frequency of luc$^+$ plants in the initial screen warranted consideration of all possible explanations for this outcome. The first possibility is that the *synthRPP8* gene cluster is very recombinagenic. The second explanation is that the genomic position of the transgene locus integration may have led to the luc$^+$ phenotype. For example, integration near a promoter may lead to transgene activation and this would

suggest a positional effect. Third, the luc$^+$ plants were contaminants from the luc$^+$ control seedlings. Fourth, the luc$^+$ plants were the result of the promoter mutation reverting back to wild type. The final possible explanation was that the point mutation in the promoter was not sufficient for promoter inactivation under the environmental conditions used in the screen.

If the *synthRPP8* gene cluster was very recombinagenic, then the luc$^+$ plants isolated from the assay should contain a recombinant chimeric transgene. To assess the genotype of the luc$^+$ plants, Southern blot analysis was performed on 10 lines randomly chosen from the collection of luc$^+$ plants. Recombinant chimeric gene formation produces two copies of the luciferase gene. Therefore two hybridizing bands of known size (~5 Kb and ~9 Kb) and a 3rd band (~7.3 Kb) (produced from the recombinant chimeric gene) is expected if recombination by unequal crossing over had occurred (Fig. II.12). Southern blot analysis revealed there was not a 2nd copy of the *LUC* gene and it showed the luc$^+$ plants produce the same size hybridizing fragment from the progenitor *synthRPP8* line (Fig. II.13).

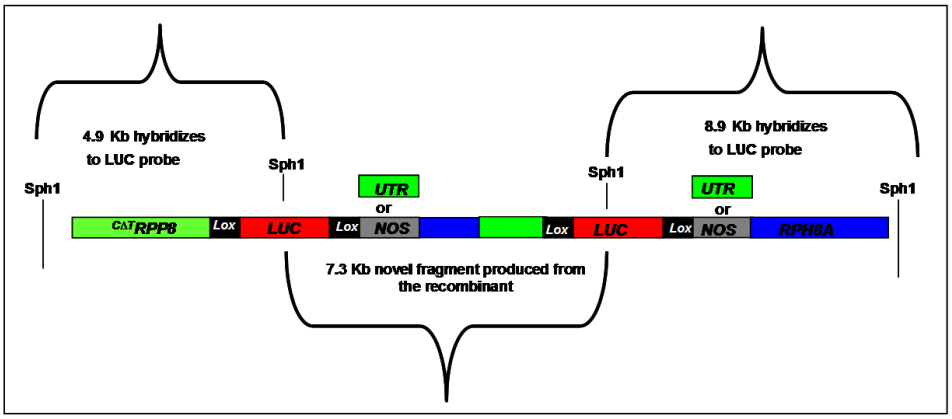


Fig. II.12 Southern Blot with LUC probe: Recombinant chimeric gene formation produces two copies of the luciferase gene. Therefore 2 hybridizing bands (~5 Kb and ~9 Kb) and a 3rd novel ~7.3 Kb band (produced from the recombinant chimeric gene) is expected if recombination by unequal crossing over has occurred

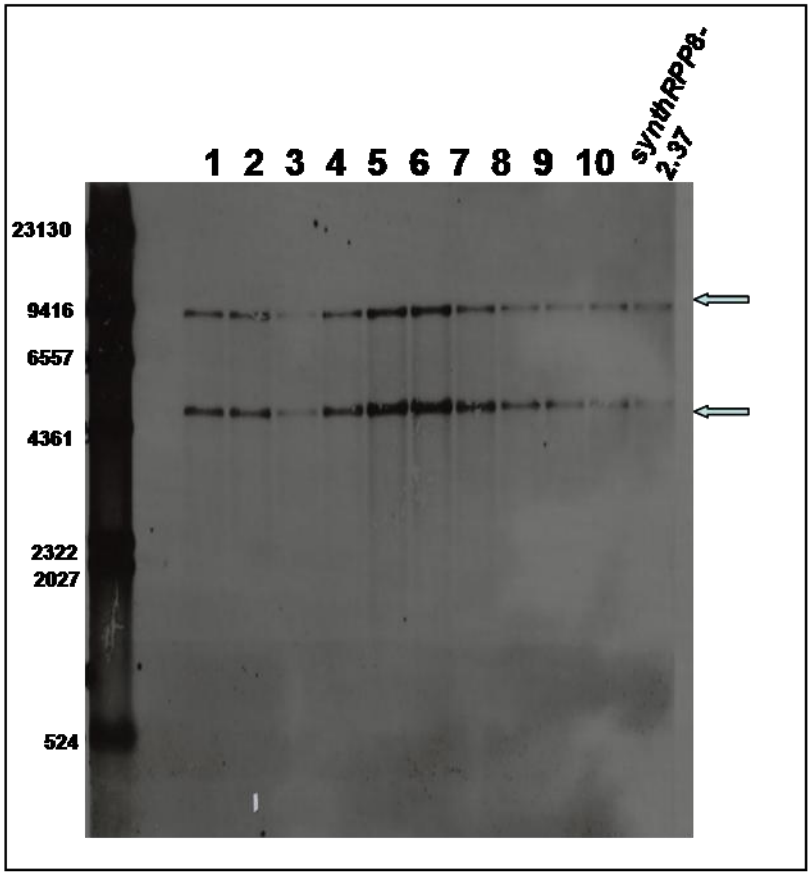


Fig. II.13 Southern Blot for *synthRPP8* recombinants: Recombinant chimeric gene formation produces a ~5 Kb, ~9 Kb and a 3rd band (~7.3 Kb) (produced from the recombinant chimeric gene). 10 plants that displayed the luc$^+$ phenotype produce the same size hybridizing fragment from the progenitor *synthRPP8-2.37*.

The luc$^+$ phenotype was observed in the two independent transgenic lines, *synthRPP8-1.21* and *synthRPP8 -2.37*. This observation indicates that the luc$^+$ phenotype is not the result of a positional effect.

To eliminate the possibility that the luc$^+$ plants were contaminating control seedlings (e.g. *RPP8::Lox::LUC-Lox-NOS* or *RPH8A:: Lox::LUC-Lox-NOS)* or that the promoter mutation reverted back to the wildtype promoter genotype, a diagnostic restriction marker assay was used. The assay differentiates a wild type PCR amplified promoter fragment from a mutated PCR amplified promoter fragment using the

restriction enzyme *Hae*III. The recognition sequence for *Hae*III has been altered in the mutated promoter due to the point mutation introduced within the putative cis-element upstream of the *RPP8* coding sequence. A 300 bp PCR product is produced in a wildtype promoter. *Hae*III cleaves this product and produces fragment sizes of 113 bp and 187 bp. PCR products that contain the mutated promoter are not cleaved by *Hae*III so the fragment size remains at 300 bp. Luc^+ plants were analyzed using this assay and all of the plants contained the promoter mutation (Fig. II.14). The luc^+ plants did not display the wildtype promoter genotype, thus seed contamination from the control seedlings *RPP8::Lox::LUC-Lox-NOS* or *RPH8A:: Lox::LUC-Lox-NOS* could also be eliminated as a possible source of the luciferase activity.

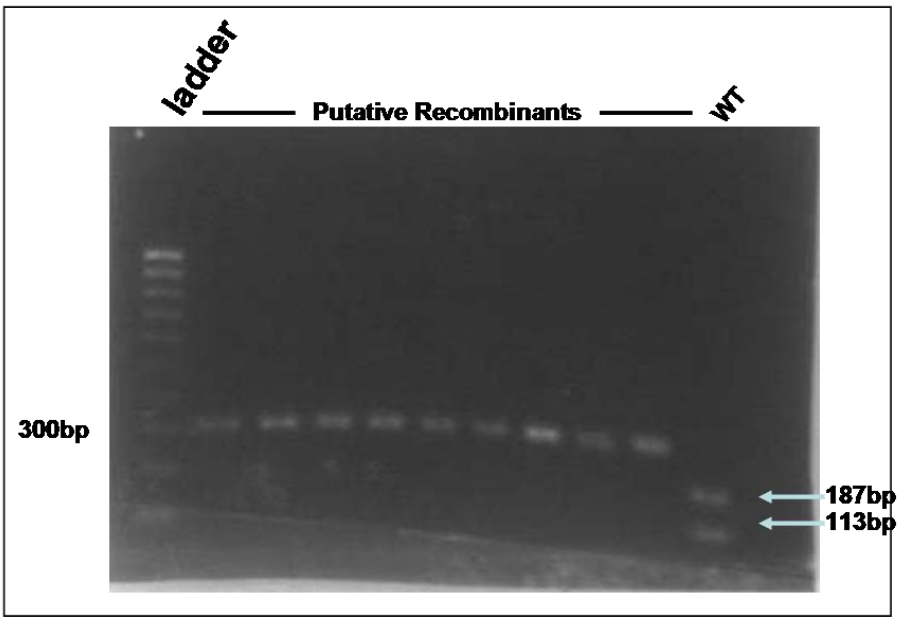


Fig. II.14 Cleaved Amplified Polymorphic Sequence Assay: A 300 bp PCR product is produced in wildtype *RPP8* promoter. HaeIII cleaves this product and produces fragment sizes of 113 bp and 187 bp. PCR products that contain the $^{C\Delta T}$*RPP8* promoter are not cleaved by HaeIII.

The effectiveness of the promoter mutation was previously assayed and confirmed to impart a luc^- phenotype in transgenic plants (described in Mutation of the *RPP8*

promoter) (Fig. II.3). In addition, both transformed lines, *synthRPP8-1.21* and *synthRPP8 -2.37*, were confirmed to be luc^- in every generation preceding the T_5 seeds that were used in the luciferase screen. Therefore, background luciferase activity from the $^{C\Delta T}$*RPP8::LUC* gene fusion with a constitutively active promoter was determined to be unlikely because if the promoter mutation did not successfully inactivate *RPP8* promoter function then one would expect all the plants screened to exhibit luciferase activity. We observed that only a variable number of the seedlings screened expressed luciferase at relatively low frequencies (~1 in 10^3) (Fig. II.11). The non-uniform luciferase expression suggests some other factor was contributing to the luciferase activity that was observed.

Based on the above results, we were able to rule out all the postulated explanations for the unexpectedly high frequency of luc^+ plants. The only other probable cause that was not ruled out was inducible background activity from the promoter. Thus, our provisional conclusion is that the promoter mutation did not completely inactivate the gene, and that the gene is responding to some sort of environmental cue that did not come into play when we were assaying the *synthRPP8-1* and *synthRPP8 -2* lines (or control *RPP8::Lox::LUC-Lox-NOS* or *RPH8A:: Lox::LUC-Lox-NOS* lines) in previous generations. Thus the next step was to test whether we could eliminate background activity.

Attempts to reduce the background of bioluminescence in the *synthRPP8* transgenic lines

The bioluminescence assay relies on the ability to distinguish *bona fide* luciferase expression from background photon emission levels (i.e. infrared heat photons). Our ability to conduct an effective screen for chimeric *R* gene formation was impeded by the residual luciferase activity. Therefore, several steps were taken to reduce interfering background photon emission and the identification of luc^+ plants that do not really express luciferase when screening *synthRPP8* seedlings. As previously mentioned in Optimizing the Luciferase Bioluminescence Assay, the advantage i.e. less background photon emission of performing the screen on soil versus Whatman 3MM chromatography paper moistened with 1X Hoagland's solution was not significant. However, considering

the dilemma described above, the slight reduction in background photon emission when the screen is performed on soil warranted trying to perform the screen on soil rather than the Whatman 3MM chromatography paper moistened with 1X Hoagland's solution. However, additional steps to confirm the presence of luciferase activity were taken following the isolation of putative luc^+ recombinant plants identified on soil. After a putative luc^+ recombinant plant was identified on soil, it was transplanted to a water-agar plate to verify the right luc^+ plant was chosen. Next, the luc^+ plant was transferred to a 2 ½ in. pot for further growth. After ~2 weeks, the luc^+ plant was re-screened for luciferase expression. At this stage, luc^- plants were discarded and luc^+ plants were subjected to the molecular analysis described above for identifying recombinant chimeric gene formation. With the additional steps taken, there was a reduction in background photon emission and there was a reduction in the number of false luc^+ plants that were identified. However, we were not able to completely eliminate the residual luciferase activity that was often present during screening.

synthRPP8 and Abiotic Stress Assay

The luc^+ plants were not the products of an unequal recombination event because they contained the progenitor *synthRPP8* genotype and configuration. Nevertheless, they produced elevated levels of luciferase expression. To deduce a plausible explanation for the luciferase activity, an experiment to assess the effect of abiotic stress on the luciferase expression from the *synthRPP8* gene cluster was performed. T_6 seedlings derived from *synthRPP8-1.37* plants that were confirmed to be non-recombinant (refer to Molecular Analysis of Luc^+ Plants) were germinated on soil and screened for luciferase activity at 7 days. These plants were confirmed as luc^-. The plants were then subjected to two abiotic stresses: water deprivation/drought and constant light. At three weeks the plants were noticeably stressed as evidenced by yellow coloring, small size, and early bolting. The three week-old plants were re-imaged for luciferase activity. A high level of luciferase expression was detected in the plants (Fig. II.15). The level of activity was comparable to that produced by the control seedlings *RPP8::Lox::LUC-Lox-NOS* and *RPH8A:: Lox::LUC-Lox-NOS*. It appears that the $^{C\Delta T}$*RPP8::Lox::LUC* expression is a stress-induced phenomenon. Therefore the point mutation in the *RPP8* promoter ($^{C\Delta T}$*RPP8*)

does not provide reliable inactivation of *RPP8::Lox::LUC* gene fusion under the conditions used in this screen. In addition to the leaky promoter, there may be a stress response element that allows for gene expression.

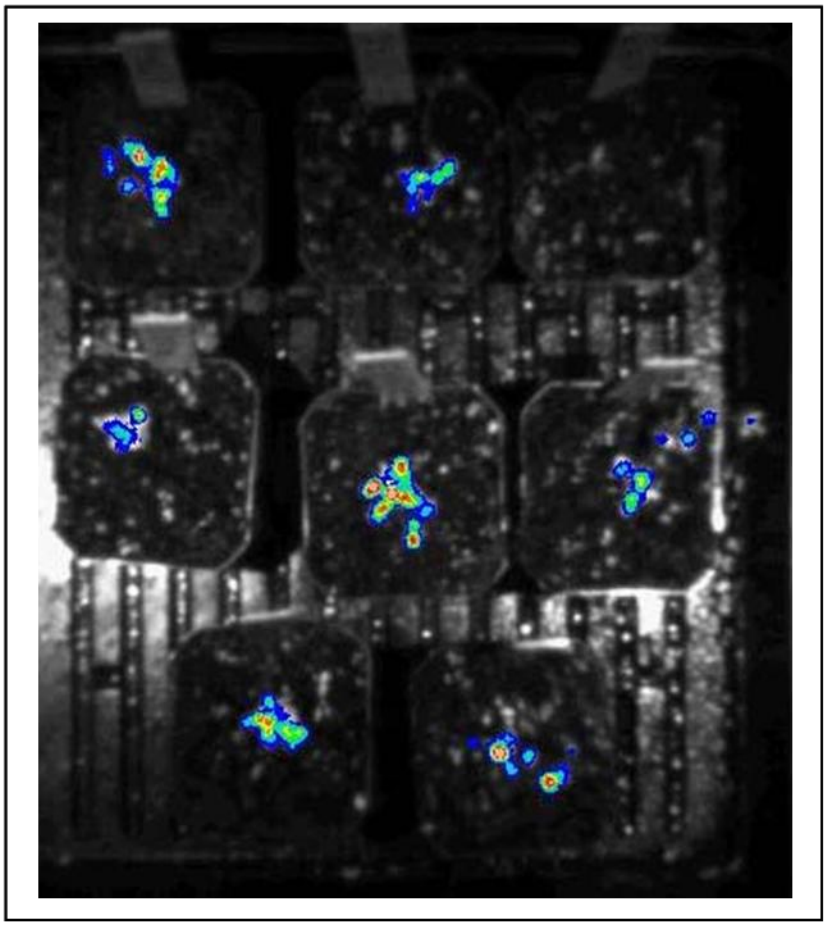


Fig. II.15 *synthRPP8* and Abiotic Stress Assay: Seedlings derived from *synthRPP8-2.37* plants that were previously confirmed to be luc$^-$ were subjected to water deprivation/drought and constant light then re-imaged for luciferase activity.

Assembly of *synthRPP8-3* and *synthRPP8-4* and Selection of Single-Copy, Luc$^-$ Transgenic Plants

Because the point mutation was insufficient to inactivate the *RPP8* promoter, we explored an alternate strategy in which we deleted large portions of the promoter from the *synthRPP8* constructs. The disadvantage of this approach is that it significantly alters the structure of *RPP8* compared to the endogenous gene; the deleted region would not be available as a recombination substrate. However, we expected that this alteration would provide more efficient inactivation of *RPP8::LUC* transcription.

Two modified *synthRPP8* (*synthRPP8-3* and *synthRPP8-4*) gene clusters were constructed. *synthRPP8-3* was derived from pSS35 ($^{535\Delta}$*RPP8::Lox::LUC-Lox-NOS-RPH8A*) (Fig. II.16B). The *RPP8* sequence in pSS35 begins 151 bp 5' to the ATG start site in *RPP8*). From the *RPP8* promoter in the original construct pSS16, 535 bp was deleted to create pSS35. *SynthRPP8-4* was derived from pSS36 ($^{679\Delta}$*RPP8::Lox::LUC-Lox-:NOS-RPH8A*) (Fig. II.16C). The *RPP8* sequence in pSS36 begins 7 bp 5' to the ATG start site in *RPP8*. From the *RPP8* promoter in the original construct pSS16, 679 bp was deleted to create pSS36.

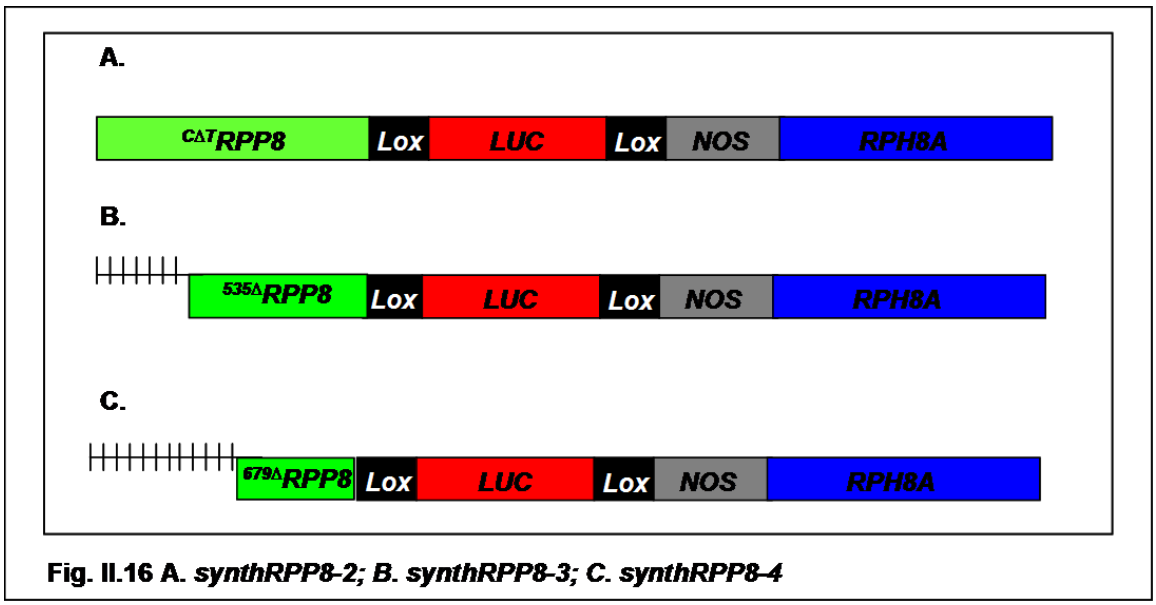


Fig. II.16 A. *synthRPP8-2; B. synthRPP8-3; C. synthRPP8-4*

Identification of CW84 transgenic lines derived from *synthRPP8-3* and *synthRPP8-4* with single copy inserts are currently underway using the procedures described in Materials and Methods. To rapidly identify single-copy lines, the Southern blot screening has been optimized. Instead of screening for single-copy lines in the T_2 generation, we are screening in the T_1 generation. Based on our previous experience with the low frequency of single copy transgenic lines (~1 in 50), we are screening much larger populations of T_1 plants. One transgenic line from *synthRPP8-4* (*synthRPP8-4.64*) was identified as a single transgenic locus but requires further analysis to confirm its utility for recombination assays. More lines (200-400) from *synthRPP8-3* and *synthRPP8-4* will need to be screened so that multiple single copy lines can be obtained.

This research will be continued with the goal of isolating 5-10 single-copy lines from both *synthRPP8-3* and *synthRPP8-4*. As previously described, luciferase activity will be assayed in every generation but a stress stimulus, such as heat shock, will also be performed to serve as a treatment to assay for stress responsiveness.

We predict that removal of a large proportion of the *RPP8* promoter will effectively prohibit luciferase expression from the Δ*RPP8* promoter. *SynthRPP8-3* and *synthRPP8-4* should be more suitable for recapitulating meiotic recombination within a multigenic *R* gene cluster.

II.2.4 Discussion

Although the initial development of *synthRPP8* gene clusters utilizing a single point mutation in the *RPP8* promoter did not come to fruition, the steps taken to create a functional *R* gene cluster recombination assay led to very important lessons learned about constructing synthetic clusters as well as other interesting discoveries.

We anticipated that the point mutation used to inactivate *RPP8* promoter function in *synthRPP8* would perform as expected based on the evidence of reduced mRNA accumulation provided by Holt et al (2002) and our observations (refer to Mutation of the *RPP8* Promoter). We speculate that the leakiness of the point mutation used to inactivate *RPP8* promoter function is the result of a stress response element that allowed for *RPP8::Lox::LUC* expression. We think the stress response that led to luciferase activity was not observed previously in the T_1, T_2, and T_3 generation of transgenic lines *synthRPP8-1 and synthRPP8-2* because the growth conditions used to assay these plants were more conducive to healthy growth. Plants assayed on the Whatman 3MM chromatography paper matrix are more apt to becoming stressed. High heat and humidity can accumulate under the dome covering the plants thereby creating an unfavorable environment for plant growth. Notably the luciferase expression was not observed in all plants but rather it was punctate on a given tray. The punctate appearance of the luc^+ plants suggests an extremely localized stressor. The punctate appearance could not be recapitulated on soil but rather a homogenous phenotype was produced when plants were subjected to water deprivation/drought and constant light (Fig. II.15). Nearly all of the plants supplied with the homogenous stress produced luciferase activity. The punctate appearance of the luc^+ plants corroborates a local, micro-stress environment. We have speculated that the high humidity under the dome creates an environment conducive to infection by a microorganism and such an infection may be the local, micro-stressor. This speculation may be corroborated by our observation that when the luc^+ plants are transferred to soil and grown in a more conducive environment, the plants no longer express detectable levels of luciferase.

We anticipate that the removal of majority of the *RPP8* promoter in *synthRPP8* will greatly lessen the potential for *RPP8* promoter function because this strategy was highly effective in the *synthRBCSB* gene clusters (Jelesko et al., 1999, 2004), albeit at a

cost of biasing the outcome of the screen. The undesirable effect that stress had on the original *synthRPP8* was disappointing but we are optimistic that the *synthRPP8-3* and *synthRPP8-4* gene clusters will serve as useful models for assaying the frequency, character, and functional consequences of meiotic recombination on R gene evolution.

Chapter III

UV-C Irradiation and its Effect on Meiotic Recombination at a *synthRBCSB* Gene Cluster in *Arabidopsis thaliana*

III.3 Introduction

There is a long-standing hypothesis that the frequency of recombination can be elevated in unfavorable environments, thereby increasing the potential to create genetic variation that could facilitate adaptation (McClintock, 1984). There are DNA-damaging agents, both chemical and physical (Kovalchuk et al., 2000) that induce several DNA repair mechanisms. Homologous recombination is utilized by DNA repair mechanisms during repair of the lesion. Already, there are several lines of evidence that suggest plants respond to stressful conditions by increasing the rate of recombination (Lucht et al., 2002; Kovalchuk et al., 2003). For example, an increase in somatic recombination was observed in plants treated with DNA damaging agents i.e. ionizing radiation doubled the frequency of recombination, mitomycin C increased the frequency of recombination by 9-fold recombination, and heat shock increased the frequency of recombination by 6.5-fold (Lebel et al., 1993). The frequency of recombination can also be enhanced by pathogen infection. Kovalchuk et al (2003) demonstrated that infection with TMV induced a three to four-fold increase in somatic recombination. In another study, the oomycete pathogen *Hyaloperonospora parasitica* was found to stimulate a 1.8-fold increase in somatic recombination in *Arabidopsis* (Lucht et al., 2002). Less is known about the effects of stress on meiotic recombination, especially with regards to endogenous gene clusters compared to transgenic bacterial reporter genes, which have been the primary substrates used to evaluate recombination and stress.

The increase in somatic recombination due to stress-induced stimuli suggests that meiotic recombination may also be affected by stress. Notably, the somatic recombination events have little evolutionary significance because the recombinant genes are generally not passed on to the next generation. In contrast, the meiotic recombination events are the evolutionarily important events as these are passed on. The extent of the effect of the stress-induced stimulus on the sequence characteristics and rate of meiotic recombination may have important implications for the evolution of plant gene clusters, but this remains to be investigated. The influence of abiotic agents on somatic recombination in affected plants has been reported but the data is still lacking for meiotic recombination frequencies that reflect specific types of DNA damage. An increase in

somatic recombination was previously observed after irradiation with UV-C (Filkowski et al., 2004). UV-C irradiation damages the DNA by creating photoproducts like pyrimidine dimers. The damage can be repaired by a variety of mechanisms. Photoreactivation, which uses photolyase, repairs the photoproducts and restores the original sequence and base excision repair (BER) or nucleotide excision repair (NER) substitutes the damaged nucleotides with new ones, thereby possibly leading to mutation (Britt, A.B., 1999). Additionally, double strand break (DSB) repair, which can include either homologous recombination or NHEJ, can be used to repair the DNA damage caused by UV-C irradiation (Jansen et al., 1998). It will be of interest to determine the extent that UV-C-induced stress physiologies integrates meiotic recombination and how this impacts gene cluster evolution in plants.

In this chapter, I report the implementation of a UV-C irradiation experimental system that is used in conjunction with a *synthRBCSB* recombination assay in *Arabidopsis thaliana*. The transcriptional profile of genes (*MYB10*, *PR-1* and *HSF-3*) demonstrated to be influenced by UV-C irradiation was assessed. Additionally, the frequency of recombination for UV-C irradiated and non-UV-C irradiated plants was evaluated for statistically significant differences. The frequency of recombination from UV-C irradiated plants was not significantly different than non-UV-C irradiated plants. However, an increase was observed suggesting there is potential for UV-C irradiation to be used as a stress-induced stimulus for recombination at a *synthRBCSB* locus.

III.3.2 Materials and Methods

Recombination Substrate: The creation of *synthRBCSB* has been described previously in detail (Jelesko et al., 1999).

Plant Growth Conditions:

Growth Conditions

Plants were grown under fluorescent lights (Philips Universal/H-Vision, F32T8/TL741) on a 16-hr light/8-hr dark cycle in a growth chamber set to 65% relative humidity with a daytime temperature of 23°C and a nighttime temperature of 20°C.

UV-C Treatment

For the UV-C treatment, a shortwave (254nm) mineral light lamp was used (Model UVS-225D, Ultra-Violet Products Inc., Upland, CA USA). Ultraviolet measurements were made with a hand held photovoltaic device used for measuring the intensity of ultraviolet energy emitted from ultraviolet lamps (Model J-225 BLAK-RAY®, Ultra-Violet Products Inc., Upland, CA USA) (Fig. III.1A). 21 day–old plants grown on soil were irradiated for 14 sec at 440$\mu W/cm^2$, using an irradiation chamber that is illustrated in Fig. III.1B. Control plants were handled identically, except that the UV-C treatment was omitted. Plants that were either treated with UV-C irradiation or not treated with UV-C irradiation were placed back into the growth chamber and grown using the growth conditions described above until the plants set seed and were ready for harvesting.

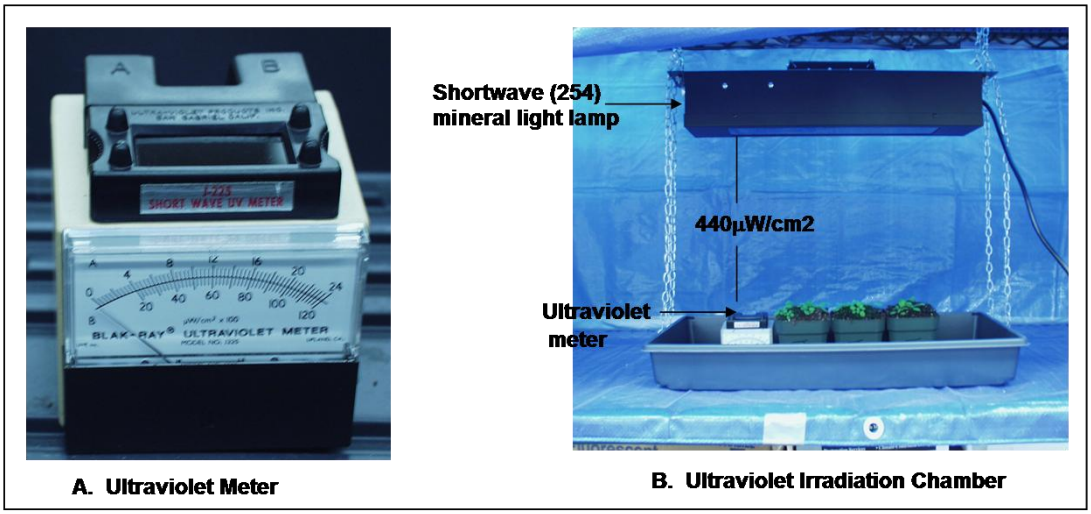


Fig. III.1A Ultraviolet meter to measure intensity of ultraviolet energy; B. Ultraviolet irradiation chamber

T_5 seed obtained from UV-C irradiated and non-UV-C irradiated plants homozygous for *synthRBCSB* (AtJGJ203.10 (*ΔRBCS1B::LUC-Nos-RBCS2B-RBCS3B*)) were collected into 10 independent lots. Each lot contains seed from 5 to 6-$3^{1/2}$ in. pots with ~9 plants per pot (each plant yields an estimated 2500 seeds).

Semiquantitative RT-PCR Analysis of Transcription

Whole plants at 21 days of age were harvested before and 30 min, 2, 6, and 24 hours after UV-C treatment. Non-UV-C irradiated plant material was also collected at the same time points. The plant material was stored at -80°C until further analysis. RNA was extracted from whole plants (roots included) using TRIzol® Reagent, following the manufacturer's protocol (Invitrogen Corporation, Carlsbad, California). Reverse transcription was performed according to the manufacturer's protocol (Omniscript RT Kit, Qiagen). *Actin2* (Brunneret al., 2004) (133bp) was used as a constitutive control and the transcript levels of *HSF3* (675bp), *MYB10* (552bp), and *PR1* (411bp) from UV-C irradiated and non-UV-C irradiated plants was evaluated. Primers used: Actin2-F (5'-TCGGTGGTTCCATTCTTGCT-3') and Actin2-R (5'-GCTTTTTAAGCCTTTGATCTTGAGAG-3') at 50°C; HSF3 F (5'-CTTGCCCAAGTATTTCAAGCACAACA-3') and HSF3 R (5'-

GAACTGTGTTGGGTGAAAACTCGGCC-3') at 50°C; PR-1 F (5'-TTCTTCCCTCGAAAGCTCAA-3') and PR-1 R (5'-CGTTCACATAATTCCCACGA-3') at 50°C; MYB10 F (5'-GATTGATGAGATGCGGAAAGAGTTGTCG-3') and MYB10 R (5'-GAGCCACTCATTGTAATCATCGAACCCT-3') at 53°C. Amplification consisted of one cycle of 94°C for 3 min; 35 cycles (except for actin, used 25 cycles) of 30 sec at 94 °C, 30 sec at the annealing temperature appropriate to each oligonucleotide pair, 1 min/kb of expected size fragment at 72°C; followed by a 10 min extension at 72°C.

Assay for Bioluminescence LUC Detection

Growth and imaging conditions for LUC assay

Approximately 7500 T_5 seedlings from a single independent seed lot were germinated on 20 cm X 20 cm Whatman 3MM chromatography paper moistened with 1X Hoagland's solution (plant nutrient solution). The seedlings used in the screen were obtained from independent seed lots to ensure that the luc^+ plants isolated would truly represent independent recombination events. An aberrant distribution of luc^+ seedlings in a particular seed lot would suggest a somatic recombination event. Somatic recombination in the inflorescence meristem gives rise to flowers/gametes with mitotic recombinant alleles. Therefore recombinants obtained from a somatic event will all have the same recombination resolution sites.

The seedlings were stratified for two nights at 4°C and then placed under fluorescent lights (Philips Universal/H-Vision, F32T8/TL741) on a 16-hr light/8-hr dark cycle for 7 days at 23°C. T_5 seedlings were then assayed for *in vivo* luciferase activity as follows: 20 minutes prior to imaging, seedlings were sprayed with 1 mM synthetic D-luciferin (Biosynth, Basel)/0.01% Triton X-100 solution. The tray was imaged for 20 min in photon-counting mode using an intensifier charge-coupled device video camera (model C2400 47), an Image Intensifier Controller (model M4314), an Image Processor (Argus 50), and an imaging chamber (model A417) containing a Xenon CM 120 lens (Schneider, Bad Kreuznach, Germany). Unless otherwise stated the imaging equipment are products of Hamamatsu Photonics, Hamamatsu City, Japan (refer to Jelesko et al., 1999 for more detailed information on imaging equipment). If a putative luc^+ plant

appeared, the tray was imaged for an additional 10 min. The approximate region on the tray where the luc^+ signal appeared was estimated and all of the seedlings in this area were transferred to a water-agar (Difco™ Agar, Becton, Dickinson and Company, Sparks, MD) plate and re-imaged to identify the individual luc^+ seedling. Luc^+ seedlings were then transferred to soil and grown under standard conditions as described above.

Genotyping luc^+ plants by PCR

Genomic DNA was isolated using a CTAB miniprep protocol, as described in sections 2.3.3-2.3.7 of Current Protocols in Molecular Biology (Ausubel et al., 1996). Recombinant chimeric gene formation was determined by PCR analysis using a *RBCS* promoter specific oligonucleotide primer that binds to a 14-bp sequence present in all *RBCS* promoters oJGJ13 (5'-CAAAAGAAAGATAAGATAAGGGTGTCAA-3') or a *RBCS3B* specific primer oJS112 (5'-AATCCTGTGGCAGTAAACGACG-3') in conjunction with a *LUC*-specific oligonucleotide primer oJGJ14 (5'-CCTTTCTTTATGTTTTTGGCGTCTTC-3').

Characterization of *RBCS3B/1B::LUC* chimeric gene sequences

The PCR amplified products created when genotyping the luc^+ plants were purified using the QIAquick PCR Purification Kit (Qiagen) and sequenced using the following oligonucleotide primers: oJGJ13, oJGJ14, oJS112, oJS113 (GTTTTTGGCGTCTTCCAGCTTG-3'), oJGJ50 (5'-CTATCTTACCTCCCTGAC-3'), oJGJ52 (5'-TAATAATGATTAGTAGAC-3'), oJGJ53 (5'-GAATGGAGCGACCATGGT-3'), oJGJ54 (5'-CTTCTCCGCAACAAATGGATTC-3'), and oJGJ55 (5'-TTGTCCAGTACCGTCCATCGTAG-3'). The overlapping contigs were assembled and analyzed using the Lasergene DNA Star software package (Madison, WI).

Calculation of meiotic recombination frequency

The statistical significance of differences in the frequency of luc^+ seedlings isolated from UV-C irradiated plants versus non-UV-C irradiated plants was assessed with a two-proportion test of significance for recombination frequency using the

MINITAB program, Windows version 13.1 (Minitab, State College, PA). P-Values were calculated using a test of two binomial proportions (two-tailed test), which performs a hypothesis test of the difference between two proportions (P-Value ≤ 0.05 is considered statistically significant) (Jelesko et al., 2004).

Test and CI for Two Proportions

Sample	X	N	Sample p
1	3	2175000	0.000001
2	1	1385500	0.000001

Difference = p (1) - p (2)
Estimate for difference: 6.575492E-07
95% CI for difference: (-1.44894E-06, 2.764035E-06)
Test for difference = 0 (vs not = 0): Z = 0.61 P-Value = 0.541

III.3.3 Results

Overview of Experimental Design

The *synthRBCSB* cluster was derived from the *RBCSB* locus in *Arabidopsis thaliana*. This cluster contains three genes: *RBCS1B, RBCS2B,* and *RBCS3B* (Fig. III.2A). *SynthRBCSB* is composed of a silent *ΔRBCS1B::LUC* chimeric gene fusion, lacking all 5' transcription and translation signals, followed by *RBCS2B* and *RBCS3B* genomic DNA (Fig. III.2B). The paralogous *RBCSB* genes could misalign and undergo unequal crossing-over between the inactive *ΔRBCS1B::LUC* gene and either *RBCS2B* or *RBCS3B* genes, to yield a recombinant chimeric gene that expresses luciferase activity. Notably, chimeric *RBCS2B/1B::LUC* gene fusions will most likely be undetectable because only 133 bp of the 5' *RBCS2B* sequences upstream of the *RBCS2B* are present and this may not be sufficient for activation of *RBCS2B/1B::LUC* gene fusions. Transcription initiation of a *RBCS2B/1B::LUC* recombinant may require more promoter domains than those included in this synthetic cluster. To date, no recombinant *RBCS2B/1B::LUC* chimeras have been detected. Therefore, we only expect recombinant *RBCS3B/1B::LUC* chimeras. Recombination between misaligned *ΔRBCS1B::LUC* and *RBCS3B* genes would create a recombinant chimeric *RBCS3B/1B::LUC* gene and a duplicated *RBCS2B* gene. A homologous recombination event between *ΔRBCS1B::LUC* and *RBCS3B* genes yields a novel recombinant chimeric gene whose expression is driven by *RBCS3B* 5' transcription and translation signals (Fig. III.2C).

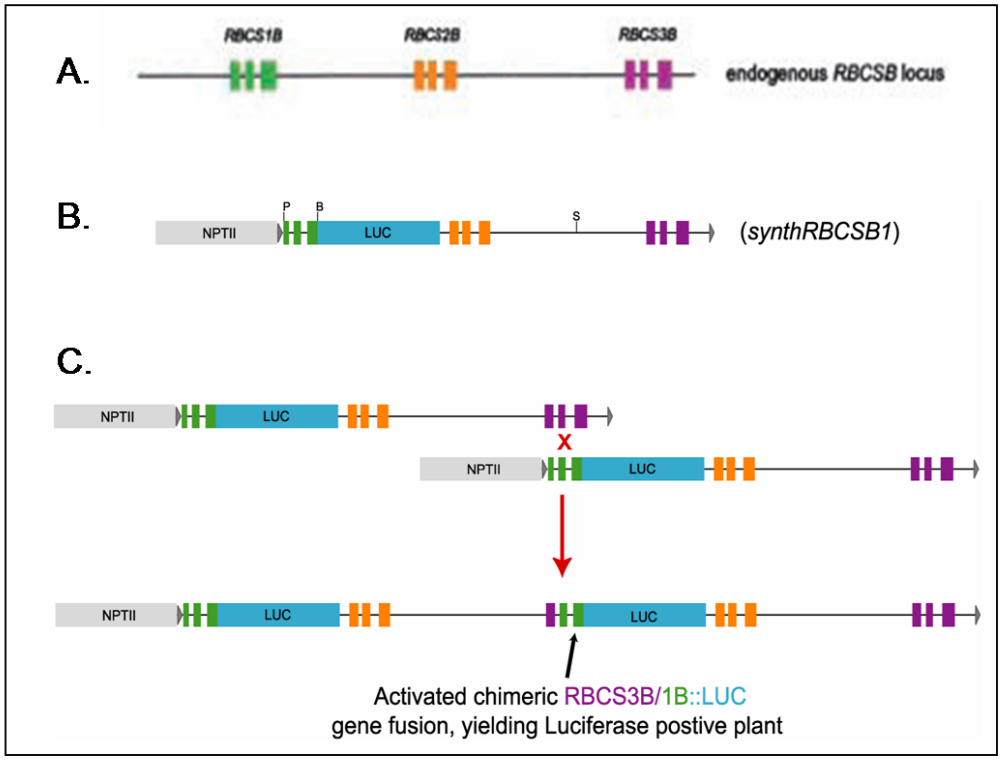


Fig. III.2A. *RBCSB* locus in *A. thaliana*; B. *synthRBCSB*; C. Recombination between paralogous *RBCSB* genes creates a recombinant chimeric *RBCS3B/1B::LUC* gene and a duplicated *RBCS2B* gene.

Below, I describe the effect of UV-C irradiation on the frequency of unequal meiotic recombination between paralogous *RBCSB* genes and on the expression of genes associated with the defense/stress response. I used the UV-C treatment protocol as described by Molinier et al (2004). This treatment regime had been previously reported to elevate the frequency of somatic recombination.

Recombination in UV-C Irradiated and Non-UV-C Irradiated Plants

The effect of UV-C irradiation on the frequency of unequal meiotic recombination between paralogous *RBCSB* genes was determined by measuring the rate at which a transgenic synthetic *RBCSB* (*synthRBCSB*) gene cluster yielded luc^+ seedlings from UV-C irradiated and non-UV-C irradiated plant populations. T_4 plants homozygous for the *synthRBCSB* recombination substrate were irradiated for 14 sec at 440$\mu W/cm^2$ using a shortwave (254nm) mineral light lamp. The irradiation conditions used were

based on conditions previously reported to induce homologous somatic recombination (Kovalchuk et al., 2000; Molinier et al., 2006). T_5 seedlings from independent seed lots from UV-C irradiated and non-UV-C irradiated plants were screened for *in vivo* luciferase activity.

Previous experiments from our lab that utilized this synthetic *RBCSB* gene cluster (Jelesko et al, unpublished) found 3 luc^+ recombinants in a collection of 1,537,500 seedlings, which is a meiotic recombination frequency of 1.9×10^{-6}. We used this meiotic recombination frequency as a reference point for estimating how many seedlings from UV-C irradiated and non-UVC irradiated plants that would need to be screened to identify a luc^+ seedling.

Two trials were performed to assess the effect of UV-C irradiation on the frequency of recombination at the *synthRBCSB* locus (Table 4). In the first trial, ~780,000 T_5 seedlings from UV-C irradiated and ~433,000 T_5 seedlings from non-UV-C irradiated plants were screened for *in vivo* luciferase activity. Three luc^+ T_5 seedlings (*synthRBCSB1-10.R64*, *synthRBCSB1-10.R 68*, and *synthRBCSB1-10.R75*) were obtained from UV-C irradiated plants, which suggests a meiotic recombination frequency of 3.8×10^{-6} and no luc^+ T_5 seedlings were obtained from non-UV-C irradiated plants. Luc^+ seedlings from UV-C irradiated plants occurred ~1 per 260,000 seedlings screened for *in vivo* luciferase activity.

Table 4 **Frequency of Luc+ seedlings**

Line	Trial	UV-C treatment	Total seedlings screened	Luciferase positive seedlings	Meiotic recombination frequency	chimeric *RBCS3B/RBCS1B::LUC* alleles
JGJ203.10	1	No	433,000	0	N/A	N/A
JGJ203.10	1	Yes	780,000	3	3.8×10^{-6}	*synthRBCSB1-10.64, synthRBCSB1-10.68, synthRBCSB1-10.75*
JGJ203.10	2	No	952,500	1	1×10^{-6}	*synthRBCSB1-10.84*
JGJ203.10	2	Yes	1,395,000	0	N/A	N/A
JGJ203.10	1 and 2	No	1,385,500	1	7.2×10^{-7}	Described above
JGJ203.10	1 and 2	Yes	2,175,000	3	1.4×10^{-6}	

To obtain additional measures of the effect of UV-C irradiation on the frequency of unequal crossing over at the *synthRBCSB* locus, a second trial was performed. Approximately 1,395,000 T_5 seedlings from UV-C irradiated and ~952,500 T_5 seedlings from non-UV-C irradiated plants were screened for *in vivo* luciferase activity.

Surprisingly, no luc$^+$ T_5 seedlings were obtained from UV-C irradiated plants and one luc$^+$ T_5 seedling (*synthRBCSB1-10.R84*) was obtained from non-UV-C irradiated plants, which suggests a meiotic recombination frequency of 1×10^{-6}. Collectively, 1,385,500 seedlings from non-UVC irradiated plants and 2,175,000 seedlings from UV-C irradiated plants were screened for *in vivo* luciferase activity. One luc$^+$ seedling was obtained from 1,385,500 seedlings from non-UVC irradiated plants, which suggest a meiotic recombination frequency of 7.2×10^{-7}. Three luc$^+$ seedlings were obtained from 2,175,000 seedlings from UV-C irradiated plants, which suggest a meiotic recombination frequency of 1.4×10^{-6}.

The significance of the difference in the frequency of luc$^+$ seedlings isolated from UV-C irradiated plants versus non-UV-C irradiated plants was assessed with a two-proportion test. The two-proportion test revealed the difference in meiotic recombination frequency between UV-C irradiated plants and non-UV-C irradiated plants is not statistically significant. The P-Value was equal to 0.541. The meiotic recombination frequency rate suggests a small increase (~2-fold) in luc$^+$ seedlings isolated from UV-C irradiated plants versus non-UV-C irradiated plants. The small increase (~2-fold) suggests that UV-C irradiation may have a minor effect on the frequency of recombination. However, the limited sampling size may not have been sufficient to accurately capture the mean. Therefore, additional trials are necessary to confirm this.

Confirmation of recombinant chimeric gene formation by PCR and sequence analysis

Genomic DNA from luc$^+$ plants was subjected to PCR analysis to determine whether a homologous recombination event had positioned a *RBCSB* promoter upstream of the previously inactive *ΔRBCS1B::LUC* reporter gene. Recombinant chimeric gene formation was determined by using either a *RBCS* promoter-specific primer that binds to a 14-bp sequence present in all *RBCS* promoters or a *RBCS3B*-specific primer in conjunction with a *LUC*-specific primer. These primer combinations should only yield a PCR product from plants that show the luc$^+$ phenotype resulting from the fusion of a *RBCSB* promoter to the *LUC* reporter gene.

Luc$^+$ plants with recombinant chimeric gene formation yielded a ~0.9 Kb PCR fragment (Fig. III.3A and B). This fragment was used as the template for sequence analysis to confirm that the luc$^+$ plants contained chimeric *RBCSB* genes.

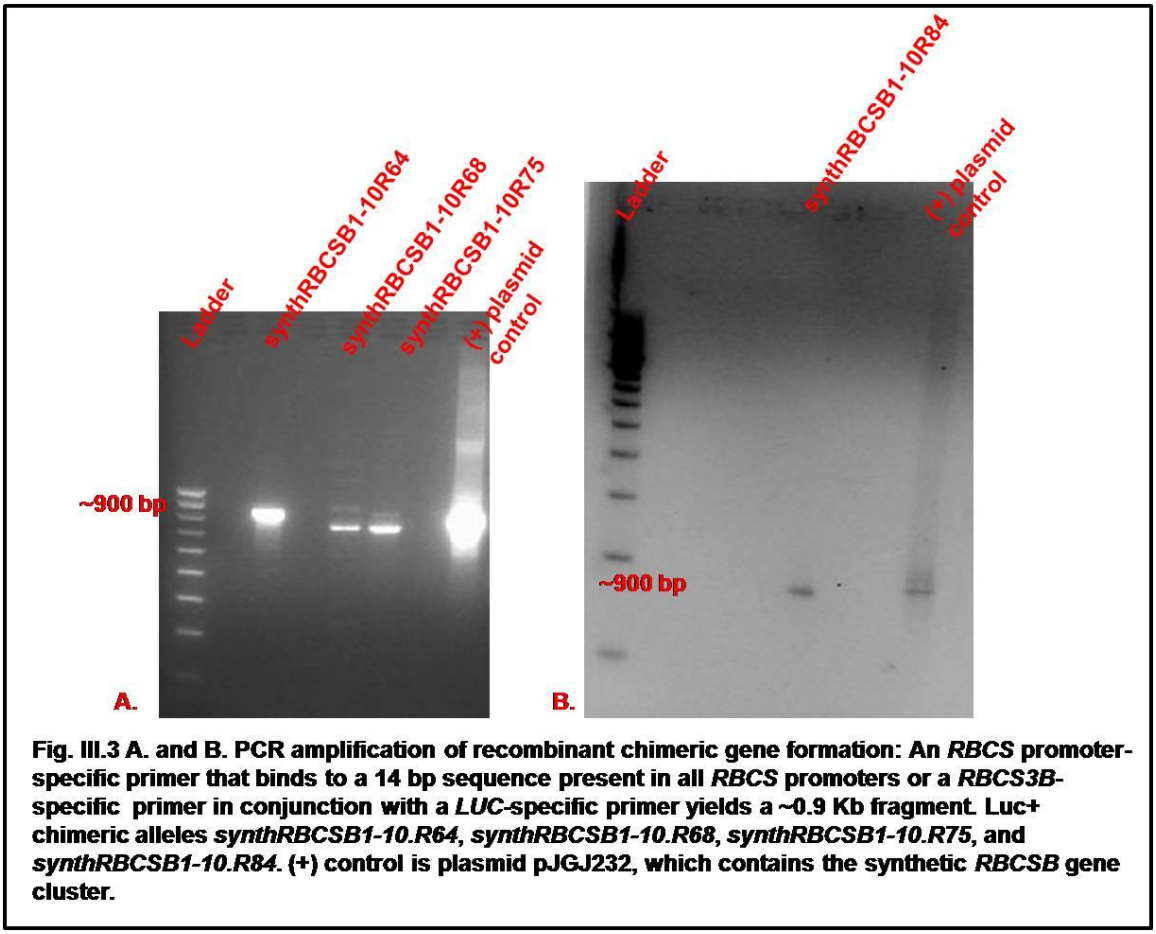


Fig. III.3 A. and B. PCR amplification of recombinant chimeric gene formation: An *RBCS* promoter-specific primer that binds to a 14 bp sequence present in all *RBCS* promoters or a *RBCS3B*-specific primer in conjunction with a *LUC*-specific primer yields a ~0.9 Kb fragment. Luc+ chimeric alleles *synthRBCSB1-10.R64*, *synthRBCSB1-10.R68*, *synthRBCSB1-10.R75*, and *synthRBCSB1-10.R84*. (+) control is plasmid pJGJ232, which contains the synthetic *RBCSB* gene cluster.

Sequence analysis revealed all of the recombinants were *RBCS3B/1B::LUC* gene fusions, with 5' *RBCS3B* sequences. No chimeric *RBCS2B/1B::LUC* gene fusions were obtained and this is consistent with data from Jelesko et al (1999, 2004) that utilized the same *synthRBCSB* gene cluster. Presumably, no chimeric *RBCS2B/1B::LUC* gene fusions were obtained because only 133 bp of the 5' *RBCS2B* sequences upstream of the *RBCS2B* are present and this may not be sufficient for activation of *RBCS2B/1B::LUC* gene fusions.

Mapping the Recombination Breakpoints in *synthRBCS* Recombinants

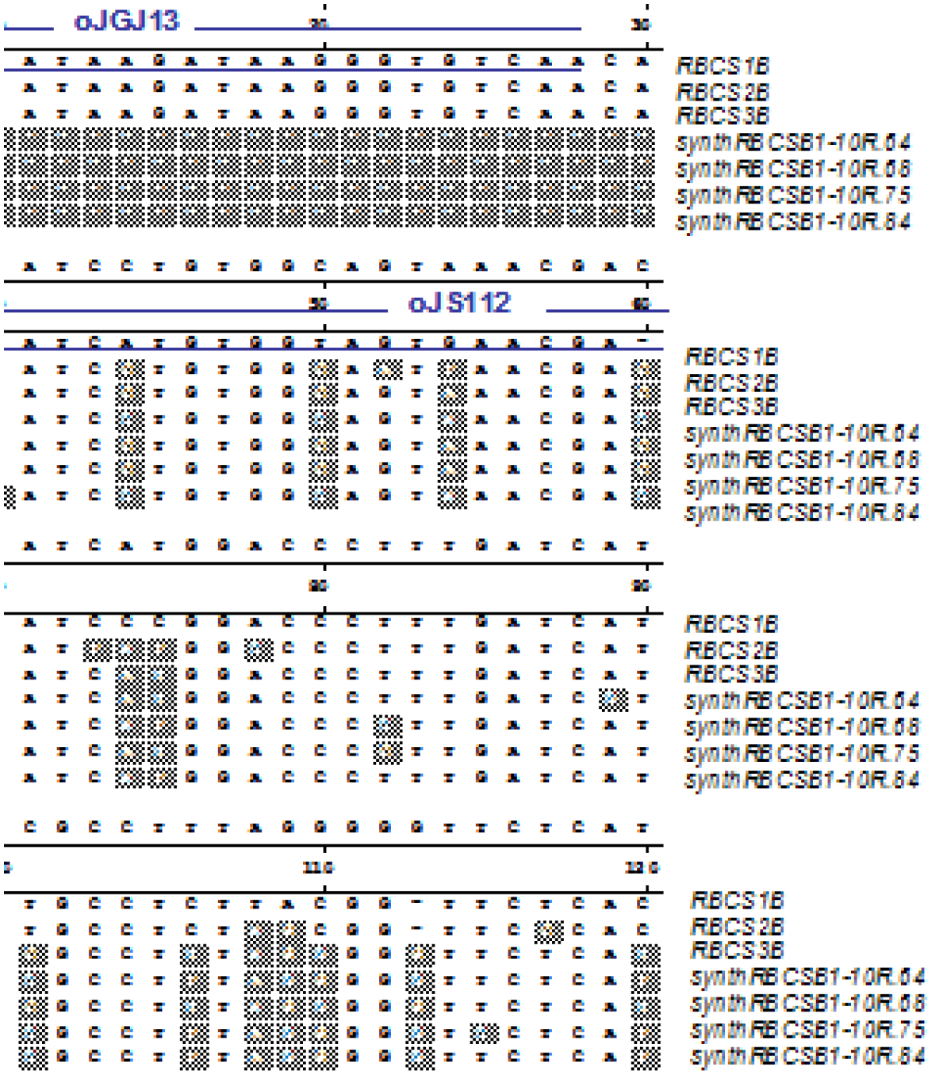
Sequences from the luc$^+$ chimeric *RBCS3B/RBCS1B::LUC* alleles *synthRBCSB1-10.R64*, *synthRBCSB1-10.R 68*, *synthRBCSB1-10.R75*,and *synthRBCSB1-10.R84* were aligned to the genomic sequence of genes *RBCS1B*, *RBCS2B*, and *RBCS3B*. (FIG. III.4). Using polymorphisms between *RBCS1B* and *RBCS3B*, the recombination resolution break points were localized. Mapping the region where recombination resolution has occurred provides definitive evidence that a recombinant chimeric *RBCS3B/RBCS1B::LUC* gene was responsible for the luc$^+$ phenotype. Additionally, mapping the resolution sites differentiates meiotic recombinants from somatic recombinants. Somatic recombination in the inflorescence meristem gives rise to flowers/gametes with mitotic recombinant alleles. Therefore recombinants obtained from a somatic event will all have the same recombination resolution sites in a given seed lot.

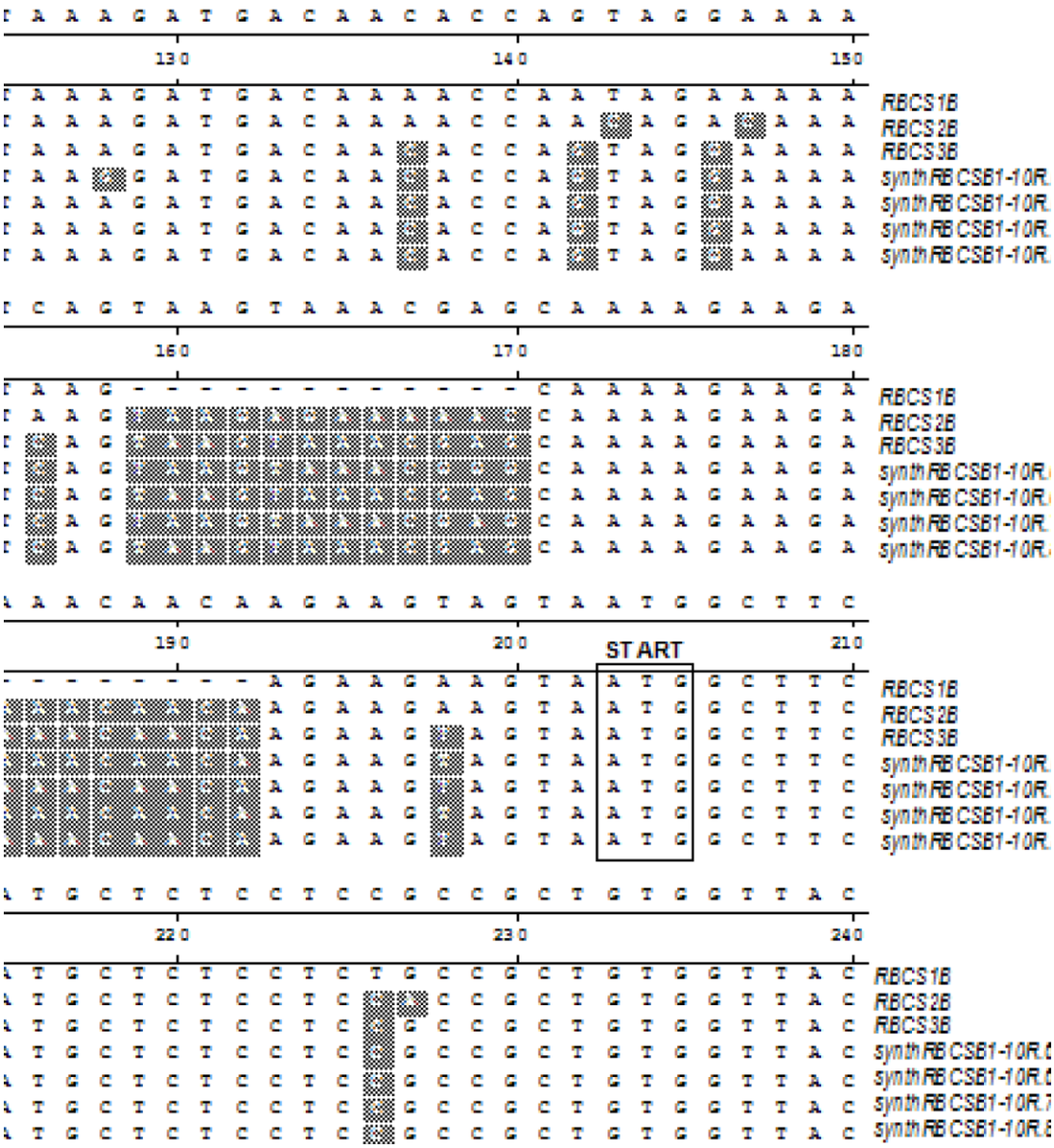
The recombination resolution breakpoints were localized to position 690 for *synthRBCSB1-10.R75*, position 703 for *synthRBCSB1-10.R64* and *synthRBCSB1-10.R84* and position 887 for *synthRBCSB1-10.R 68*. *synthRBCSB1-10.R68* and *synthRBCSB1-10.R75* are meiotic recombinants and *synthRBCSB1-10.R64* and *synthRBCSB1-10.R84* are likely meiotic recombinants since they were isolated from independent seed lots. The same recombination resolution site in *synthRBCSB1-10.R64* and *synthRBCSB1-10.R84* may reflect a preferential site for the final resolution of the Holliday junction. However, the limited sampling of recombinants makes it difficult to determine whether recombination hotspots exist. No gene conversion tracts were identified.

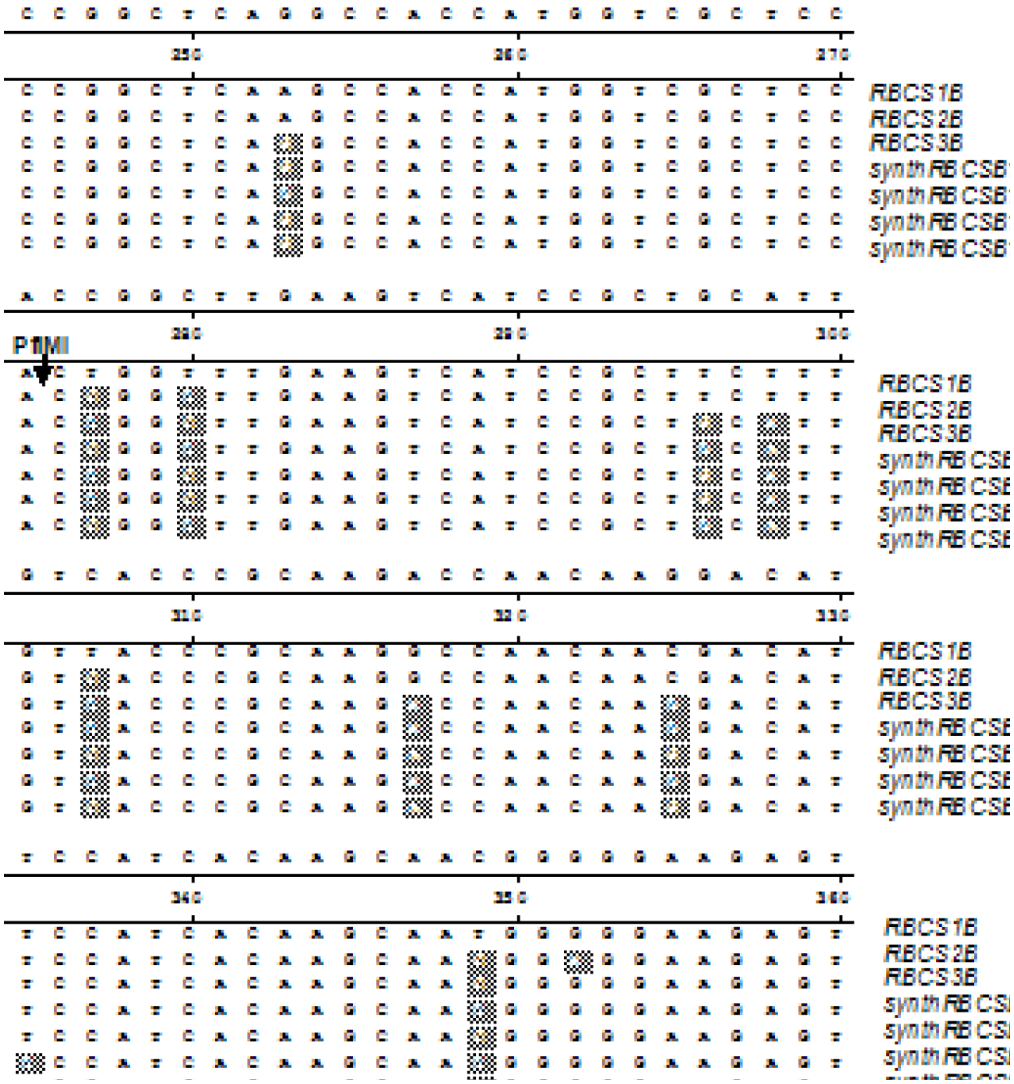
The chimeric *RBCS3B/1B::LUC* genes were consistent with a single resolution site responsible for formation of the chimeric gene. This pattern is consistent with a simple unequal crossover. Complex recombinants may have more than one resolution site i.e. the chimeric allele may show more than one alternating interval of *RBCS3B*-specific sequence and *RBCS1B*-specific sequence. There were no additions or deletions of nucleotides within the recombinant chimeric *RBCS3B/RBCS1B* alleles. This suggests that only the parental *RBCS3B* and *RBCS1B* sequences contributed to the sequence within the chimeric *RBCS3B/RBCS1B::LUC* alleles. The recombinants contained *RBCS3B* promoter and *RBCS3B* exon 1 sequences at the 5` end of the chimeric gene. The recombination resolution site for *synthRBCSB1-10.R64*, *synthRBCSB1-10.R75* and *synthRBCSB1-10.R84* mapped to the middle of intron 2 and the recombination resolution

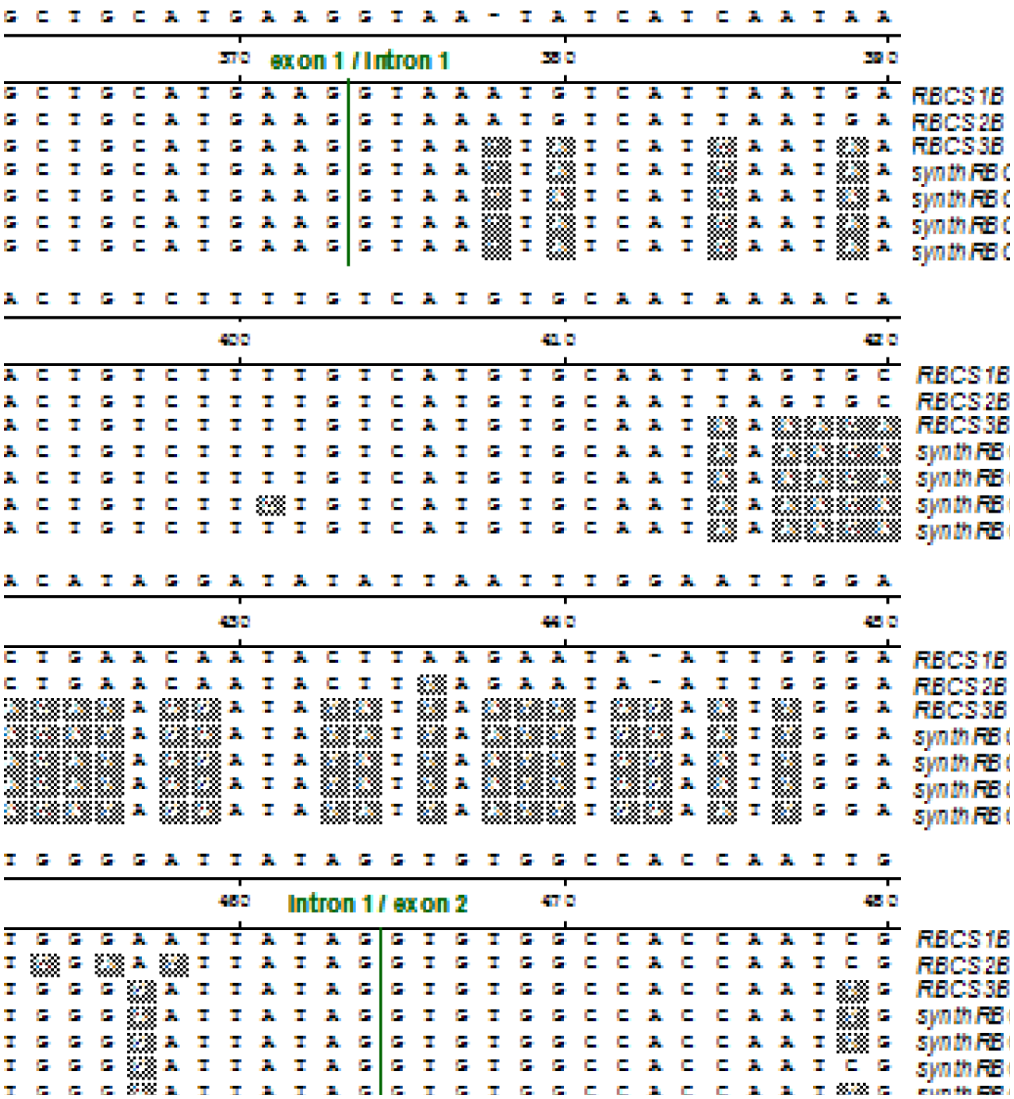
site for *synthRBCSB1-10.R 68* mapped to exon 3, 15bp away from the *LUC* gene (Fig. III.4). The chimeric *RBCS3B/RBCS1B::LUC* alleles mapped to regions previously identified by Jelesko et al (1999, 2004) (Fig. III.5).

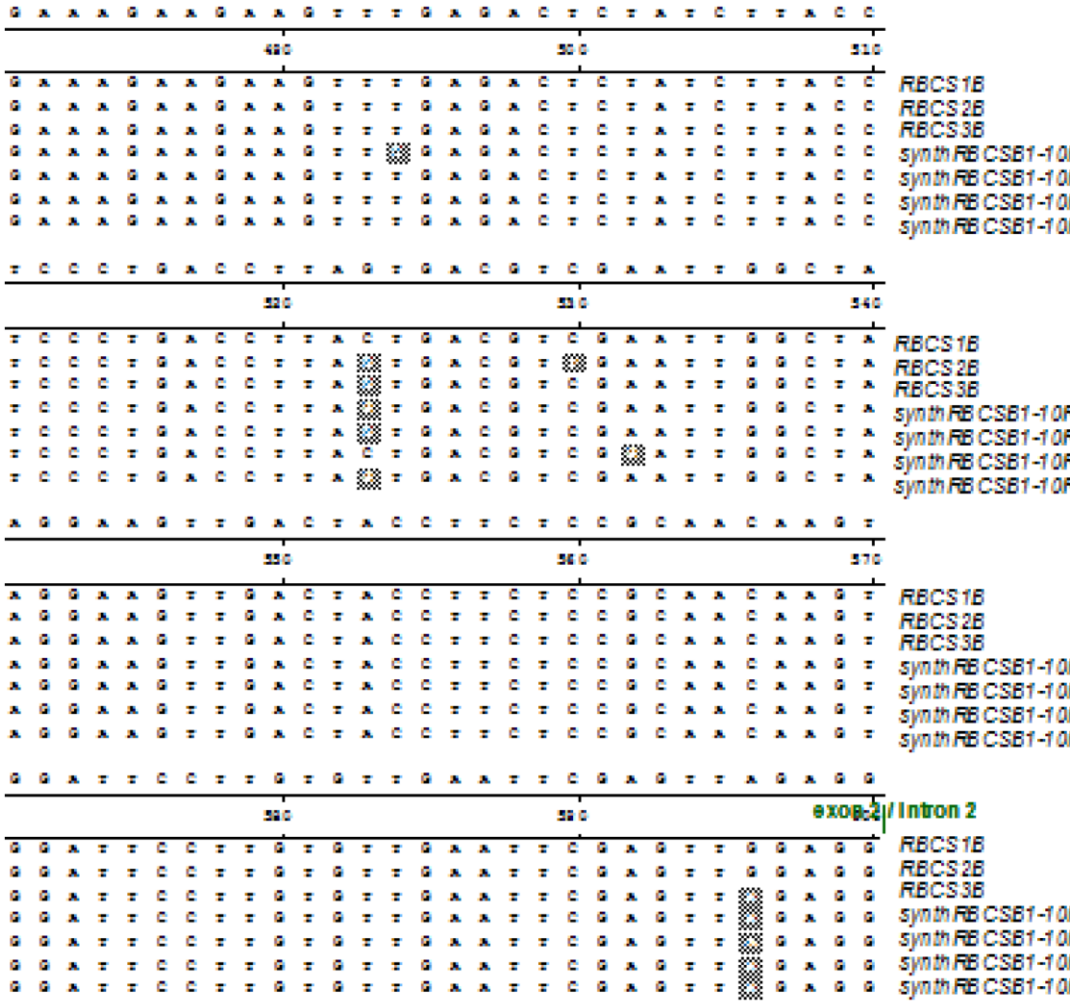
Fig. III.4 Sequence of the chimeric *RBCS3B/1B::LUC* genes, *synthRBCSB1-10.R64, synthRBCSB1-10.R 68, synthRBCSB1-10.R75*,and *synthRBCSB1-10.R84*. ~900 bp of DNA sequence were aligned with genomic *RBCS1B, RBCS2B,* and *RBCS3B* DNA sequences. oJGJ13 and oJS112 are the oligonucleotide sequences used for PCR amplification of the gDNA. Box indicates translation initiation codon in exon 1. Arrow indicates PflMI restriction site that is the 5' boundary of the synthetic *RBCSB* gene cluster. Vertical lines define intron-exon boundaries. Shaded areas define the regions in which crossovers occurred. Asterisk indicates where the recombination resolution breakpoint must have occurred.

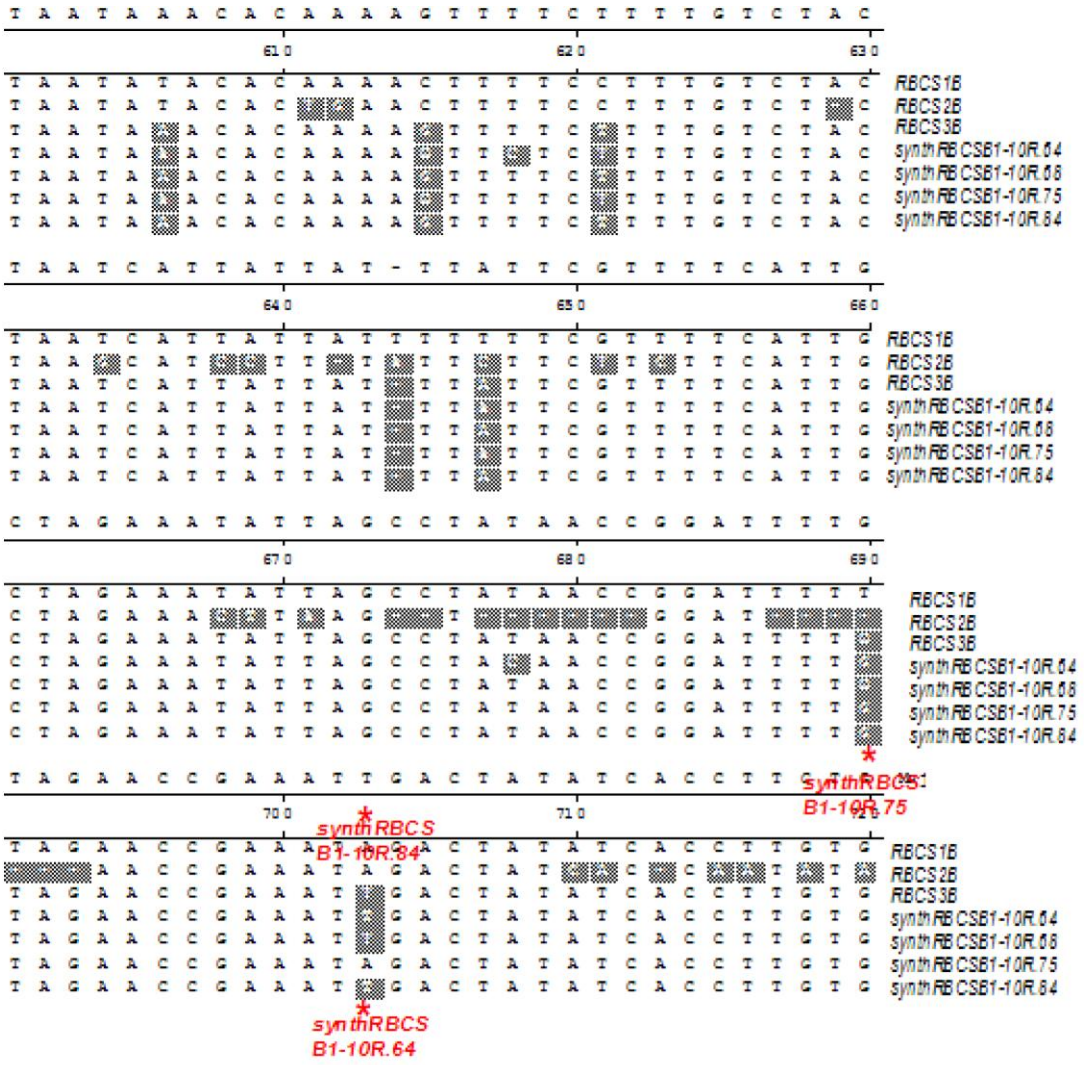


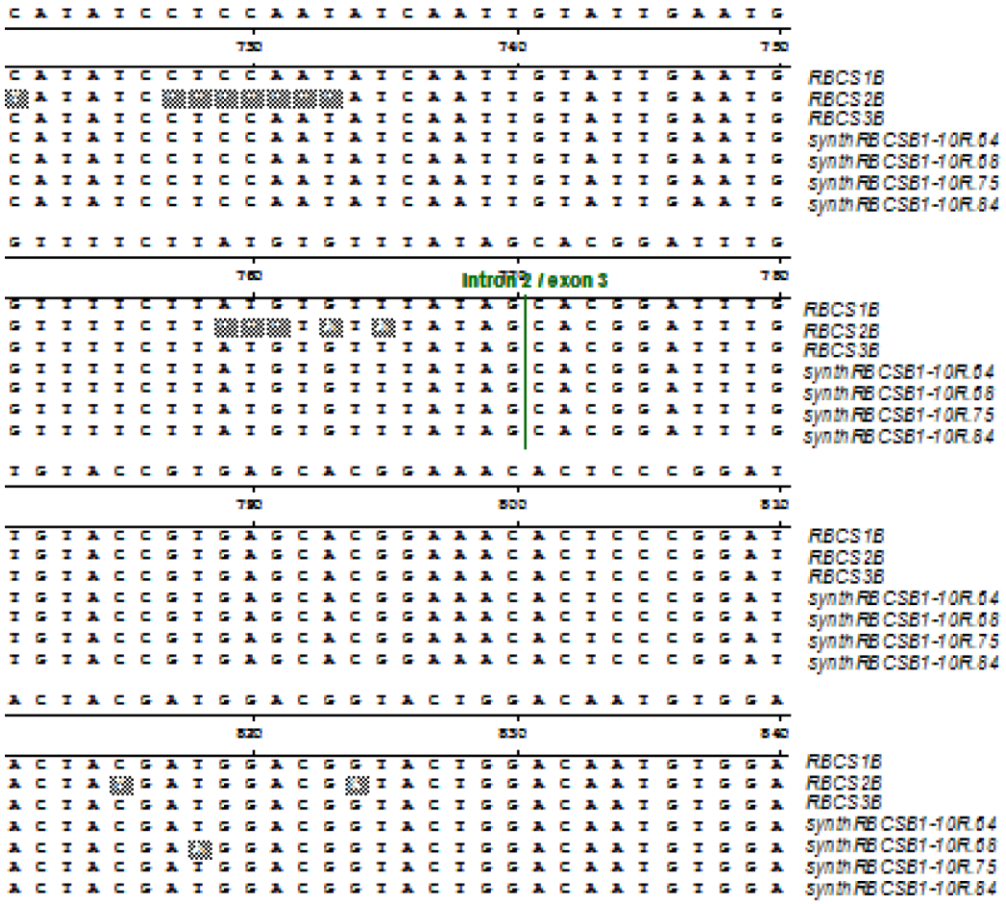

START
RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10R.
synthRBCSB1-10R.
synthRBCSB1-10R.
synthRBCSB1-10R.
synthRBCSB1-10R.
synthRBCSB1-10R.
synthRBCSB1-10R.

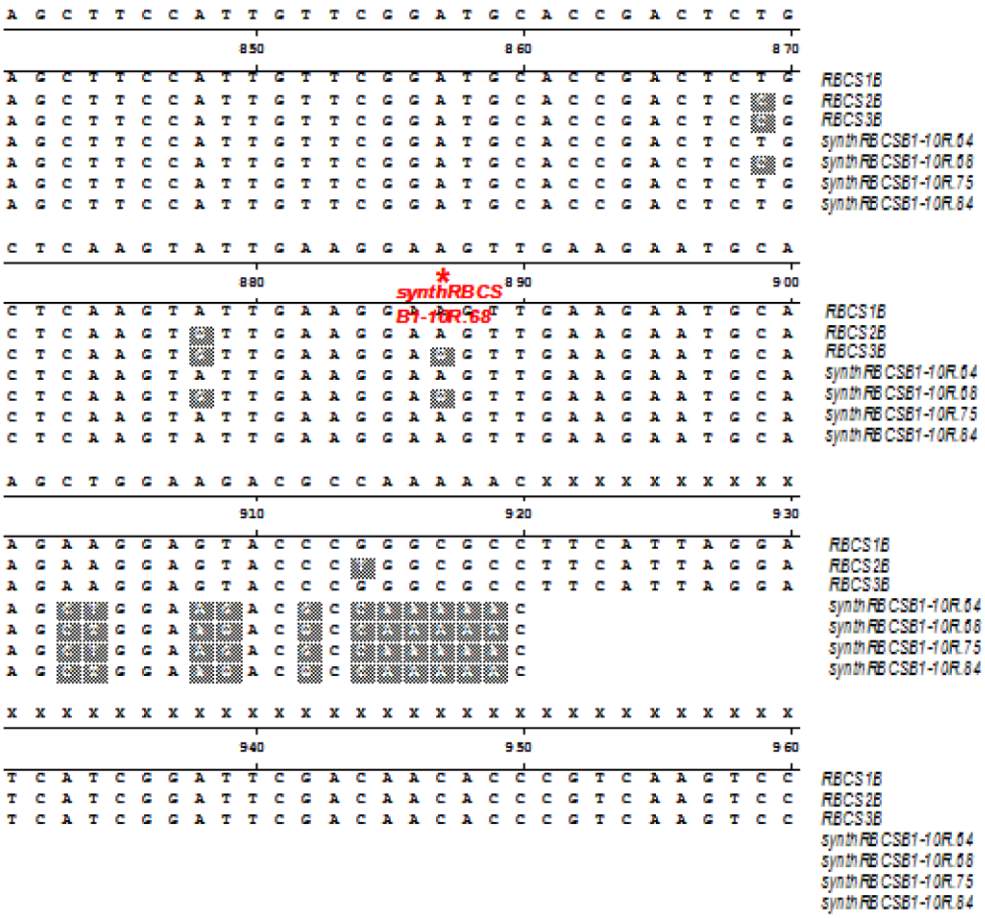

250
260
270
RBCS1B
RBCS2B
RBCS3B
synthRBCSB'
synthRBCSB'
synthRBCSB'
synthRBCSB'
PflMI
280
290
300
RBCS1B
RBCS2B
RBCS3B
synthRBCSB
synthRBCSB
synthRBCSB
synthRBCSB
310
320
330
RBCS1B
RBCS2B
RBCS3B
synthRBCSB
synthRBCSB
synthRBCSB
synthRBCSB
340
350
360
RBCS1B
RBCS2B
RBCS3B
synthRBCS
synthRBCS
synthRBCS


exon 1 / Intron 1
Intron 1 / exon 2
RBCS1B
RBCS2B
RBCS3B
synthRB
synthRB
synthRB
synthRB
synthRB


RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
exon 2 / Intron 2
RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10
synthRBCSB1-10


RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10R.64
synthRBCSB1-10R.68
synthRBCSB1-10R.75
synthRBCSB1-10R.84
synthRBCS B1-10R.75
synthRBCS B1-10R.84
synthRBCS B1-10R.64


730
740
750
760
Intron 2 / exon 3
780
790
800
810
820
830
840
RBCS1B
RBCS2B
RBCS3B
synthRBCSB1-10R.64
synthRBCSB1-10R.66
synthRBCSB1-10R.75
synthRBCSB1-10R.84



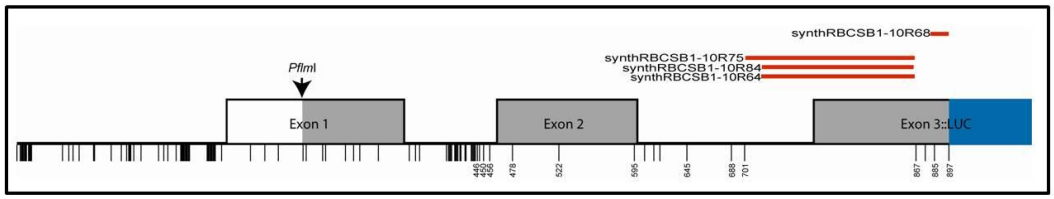


Fig. III.5 Mapping of recombination resolution sites. **Gray regions indicate DNA sequences available for homologous recombination between the *RBCS3B* and *ΔRBCS1B* genes. The black arrow indicated the PflMI site used to generate the 5' end of the *ΔRBCS1B::LUC* gene. Vertical lines indicate single polymorphic bases between the *RBCS3B* and *ΔRBCS1B* genes used to map the resolution sites. Red horizontal lines define where recombination resolution occurred.**

UV-C Irradiation and Regulation of Transcription Factor and Defense Related Genes

Genes regulated after a stress treatment are thought to reflect a part of the physiological adaptive response to stress. Plants treated with UV-C that leads to DNA damage are candidates for a coordinated stress response that includes both the regulation of stress-responsive genes and DNA damage repair by homologous recombination. To assess the effect of the UV-C treatment, genes previously identified to be associated with the stress response were analyzed for changes in their expression. Molinier et al (2004) used microarray technology to identify genes that responded to UV-C irradiation. Defense/stress response genes (*PR-1*, disease resistance proteins (*RPS2*)), genes encoding components of signaling pathways (receptor protein kinases), as well as transcription factors (*MYB10* and *HSF3*), were among the genes found to be up-regulated by UV-C (Molinier et al., 2004). The twenty-four hour time point showed the largest fold-change in gene expression. Twenty-four hours after UV-C irradiation, the transcript level of *PR-1* increased ~2-fold, transcription factor *MYB-10* increased ~25-fold and *HSF3* increased ~174-fold. The expression profile of these genes lead us to believe that a similar UV-C irradiation treatment could be used to replicate the differential expression of genes *PR-1*, *MYB10*, and *HSF3*. *HSF3* also served as an internal control for the UV-C irradiation treatment. *HSF3* differentially responds to temperature stimuli above the optimal temperature. We speculated that *HSF3* might respond to heat damage directly affiliated with the use of the mineral light lamp. Thus, the differential expression of the genes would serve as an indicator that the UV-C treatment was successful.

We evaluated RNA transcript levels of *PR-1*, *MYB10*, and *HSF3* from *Arabidopsis* plants exposed to UV-C irradiation by semiquantitative RT-PCR and compared to the transcript levels of non-UV-C irradiated plants (Fig. III.6). The level of *PR-1* expression fluctuated over the course of 24 hours. From UV-C treated plants, at 30 min and 6 hrs peak expression of *PR-1* was observed. By 24 hours the expression level of *PR-1* dropped but not below the detection limit, as it was still above the gene expression level observed before UV-C irradiation. Molinier et al (2004) observed a 2-fold induction by 30 min, a 1-fold induction by 6 hrs and a 2-fold induction by 24 hrs. Our *PR-1* expression data follows the general trend of an increase in gene expression, as was observed by Molinier et al (2004). We observed that *MYB10* expression levels

stayed fairly constant over the time course of twenty-four hours after UV-C treatment. This is in contrast to the increase in *MYB10* expression levels observed by Molinier at el (2004). Over the course of 24 hrs, Molinier et al (2004) observed no change in transcript levels through 6 hrs then a 25-fold induction in *MYB10* expression by 24 hrs. Within our experiment, we observed a difference in *MYB10* expression between non-UV-C irradiated plants and UV-C irradiated plants. In contrast to the fairly steady levels of *MYB10* expression in UV-C irradiated plants, *MYB10* expression in non-UV-C irradiated plants fluctuated. *MYB10* expression levels were steady until the 2 hr time point. At 2 hrs it dropped substantially. By 6 hrs *MYB10* expression spiked upward then fell dramatically to an undetectable level by twenty-four hours. One of the most striking differences we observed was between the *HSF3* expression data obtained by Molinier et al (2004) and our expression data. Molinier observed no change in induction in *HSF3* expression through 6 hrs then by 24 hrs the expression level spiked to a 173-fold induction. This is in marked contrast to our expression data. We observed subtle fluctuations in *HSF3* expression through 6 hrs and there was a slight increase at 6 hrs. However, there was not a strong induction in the expression level of *HSF3* from UV-C irradiated plants by 24 hrs. Notably, at the end of the time course *HSF3* expression dropped dramatically. We observed variable expression from *HSF3* in non-UV-C irradiated plants. However, we did not see the general trend of increased gene expression in UV-C irradiated plants versus non-UV-C irradiated plants. We were interested in replicating the expression data from Molinier et al (2004) because we wanted a bit of assurance that the UV-C treatment was effective. The expression data I gathered provided minimal support for whether the UV-C treatment was an effective DNA damaging agent.

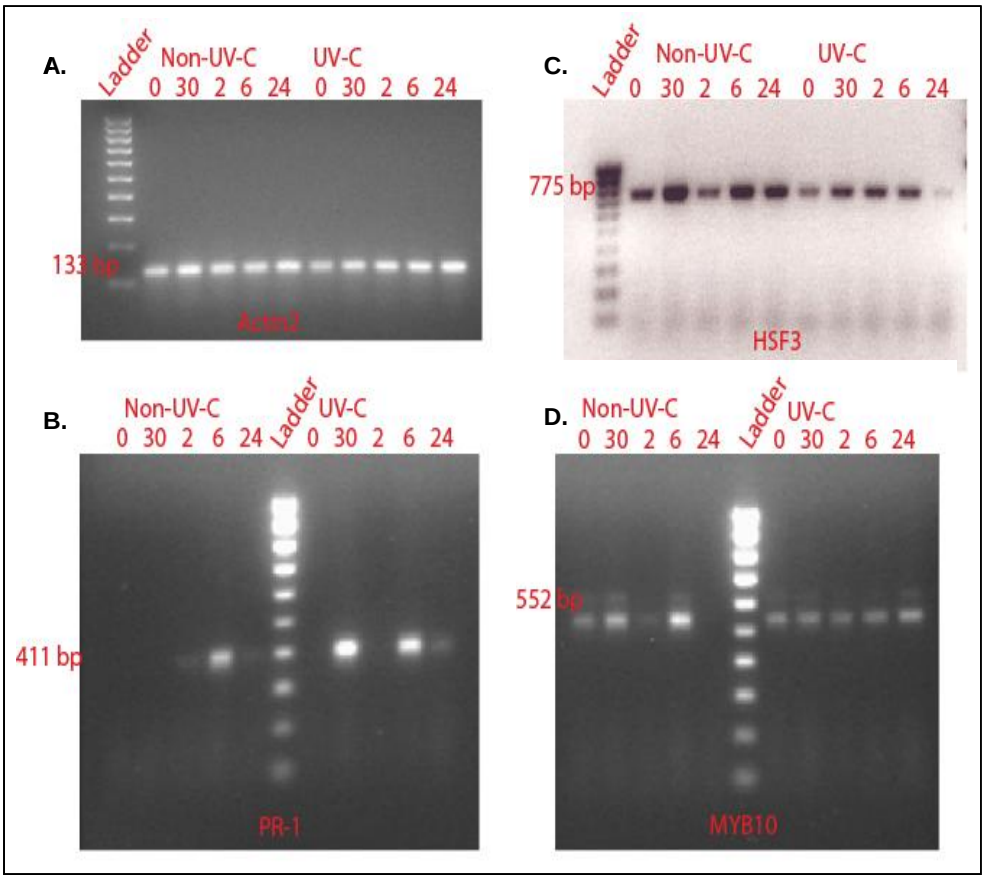


Fig. III.6 Semiquantitative RT-PCR analysis of transcription from non-UV-C irradiated and UV-C irradiated *Arabidopsis* plants containing the synthetic RBCSB gene cluster. A. constitutive control *Actin2*; B. *PR-1*; C. *HSF3*; D. MYB10

III.3.4 Discussion

Plants can recognize and respond to stimuli that alter their physiological state with a battery of mechanisms whose aim is to remedy the situation and/or acclimate accordingly. As a part of the adaptive response to stress, the expression of genes that contribute to various physiological processes in the plant can be differentially regulated. The transcription factor *MYB10* is a part of a superfamily of transcription factors that play regulatory roles in development and defense responses in plants (Yanhui et al., 2006). *MYB10* has been described to be responsive to salicylic acid (SA), which is a compound shown to accumulate when there is an increase in the production of reactive oxygen species (ROS), which can occur as a result of pathogen attack. Notably, UV-C irradiation can lead to the production of destructive free radical species that can damage DNA. In the plant defense pathway SA functions as a signaling component and it has been shown to lead to the up-regulation of pathogenesis-related gene *PR-1*. UV-B irradiation lead to an increase in transcripts for gene *PR-1* (A.-H.-Mackerness, 2000), therefore a similar effect on gene expression was speculated to occur with UV-C irradiation. Additionally, since ROS have already been implicated in the regulation of gene expression in response to UV-B irradiation (Green and Fluhr, 1995; Surplus et al., 1998; A.-H.-Mackerness, S., 1998), UV-C irradiation may also affect gene expression. If the damaging effects of UV-C irradiation were to similarly regulate the expression of genes *MYB10*, and *PR-1* then this would provide some evidence for possibly common or shared components between the defense and recombination repair pathways utilized by plants.

In these studies, the UV-C gene regulation data obtained did not demonstrate induction of *MYB10*, *PR-1* and *HSF-3* in the response to UV-C irradiation. This is in contrast to the report by Molinier et al (2004), who found strong induction of these genes at twenty-four hours after UV-C irradiation. We did not observe large fold changes at this time-point that would suggest UV-C irradiation directly affected the expression of these genes. The ROS produced from the DNA damage caused by UV-C irradiation do not appear to be mobilized by the same components associated with the defense pathway. This may explain the stability observed with *MYB10* transcript expression and the minimal induction of pathogenesis-related gene *PR-1*. However, for *HSF3*, the stable

transcript levels we observed are not unusual because northern blot analysis has shown that *HSF3* mRNA is not affected by heat shock (37°C heating block for 1 hour) (Prandl et al., 1998) and it is plausible that it would take less than optimal conditions i.e. sublethal heat stress to see a strong increase in *HSF3* transcript levels.

A number of variables may have influenced some of the differences we observed compared to Molinier et al (2004). The effect of UV-C irradiation on gene expression was assayed in wild-type *Arabidopsis thaliana* plants ~13 days of age (Molinier et al., 2004), whereas our plants were 21 days of age. The difference in age and genotype may have influenced how the plants respond to stress. Molinier et al (2004) UV-C irradiated plants germinated on solid plant media then transferred to soil, whereas we utilized plants germinated and grown on soil. Notably, our experiments utilized the same UV-C mineral light lamp utilized by Molinier et al (2004). We also used semiquantitative PCR to assess the effect of UV-C irradiation on the expression of genes *PR-1, MYB10,* and *HSF3* whereas Molinier et al (2004) used microarray technology. We believe the large fold changes Molinier et al (2004) observed are within the limits of detection for semiquantitative PCR. However, only a direct comparison using microarray technology would confirm this.

An important question that remains is whether the UV-C treatment we used was effective. Molinier et al (2004) UV-C irradiated plants at 6 kerg/cm^2. The UV-C dosage we utilized (440µW/cm^2 for 14 seconds is equal to 61.6 kerg/cm^2) was ten times the amount used by Molinier et al (2004). The increased dosage we used was the result of a miscalculation. One may speculate that the larger dosage used in our experiments may explain why we were unable to recapitulate the gene expression data and increased recombination demonstrated by Molinier et al (2004). However, the UV-C dosage we utilized is lower than other treatments that successfully induced recombination in *Arabidopsis* and tobacco (Kovalchuk et al., 2003; Filkowski et al., 2004). Overall, our results do not confirm common upstream regulators between the ROS induced transcription factors and stress response genes and the expression data we gathered provides minimal support for the effect of UV-C irradiation on the plants' adaptive response to stress. However, the insignificant differential regulation observed with these

genes does not abrogate a potential role for effects of UV-C irradiation on recombination at the *synthRBCSB* locus through the action of other gene products.

After UV-C irradiation, we did not observe a statistically significant increase (~2-fold) in the frequency of meiotic recombination. We anticipated that we might obtain recombinants at an estimated frequency of 1.9×10^{-6} (based on previously established measures of recombination with the same synthetic *RBCSB* gene cluster (Jelesko et al., 1999, 2004)) or higher from UV-C irradiated plants. The UV-C irradiation treatment did not have a significant effect on the frequency of meiotic recombination. This is in contrast to the rates of somatic recombination following UV-C irradiation. Filkowski et al (2004) observed somatic recombination to be 3.1-fold higher in irradiated plants than in untreated controls. It is possible that the observations by Molinier et al (2004) and Filkowski et al (2004) indicate that UV-C irradiation affects somatic recombination and not meiotic recombination. Filkowski et al (2004) also observed a smaller 1.6-fold increase in somatic recombination. The two somatic recombination frequencies obtained from Filkowski et al (2004) are measurements from two independent transgenic reporter lines at different genomic loci.

The site of transgene insertion is another factor to consider. It is becoming more apparent that where the recombination substrate integrates in the genome influences the frequency of recombination (Puchta et al., 1995; Kovalchuk et al., 2000; Filkowski et al., 2004) and this may apply to recombination at the *synthRBCSB* locus we used in our experiments. Additionally, plants that are either hemizygous or homozygous for the transgene locus have been shown to exhibit different recombination rates (Molinier et al., 2004). In hemizygous plants recombination can only occur between sister chromatids. In homozygous plants recombination can occur between sister chromatids or between homologous chromosomes. Higher somatic recombination frequencies were observed in plants homozygous for the transgene compared to plants hemizygous for the transgene (Molinier et al., 2004) with multiple transgenic lines that were at different genomic loci. However, following DNA damage, somatic recombination in hemizygous plants and homozygous plants was differentially stimulated and the differences observed were dependent on the type of DNA damage, as well as the zygosity state of the transgene (Molinier et al., 2004). We may speculate that the homozygosity of *synthRBCSB* may

suppress meiotic recombination upon DNA damage (Simon, unpublished). Another potential cause for the low induction of UV-C stimulated HR may be that some chromosomal regions might contain higher numbers of UV-induced DNA lesions than others. The cyclobutyl pyrimidine dimers (CPDs) and photoproducts (PP) created by UV-C irradiation depend on the chromatin structure surrounding the target sequence (Mitchell et al., 1992). Therefore, the genomic position of the transgene locus is important for examining recombination.

Notably, our data may not account for an increase in recombinatorial repair. It is possible that due to the efficient repair of the DNA damage there were no detectable rearrangements in gene structure thus prohibiting an assessment of these recombination events. Recombination frequency estimates are subject to the limitations of the detection strategy utilized and the UV-C assay we utilized was performed to assess the influence of abiotic stress on DNA recombination events that yield gene rearrangements.

Our data suggest recombination frequencies are lower for meiotic recombination versus somatic recombination. We can speculate that the low induction of meiotic recombination may be the result of maintenance of genome integrity. Further examination of stress stimuli and its influence on recombination at the *synthRBCSB* locus will provide more information about a positive or negative correlation between the recombinagenic behavior of multigenic loci and stress. We may begin to see how UV-C irradiation can be used as a tool to increase the frequency of recombination, which can increase our chances of generating diversity within plant gene clusters.

Chapter IV
Conclusions and Future Prospects

IV.4 Conclusion

DNA recombination impacts plant fertility, DNA damage repair, and genetic mutations that can contribute to gene evolution and functional diversity. As stated previously, some of the most relevant studies of the different types of recombination have utilized yeast as a model. However, studies of the meiotic behavior of endogenous gene clusters in yeast may not provide an accurate depiction of recombination in plants because plants have more and larger gene families. In plants, numerous recombination studies have been performed using reporter gene-based recombination substrates but again these studies lack the detailed information about the sequence characteristics of recombination; therefore, many of these studies may not accurately reflect the mechanisms responsible for the evolution of gene clusters in multicellular organisms. A more accurate analysis of the meiotic behavior of plant gene clusters will require a significantly improved model of recombination utilizing endogenous gene clusters found in multicellular organisms. Careful characterization of the recombination patterns and rates would provide more information about the meiotic events that lead to gene duplications and recombinant genes and how these can be evolutionarily advantageous.

There is broad diversity in the evolutionary trajectories of *R* genes. For this reason, it has been difficult to formulate a universally applicable model for *R* gene evolution. There are differences in the mechanism that which some *R* genes proliferate, as well as inherent differences in the tempo and mode of genetic exchanges observed with some *R* gene clusters. Although recombination has the potential to influence *R* gene evolution, the actual relevance of recombination to *R* gene evolution is not yet known. The *RPP8* locus is a good example of divergence and evolution of an *R* gene cluster. Among the *RPP8* family members, the sequence patterns that have demonstrated recombination has occurred and the functionally divergent *RPP8* alleles have supported the relevance of recombination to *R* gene evolution. However, there are few other examples that have provided such strong support on both the structural and functional level. There is a current lack of knowledge about the relative frequency of recombination and whether the recombination events were integral to the evolution of new resistance

specificities. Studies that expand our understanding of the mechanistic details of recombination will provide some of the data that is currently lacking.

The development of a genetic screening system that utilizes synthetic gene cluster technology to investigate meiotic unequal crossing-over at a synthetic *RPP8* locus (*synthRPP8*) in *Arabidopsis thaliana* was a first step towards beginning to understand recombination and *R* gene evolution. The genetic screen using *synthRPP8* was not successfully implemented. However, the optimization of the bioluminescence assay and the newly constructed *synthRPP8* gene cluster represent substantial progress towards implementing the genetic screen and we anticipate only minor fine-tuning of the genetic screen. Soon we can begin to address aspects of recombination at the *synthRPP8* locus that may have contributed to allelic diversity at the *RPP8* locus. This will necessitate having multiple independent transgenic lines in both the hemizygous and homozygous state. It's important that we learn more about how zygosity of a locus affects recombination. Most of the research that has addressed this issue has utilized reporter gene constructs and we would like to address the zygosity issue with endogenous gene clusters. We also want to compare recombination frequencies with lines containing the transgene at different chromosomal locations. This will provide information on the influence of insertion site on the frequency of recombination. For example, we do not know if a *synthRPP8* locus positioned near telomeres may demonstrate a different rate of recombination than a *synthRPP8* locus located in a large block of euchromatin. Having multiple transgenic lines will also be important for assessing the influence of stress on meiotic recombination at the *synthRPP8* locus because we may find that certain chromosomal regions respond differently to stress stimuli. The UV-C irradiation performed with the *synthRBCSB* locus provided preliminary data for stress stimuli and recombination. However, an important parameter that should be addressed is the efficiency of the stress stimuli. This can be done by assaying for somatic recombination in UV-C treated plants, to provide some level of assurance that the UV-C stimulus used sufficiently damages the DNA thus increasing the likelihood that repair mechanisms i.e. HR will be initiated. As we learn more about the efficiency of UV-C stress stimuli we can broaden this to other types of stress such as pathogens, hormones, and physical wounding.

We anticipate that using stress as a stimulus to induce HR in conjunction with *synthRPP8* will produce a large collection of recombinants. We will to map the recombination resolution sites of the chimeric genes and compare to the parental genotype to determine whether highly homologous or divergent regions of sequence are a stimulus for recombination. We can also assess whether there is a bias in the location of the resolution site and whether this reflects a recombination "hot-spot." High-resolution mapping will also help determine whether recombinatorial regions correlate with putative functional domains in the encoded protein. This could provide insight into how recombination leads to the evolution of novel resistance specificities. For example, recombinatorial events that increase or decrease the length of the LRR may contribute to *R* gene diversification since the LRR is predicted to be the determinant for *R* gene specificity. As postulated by Ellis et al (2000), expansion and contraction of LRR repeats could change the LRR's specificity for ligands, which could diversify the repertoire of recognition specificities.

The objectives set forth in this dissertation have made advances toward implementing a genetic screening system that can be used with other resistance genes to model recombination and resistance gene evolution. We can look forward to broadening our understanding of the various modes of recombination when this study is expanded to larger *R* gene clusters i.e. the 3 gene cluster of *RPP1*. Additionally, we may be able to answer the question of whether novel resistance specificities can be generated *in planta* by meiotic recombination and whether this will lead to genes created *ex planta* that provide more effective resistance to pathogens.

The unexpected discovery of a possible stress response element in the promoter of *RPP8* adds a new dimension to understanding the stress response and the effects associated with stress regulation. The point mutation within the putative cis-element upstream of the *RPP8* coding sequence did provide reliable inactivation of the gene under optimal growth conditions. However, under conditions that were not optimal, the point mutation did not provide reliable inactivation of the gene. Further insight into the regulation of the *RPP8* promoter may come from examining how this putative stress response element mediates the response to various stress signals. Analysis of the cis-acting element and the associated transcription factors may help to understand the altered

RPP8 gene expression. Additionally, it will be interesting to see if there are other functional consequences associated with changes in this putative cis-element.
A future objective of this research is to assess the functional consequences of recombination on chimeric *R* genes. To do this, the recombinant chimeric *R* genes created from *synthRPP8* can be challenged with pathogens that define the recognition specificities of the parental genes as well as a panel of virulent pathogens to test for novel recognition specificities. We may be able to correlate the transfer of recognition function to chimeric gene formation if the chimeric gene acquires the same resistance specificity as *RPP8.*

Many of the objectives addressed in this dissertation will lead to the implementation of a genetic system that provides an evolutionary interpretation of how *R* gene clusters diversify and evolve novel recognition specificities. Additionally, implementation of the genetic system may lend an important insight into the ability of plants to adapt to stress.

References

Abramovitch, R.B., Y.J. Kim, S. Chen, M.B. Dickman, and Martin, G.B. (2003) *Pseudomonas* type III effector *AvrPtoB* induces plant disease susceptibility by inhibition of host programmed cell death. EMBO Journal 22(1): 60-69.

Abramovitch, R.B. and Martin, G.B. (2004) Strategies used by bacterial pathogens to suppress plant defenses. Curr Opin Plant Biol 7: 356-364.

Abramovitch, R.B., Janjusevic, R., Stebbins, C.E., and Martin, G.B. (2006) Type III effector *AvrPtoB* requires intrinsic E3 ubiquitin ligase activity to suppress plant cell death and immunity. PNAS 103(8): 2851-2856.

Abramovitch, R.B., Anderson, J.C. and Martin, G.B. (2006) Bacterial elicitation and evasion of plant innate immunity. Nat Rev Mol Cell Biol 7(8): 601-611.

Alfano, J.R. and Collmer, A. (1997) Bacterial pathogens in plants: Life up against the wall. Plant Cell 8: 1683-1698.

A.-H.-Mackerness, S., Surplus, S.L., Jordan, B.R., and Thomas, B. (1998) Effects of supplementary UV-B radiation on photosynthetic transcripts at different stages of development and light levels in pea: role of ROS and antioxidant enzymes. Photochemistry and Photobiology 68: 88-96.

A.-H.-Mackerness, S. (2000) Plant responses to ultraviolet-B (UV-B: 280-320 nm) stress: What are the Key Regulators. Plant Growth Regulation 32: 27-39.

Assaad, F. F. and Signer, E.R. (1992) Somatic and germinal recombination of a direct repeat in *Arabidopsis*. Genetics 132: 553-566.

Assaad, F.F., Tucker, K.L., and Signer, E.R. (1993) Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. Plant Mol. Biol 22: 1067-1085.

Bai, J., Pennill, L.A., Ning, J., Lee, S.W., Ramalingam, J., Webb, C.A., Zhao, B., Sun, Q., Nelson, J.C., Leach, J.E., and Hulbert, S.H. (2002) Diversity in nucleotide binding site-leucine-rich repeat genes in cereals. Genome Res 12(12): 1871-1884.

Bakker, E.G., Toomajian, C., Kreitman, M., and Bergelson, J. (2006) A Genome-Wide Survey of *R* Gene Polymorphisms in *Arabidopsis*. Plant Cell 18: 1803-1818.

Baumgarten, A., Cannon, S., Spangler, R., and May, G. (2003) Genome-level evolution of resistance genes in *Arabidopsis thaliana*. Genetics 165: 309-319.

Bechtold, N., Ellis, J. and Pelletier, G. (1993) In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris, Life Sciences 316: 1194-1199.

Belkhadir, Y., Subramaniam, R., and Dangl, J.L. (2004) Plant disease resistance protein signaling: NBS-LRR proteins and their partners. Curr Opin Plant Biol 7(4): 391-399.

Britt, A.B. (1999) Molecular genetics of DNA repair in higher plants. Trends Plant Science 4: 20-24.

Botella, M. A., Parker, J.E., Frost, L.N., Bittner-Eddy, P.D., Beynon, J.L., Daniels, M.J., Holub, E.B., and Jones, J.D.G. (1998) Three genes of the *Arabidopsis RPP1* complex resistance locus recognize distinct *Peronospora parasitica* avirulence determinants. Plant Cell 10: 1847-1860.

Brunner, A., Yakovlev, I.A., and Strauss, S.H. (2004) Validating internal controls for quantitative plant gene expression studies. BMC Plant Biology 4: 14.

Cannon, S.B., Zhu, H., Baumgarten, A.M., Spangler, R., May, G., Cook, D.R., and Young, N.D. (2002) Diversity, distribution, and ancient taxonomic relationships within the TIR and non-TIR NBS-LRR resistance gene subfamilies. J Mol Evol 54: 548-562.

Chang, J.H., Goel, A.K., Grant, S.R. and Dangl, J.L. (2004) Wake of the flood: ascribing functions to the wave of type III effector proteins of phytopathogenic bacteria. Curr. Opin. Microbiol. 7: 11–18.

Collmer, A. (1998) Determinants of pathogenicity and avirulence in plant pathogenic bacteria. Curr. Opin. Plant Biol. 1: 329-335.

Collins, N., Drake, J., Ayliffe, M., Sun, Q., Ellis, J., Hulbert, S., and Pryor, T. (1999) Molecular characterization of the Maize *Rp1-D* rust resistance haplotype and its mutants. Plant Cell 11: 1365-1376.

Cooley, M.B., Pathirana, S., Wu, H.J., Kachroo, P., and Klessig, D.F. (2000) Members of the *Arabidopsis HRT/RPP8* family of resistance genes confer resistance to both viral and oomycete pathogens. Plant Cell 12(5): 663-676.

Dangl, J.L., and Jones, J.D.G. (2002) Plant pathogens and integrated defense responses to infection. Nature 411: 826-833.

DebRoy, S., Thilmony, R., Kwack, Y.B., Nomura, K., and He, S.Y. (2004) A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. PNAS 101: 9927-9932.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S. and Marco, Y. (2003) Physical interaction between RRS1-R., a protein conferring resistance to bacterial

wilt, and *PopP2*, a type III effector targeted to the plant nucleus. PNAS 100: 8024–8029.

Dodds, P.N., Lawrence, G.J., and Ellis, J.G. (2001) Contrasting modes of evolution acting on the complex *N* locus for rust resistance in flax. Plant Journal. 27:439–453.

Dodds P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, A. (2006) Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. PNAS 103: 8888–8893.

Durrant, W.E., Wang, S., and Dong, X. (2007) *Arabidopsis SNI1* and *RAD51D* regulate both gene transcription and DNA recombination during the defense response. PNAS 104: 4223-4227.

Ellis, J. G., Lawrence, G.J., Luck, J.E., and Dodds, P.N. (1999) Identification of regions in alleles of the flax rust resistance gene *L* that determine differences in gene-for-gene specificity. Plant Cell 11: 495-506.

Ellis, J., Dodds, P., and Pryor, T. (2000) Structure, function and evolution of plant disease resistance genes. Curr. Opin. Plant Biol. 3: 278-284.

Farrara, B.F., Ilott, T.W., and Michelmore, R.W. (1987) Genetic analysis of factors for resistance to downy mildew (*Bremia lactucae*) in species of lettuce (*Lactuca sativa* and *L. serriola*) Plant Pathology 36 (4): 499–514.

Felix, G., Duran, J.D., Volko, S., and Boller, T. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 18 (3): 265-276.

Filkowski, J., Yeoman, A., Kovalchuk, O., and Kovalchuk, O. (2004) Systemic plant signal triggers genome instability. Plant Journal 38: 1-11.

Flor, H.H. (1971) Current status of the gene-for-gene concept. Annu. Rev. Phytopatho. 9: 275-296.

Gabriel, D.W and Rolfe, B.G. (1990) Working models of specific recognition in plant-microbe interactions. Annu. Rev. Phytopatho. 28: 365-391.

Gilbertson, L (2003) Cre-Lox: Cre-ative tools for plant biotechnology. Trends in Biotechnology 21: 550-555.

Green, R., and Fluhr, R. (1995) UV-B-induced *PR1* accumulation is mediated by active oxygen species. Plant Cell 7: 203-212.

Greenberg, J.T. and Vinatzer, B.A. (2003) Identifying type III effectors of plant pathogens and analyzing their interaction with plant cells. Curr. Opin. Microbiol. 6: 20-28.

Gumucio, D.L., Wiebauer, K., Caldwell, R.M., Samuelson, L.C., and Meisler, M.H. (1988) Concerted evolution of human Amylase genes. Molecular and Cellular Biology 8: 1197-1205.

Ham, J.H., Kim, M.G., Lee, S.Y., and Mackey, D. (2007) Layered basal defenses underlie non-host resistance of *Arabidopsis* to Pseudomonas syringae pv. phaseolicola. Plant Journal 51(4): p. 604-16.

Hammond-Kosack, K. and Jones, J.D.G. (1996) Inducible plant defense mechanisms and resistance gene function. Plant Cell 8: 1773-1791.

Hammond-Kosack, K. and Jones, J.D.G. (2000) Responses to plant pathogens. Biochemistry and Molecular Biology of Plants. B. Buchanan, W. Gruissem and R. Jones. Rockville, MD, USA, American Society of Plant Physiologists: 1102-1155.

Hann, D.R. and Rathjen, J.P. (2007) Early events in the pathogenicity of *Pseudomonas syringae* on *Nicotiana benthamiana*. Plant Journal 49(4): 607-618.

Hauck, P., Thilmony, R., and He, S.Y. (2003) A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible *Arabidopsis* plants. PNAS 100 (14): 8577-8582.

Hoess, R.H., and Abremski, K.J. (1985) Mechanism of strand cleavage and exchange in the Cre-lox site-specific recombination system. Mol Biol. 181(3):351-62.

Holliday, R. (1964). A mechanism for gene conversion in fungi. Genet. Res. Camb. 5: 282-304.

Holt, B.F., Boyes, D.C., Ellerstrom, M., Siefers, N., Wiig, A., Kauffman, S., Grant, M.R., and Dangl, JL. (2002) An evolutionarily conserved mediator of plant disease resistance gene function is required for normal *Arabidopsis* development. Dev. Cell 2: 807-817.

Holub, E.B. and Beynon, J.L. (1997) Symbiology of mouse ear cress (*Arabidopsis thaliana*) and oomycetes. Adv. Bot. Res. 24:228-273.

Hughes, A.L. and Yeager, M. (1997) Molecular evolution of the vertebrate immune system. Bioessays 19: 777-786.

Hulbert, S. H. (1997) Structure and evolution of the *Rp1* complex conferring rust resistance in Maize. Annu. Rev. Phytopathol. 35: 293-310.

Hulbert, S. H., Webb, C.A., Smith, S.M., and Sun, Q. (2001) Resistance gene complexes: Evolution and utilization. Annu. Rev. Phytopathol. 39: 285-312.

Jackson, J. A. and Fink, G.R. (1985) Meiotic recombination between duplicated genetic elements in *Saccharomyces-Cerevisiae*. Genetics 109: 303-332.

Jansen, M.A.K., Gaba, V., and Greenber, B.M. (1998) Higher plants and UV-B radiation: balancing damage, repair, and acclimation. Trends Plant Science 3: 131-135.

Jelesko, J. G., Harper, R., Furuya, M., and Gruissem, W. (1999) Rare germinal unequal crossing-over leading to recombinant gene formation and gene duplication in *Arabidopsis* thaliana. PNAS 96: 10302-10307.

Jelesko, J.G., Carter, K., Thompson, W., Kinoshita, Y., and Gruissem, W. (2004) Meiotic recombination between paralogous *RBCSB* genes on sister chromatids of *Arabidopsis thaliana*. Genetics 166: 947-957.

Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B. (2000) Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO Journal. 19: 4004-4014.

Klein, H. L. and Petes, T.D. (1981) Intrachromosomal gene conversion in yeast. Nature 289: 144-148.

Kobe, B. and Deisenhofer, J. (1994) The leucine-rich repeat: a versatile binding motif. Trends Biochem. Sci. 19, 415-421.

Kobe, B. and Kajava, A.V. (2001) The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. 11, 725-732.

Kovalchuk, I., Kovalchuk, O., and Hohn, B. (2000) Genome-wide variation of the somatic mutation frequency in transgenic plants. EMBO Journal 19, 4431-4438.

Kovalchuk, I., Kovalchuk, O., Kalck, V., Boyko, V., Filkowski, J., Heinlein, M., and Hohn, B. (2003) Pathogen-induced systemic plant signal triggers DNA rearrangements. Nature 423: 760-762.

Kuang, H., Woo, S.S., Meyers, B.C., Nevo, E. and Michelmore, R.W. (2004) Multiple genetic processes result in heterogeneous rates of evolution within the major cluster disease resistance genes in lettuce. Plant Cell, 16, 2870–2894.

Lebel, E. G., Masson, J., Bogucki, A., and Paszkowski, J. (1993) Stress-induced intrachromosomal recombination in plant somatic cells. PNAS 90: 422-426.

Leister, D., Kurth, J., Laurie, D.A., Yano, M., Sasaki, T., Devos, K., Graner, A. and Schulze-Lefert, P. (1998) Rapid reorganization of resistance gene homologues in cereal genomes. PNAS 95: 370–375.

Leister, D. (2004) Tandem and segmental gene duplication and recombination in the evolution of plant disease resistance gene. Trends Genet, 20: 116–122.

Lucht, J. M., Mauch-Mani, B., Steiner, H., Metraux, J., Ryals, J., and Hohn, B. (2002) Pathogen stress increases somatic recombination frequency in *Arabidopsis*. Nature Genetics 30: 311-314.

Luck, J.E., Lawrence, G.J., Dodds, P.N., Shepherd, K.W., and Ellis, J.G. (2000) Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. Plant Cell 12:1367-77.

Lyznik LA, McGee JD, Tung PY, Bennetzen JL, and Hodges, T.K. (1991) Homologous recombination between plasmid DNA molecules in Maize protoplasts. Molecular & General Genetics 230: 209-218.

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

McDowell, J. M., Dhandaydham, M., Long, T.A., Aarts, M.G.M., Goff, S., Holub, E.B., and Dangl, J.L. (1998) Intragenic recombination and diversifying selection contribute to the evolution of downy mildew resistance at the *RPP8* locus of *Arabidopsis*. Plant Cell 10: 1861-1874.

McDowell, J.M. and Simon, S.A. (2006) Recent insights into *R* gene evolution. Molecular Plant Pathology 7(5): 437-448.

McDowell, J. M. and Woffenden, B.J. (2003) Plant disease resistance genes: Recent insights and potential applications. Trends in Biotechnology 4: 178-183.

Mackey, D., Holt, B.F., Wiig, A., and Dangl, J.L. (2002) *RIN4* interacts with *Pseudomonas syringae* type III effector molecules and is required for *RPMI*-mediated resistance in *Arabidopsis*. Cell 108: 743-754.

Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L. (2003) *Arabidopsis RIN4* is a target of the type III virulence effector *AvrRpt2* and modulates *RPS2*-mediated resistance. Cell 112: 379-389.

Martin, G.B., Bogdanove, A.J., and Sessa, G. (2003) Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. 54:23-61.

Menke, M., Chen, I., Angelis, K.J., and Schubert, I. (2001) DNA damage and repair in *Arabidopsis thaliana* as measured by the comet assay after treatment with different classes of genotoxins. Mutation Research 493: 87-93.

Meselson, M. S. and Radding, C.M. (1975) A general model for genetic recombination. PNAS 72: 358-361.

Meyers, B. C., Chin, D.B., Shen, K.A., Sivaramakrishnan, S., Lavelle, D.O., Zhang, Z., and Michelmore, R.W. (1998) The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10: 1817-1832.

Meyers, B.C., Kozik, A., Griego, A., Kuang, H.H. and Michelmore, R.W. (2003) Genome-wide analysis of NBS-LRR-encoding genes in *Arabidopsis*. Plant Cell 15: 809–834.

Michelmore, R. W. and Meyers, B.C. (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8: 1113-1130.

Mitchell, D.L., Jen, J., and Cleaver, J.E. (1992) Sequence specificity of cyclobutane pyrimidine dimers in DNA treated with solar (ultraviolet B) radiation Nucleic Acids Res. 20: 225-229.

Molinier, J., Ries, G., Bonhoeffer, S., and Hohn, B. (2004) Interchromatid and interhomolog recombination in *Arabidopsis thaliana*. Plant Cell 16: 342-352.

Molinier, J., Oakeley, E.J., Niederhauser, O., Kovalchuk, I., and Hohn, B. (2005) Dynamic response of plant genome to ultraviolet radiation and other genotoxic stresses. Mutation Research 571: 235-247.

Moffett, P., Farnham, G., Peart, J. and Baulcombe, D.C. (2002) Interaction between domains of a plant NBS-LRR protein in disease resistance related

cell death. EMBO Journal. 21: 4511–4519.

Monosi, B., Wisser, R.J., Pennill, L. and Hulbert, S.H. (2004) Full genome analysis of resistance gene homologues in rice. Theor. Appl. Genet. 109: 1434–1447.

Nei, M. and Rooney, A.P. (2005) Concerted and birth-and-death evolution of multigene families. Annu. Rev. Genet. 39: 121–152.

Norskov-Lauritsen, N., Ebbesen, P., Demant, E.J. and Christensen, J.M. (1990) Bleomycin–iron complexes and DNA radiation damage. Cancer Biochem. Biophys. 11: 265–273.

Nürnberger, T. and Brunner, F. (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Current Opin Plant Bio. 5: 318-324.

Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L. (2004) Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. 198, 249–266.

Nurnberger T, Volker, L. (2005) Non-host resistance in plants: new insights into an old phenomenon. Mol. Plant Pathology 6(3): 335–345.

Ochman, H., Medhora, M.M., Garza, D., and Hartl, D. (1990) Amplification of flanking sequence by inverse PCR. In PCR Protocols. (M.A. Innis, D.H. Gelfand, J.J. Sninsky, and T.J. White, eds.) Academic Press, San Diego, California 219-227.

Pan, Q., Liu, Y.S., Budai-Hadrian, O., Sela, M., Carmel-Goren, L., Zamir, D. and Fluhr, R. (2000) Comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes

of two dicotyledons: tomato and A*rabidopsis*. Genetics 155, 309–322.

Parker, J.E. (2003). Plant Recognition of microbial patterns. Trends in Plant Science 8:245-247.

Parniske M, Hammond-Kosack KE, Golstein C, Thomas CM, Jones DA, Harrison K, Wulff BB, and Jones, J.D. (1997) Novel Disease Resistance Specificities Result from Sequence Exchange Between Tandemly Repeated Genes at the *Cf-4/9* Locus of Tomato. Cell 91: 821-832.

Peterhans A, Schlupmann H, Basse C, and Paszkowski, J. (1990) Intrachromosomal Recombination in Plants. EMBO Journal 9(11): 3437-3446.

Petes, T. D. (1980) Unequal Meiotic Recombination within Tandem Arrays of Yeast Ribosomal DNA Genes. Cell 19: 765-774.

Prändl, R., Hinderhofer, K., Eggers-Schumacher, G., and Schöffl, F. (1998) *HSF3*, a new heat shock factor from *Arabidopsis thaliana*, derepresses the heat shock response and confers thermotolerance when overexpressed in transgenic plants. Molecular General Genetics 258: 269-278.

Puchta, H. and Hohn, B. (1991) A Transient Assay in Plant Cells Reveals a Positive Correlation Between Extrachromosomal Recombination Rates and Length of Homologous Overlap. Nucl Acids Res. 19: 2693-2700.

Puchta, H., Dujon, B., and Hohn, B. (1993) Homologous recombination in plant cells is enhanced by in vivo induction of double·strand breaks into DNA by a site·specific endonuclease. Nucl. Acids Res. 21: 5034 5040.

Ramakrishna, W., Emberton, J., Ogden, M., SanMiguel., P. and Bennetzen, J.L. (2002) Structural Analysis of the Maize *Rp1* Complex Reveals Numerous Sites and Unexpected Mechanisms of Local Rearrangement. Plant Cell 14: 3213-3223.

Ren, T., Qu, F., and Morris, T.J. (2000) *HRT* Gene Function Requires Interaction between a NAC Protein and Viral Capsid Protein to Confer Resistance to Turnip Crinkle Virus. Plant Cell 12: 1917-1926.

Reynolds, A., Lundblad, V., Dorris, D., and Keaveney, M. (1997) Current Protocols in Molecular Biology. John Wiley & Sons, Inc. New York.

Richly, E., Kurth, J. and Leister, D. (2002) Mode of amplification and reorganization of resistance genes during recent *Arabidopsis thaliana* evolution. Mol. Biol. Evol. 19: 76–84.

Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W., and Staskawicz, B.J. (1996) Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato. Science 274: 2063-2065.

Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E., and Innes, R.W. (2003) Cleavage of *Arabidopsis PBS1* by a Bacterial Type III Effector. Science 301: 1230-1233.

Smith, S.M., Pryor, A.J. and Hulbert, S.H. (2004) Allelic and haplotypic diversity at the *Rp1* rust resistance locus of maize. Genetics, 167, 1939-1947.

Sturtevant, A. H. (1925) The Effects of Unequal Crossing Over at the *BAR* Locus in Drosophila. Genetics 10(117): 117-147.

Swoboda, P., Gal, S., Hohn, B., and Puchta, H. (1994) Intrachromosomal homologous recombination in whole plants. EMBO Journal, 13: 484-489.

Surplus, S.L., Jordan, B.R., Murphy, A.M., Carr, J.P., Thomas, B., and A.-H.-Mackerness, S. (1998) UV-B induced responses in *Arabidopsis thaliana*: role of salicylic acid and ROS in the regulation of transcripts and acidic PR proteins. Plant, Cell and Environ. 21: 685-694.

Szostak, J.W., Orr-Weaver, T.L., Rothstein, R.J., and Stahl, F.W. (1983) The double-strand-break repair model for recombination. Cell 33: 25-35.

Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A., and Cornelissen, B.J. (2002) The Tomato *R* gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. Plant Cell 14: 2929-2939.

Takahashi, H., Suzuki, M., Natsuaki, K., Shigyo, T., Hino, K., Teraoka, T., Hosokawa, D., and Ehara, Y. (2001) Mapping the virus and host genes involved in the resistance response in cucumber mosaic virus-infected *Arabidopsis thaliana*. Plant Cell Physiology 42: 340-347.

Takahashi, H., Miller, J., Nozaki, Y., Takeda, M., Shah, J., Hase, S., Ikegami, M., Ehara, Y., Dinesh-Kumar, S.P., and Sukamto. (2002) *RCY1*, an *Arabidopsis thaliana RPP8/HRT* family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism. Plant Journal 32: 655-657.

Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y., and Martin, G.B. (1996) Initiation of plant disease resistance by physical interaction of *AvrPto* and *Pto* kinase. Science 274: 2060-2063.

Thomas, M.B. (1999) Ecological approaches and the development of "truly integrated" pest management. PNAS 96: 5944-5951.

Tovar, J. and Lichtenstein, C. (1992) Somatic and meiotic chromosomal recombination between inverted duplications in transgenic tobacco plants. Plant Cell 4(3): 319-332.

Tuteja, N., Singh, M.B., Misra, M.K., Bhalla, P.L., and Tuteja, R. (2001) Molecular mechanisms of DNA damage and repair: progress in plants. Crit Rev Biochem Mol Biol. 36(4): 337-397.

Van der Biezen, E. A. and Jones, J.D.J. (1998) Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. 12: 454-456.

Yandeau-Nelson, M.D., Xia, Y., Li, J., Neuffer, M.G., and Schnable, P.S. (2006) Unequal sister chromatid and homolog recombination at a tandem duplication of the *A1* locus in maize. Genetics 173: 2211-2226.

Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., Yunfei, Z., Xiaoxiao, W., Xiaoming, Q., Yunping, S., Li, Z., Xiaohui, D., Jingchu, L., Xing-Wang, D., Zhangliang, C., Hongya, G., and Li-Jia, Q. (2006) The *MYB* transcription factor superfamily of *Arabidopsis*: expression analysis and phylogenetic comparison with the rice *MYB* family. Plant Molecular Biology 60: 107-124.

Zhao, Y., DelGrosso, L., Yigit, E., Dempsey, D.A., Klessig, D.F., and Wobbe, K.K. (2000) The amino terminus of the coat protein of turnip crinkle virus is the *AVR* factor recognized by resistant *Arabidopsis*. Molecular Plant-Microbe Interactions 13(9): 1015-1018.

Zhou, T., Wang, Y., Chen, J.Q., Araki, H., Jing, Z., Jiang, K., Shen, J. and Tian, D. (2004) Genome-wide identification of NBS genes in japonica rice reveals significant expansion of divergent non-TIR NBS-LRR genes. Mol. Genet. Genomics 271: 402–415.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D.J., Felix, G., and Boller, T. (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature 428: 764-767.

Zipfel, C. and Felix, G. (2005) Plants and animals: a different taste for microbes? Curr Opin Plant Biol 8(4): p. 353-60.

Volkov, R.A., Panchuk, I.I., and Schöffl, F. (2003) Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR. Journal of Experimental Botany 54(391): 2343-2349.

Vita

Stacey Ann Simon was born in Frederick, Maryland on December 28, 1979. She graduated from Delaware State University in 2001 with a Bachelor of Science degree in Biology. She entered Virginia Polytechnic Institute and State University in the fall of 2001.