INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700  800/521-0600
ISOLATION AND CHARACTERIZATION OF A RecA-LIKE GENE FROM SOYBEAN (GLYCINE MAX L)

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Masood Z. Hadi, M.S.

The Ohio State University
1996

Dissertation Committee
Dr. J.G. Streeter, Co-Advisor
Dr. J.J. Finer, Co-Advisor
Dr. L.M. Lagrimini
Dr. F.L. Schanbacher

Approved by

Dr. J.G. Streeter, Co-Advisor
Dr. J.J. Finer, Co-Advisor
Dr. L.M. Lagrimini
Dr. F.L. Schanbacher

Graduate Program in Agronomy
Copyright by
Masood Z. Hadi
1996
RecA protein plays a key role in prokaryotic recombination. It functions both in DNA repair and genetic recombination by catalyzing pairing and strand exchange. The RecA-like proteins DMC1 and Rad51 in yeast are required for both meiotic recombination and repair of double stand breaks; Rad51 is also required for mitotic recombination. The Lim15-like proteins of eukaryotes are also similar to RecA based on sequence similarity but, due to their ubiquitous expression, they might also be involved in functions other than recombination and repair. A soybean homolog of the *Lilium longiflorum* Lim15 gene was identified. A genomic clone was isolated from a soybean library using the lily Lim15 cDNA as a probe. This fragment was partially sequenced, and a region conserved in Lim15-like proteins was identified and used to assay for induction of the soybean Lim15-like cDNA in various tissue. A flower cDNA library was screened to isolate a cDNA. The primary sequence of the cDNA termed sblim15 was investigated by comparing the primary structure of the open reading frame to other recA-like genes. The protein exhibits strong sequence homology to the lily, Arabidopsis and mammalian Lim15-like proteins. The predicted protein of the sblim15 therefore belongs to a family of recA-like homologs that are conserved in diverse taxa. Southern hybridization analysis
indicates that the gene is present at very low copy number in soybean and other plants species. Northern hybridization analysis detected two polyadenylated transcripts in all tissue and a third transcript in field grown leaf tissue. Transcript levels are not enhanced in response to DNA damaging agents. All evidence suggests that 

To investigate the enhancement of somatic extrachromosomal recombination by constitutive over-expression of sblim15 and lily Lim15 in soybean embryogenic cultures, overlapping deletions of the β-glucuronidase (GUS) were constructed. Either of the deletions could not express GUS by themselves. Deletions were co-transformed with sblim15 or lily Lim15 into soybean somatic embryogenic cultures via particle bombardment. The resulting enhanced GUS activity over the controls would indicate that homologous recombination had taken place between the two plasmids due to the sblim15 or lily Lim15 proteins.

To investigate some of the limits and properties of co-transformation and endogenous extrachromosomal recombination, 12 different plasmids were co-transformed into embryogenic cultures of soybean via particle bombardment. The DNA used for co-transforming included 10 plasmids containing RFLP markers from maize and 2 plasmids separately encoding hygromycin resistance and β-glucuronidase. Southern hybridization analysis on 26 hygromycin resistant clones verified the presence of all of the introduced DNAs. There was no preferential uptake and integration of any of the plasmids. The copy number of individual
plasmids was approximately equal within clones but highly variable between clones. Concatemer formation indicative of homologous recombination was observed to a limited extent. Ligation of plasmid fragments was also occurring at a high rate.
Dedicated to my Ummijan and Babajan
ACKNOWLEDGMENT

I wish to thank my advisors, Dr. John J. Finer and Dr. John G. Streeter for their guidance throughout my graduate program, constant encouragement in my research and the opportunity to conduct my studies under their tutelage. I would especially like to thank Dr. John J. Finer for his ability to make me look at the brighter side of things no matter how bleak things were. I would also like to thank other members of my committee: Dr. Mark L. Lagrimini for his valuable suggestions, insights into molecular biology and opening his laboratory for my use; and Dr. Floyd L. Schanbacher for his invaluable comments and making me look at sequence data more carefully. Thanks also goes to Dr. Michael D. McMullen for his involvement in the early stages of this project.

I am especially thankful to Thomas Lanker for his help with sequencing and Mark Jones for the last box of plaque screen membranes.
VITA

June, 1963...............................................................Born in Sialkot, Pakistan


September, 1990- present.....................................Graduate Research Associate The Ohio State University.

PUBLICATIONS


FIELD OF STUDY

Major Field: Agronomy
Plant Molecular Biology and Biotechnology

Studies in Cell Biology  Dr. John J. Finer
Studies in Plant Physiology  Dr. John G. Streeter
Studies in Molecular Biology  Dr. L. Mark Lagrimini
Studies in Mammalian Cell Biology  Dr. Floyd L. Schanbacher
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>v</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>vi</td>
</tr>
<tr>
<td>Vita</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Chapter:</td>
<td></td>
</tr>
<tr>
<td>1. Transformation of 12 Different Plasmids into Soybean via Particle Bombardment</td>
<td>4</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>7</td>
</tr>
<tr>
<td>Results</td>
<td>10</td>
</tr>
<tr>
<td>Discussion</td>
<td>12</td>
</tr>
<tr>
<td>Bibliography</td>
<td>22</td>
</tr>
<tr>
<td>Abstract</td>
<td>25</td>
</tr>
<tr>
<td>Introduction</td>
<td>26</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>38</td>
</tr>
<tr>
<td>Results</td>
<td>42</td>
</tr>
<tr>
<td>Discussion</td>
<td>38</td>
</tr>
<tr>
<td>Bibliography</td>
<td>59</td>
</tr>
</tbody>
</table>
3. Isolation of a RecA-Like Gene from Soybean and its Characterization ................................................................. 67

   Abstract ................................................................................... 67
   Introduction ............................................................................... 69
   Material and Methods ............................................................. 90
   Results ....................................................................................... 96
   Discussion ............................................................................... 103
   Bibliography ............................................................................ 145

General Conclusions .......................................................................... 154
List of References .............................................................................. 156

Appendix A Protocols and Solutions .................................................. 174

Appendix B List of Abbreviations ..................................................... 199
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Plasmid copy number, GUS activity and intactness of GUS gene in transformed soybean tissue</td>
<td>21</td>
</tr>
<tr>
<td>2.1</td>
<td>Different combinations of plasmids transformed into soybean cultures</td>
<td>58</td>
</tr>
<tr>
<td>3.1</td>
<td>DNA homology calculated by Distances program for all recombinase like genes</td>
<td>141</td>
</tr>
<tr>
<td>3.2</td>
<td>DNA homology calculated by Distances program for Lim15-like genes</td>
<td>142</td>
</tr>
<tr>
<td>3.3</td>
<td>Protein homology calculated by Distances program for all recombinase-like genes</td>
<td>143</td>
</tr>
<tr>
<td>3.4</td>
<td>Protein homology calculated by Distances program for Lim15-like genes</td>
<td>144</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure

1.1 Restriction map of pUCGUS..............................................................17
1.2 Restriction map of pCIB709..............................................................18
1.3 Representative sample autoradiographs of southern hybridization analysis......................................................20
2.1 Restriction map of pUCGUS, the substrate for all the deletions...49
2.2 Deletion of the 3' end of pUCGUS....................................................50
2.3 Deletions of the 5' end of pUCGUS ...................................................51
2.4 Restriction map of lily Lim15 gene...................................................52
2.5 Restriction map of CaMV-NOS..........................................................53
2.6 Strategy for construction of Lim15 sense construct.......................54
2.7 Strategy for construction of Lim15 antisense construct...............55
2.8 Strategy for construction of sblim15 construct ..............................56
2.9 pUCGUS 5' and 3' deletions with the region of over-lapping homology.................................................................57
3.1 Epistasis groups of the yeast RAD genes.......................................113
3.2 Structure of the Lim15 gene and protein from lily........................114
3.3 Location of Exon13 in the soybean genomic fragment...............115
3.4 Sequence of the EcoR I/Spe I subclone...........................................116
3.5 Sequence of the partial cDNA isolated from flower library........117
3.6 Nucleotide sequence of the sblim15 cDNA.................................118
3.7 Pileup diagram of DNA sequences from all recombinase
like genes..............................................................................................119
3.8 Pileup diagram of DNA sequence for Lim15 genes.....................120
3.9 Growtree analysis of DNA sequence for all recombinase
like genes..............................................................................................121
3.10 Growtree analysis of DNA sequences for Lim15 like genes.....122
3.11 Deduced amino acid sequence of sblim15 gene.........................123
3.12 Pileup diagram of the deduced amino acid sequences for
all recombinase genes.........................................................................124
3.13 Pileup diagram of the deduced amino acid sequence for
Lim15 like genes..................................................................................125
3.14 Growtree analysis on the predicted protein sequence of
all recombinase like genes.................................................................126
3.15 Growtree analysis on the predicted protein sequence of
all recombinase like genes.................................................................127
3.16 Alignment of the deduced protein sequence from Lim15 like,
DMC1 and recA protein........................................................................128
3.17 High stringency southern hybridization analysis of soybean
DNA using sblim15 as a probe............................................................131
3.18 Low stringency southern hybridization analysis of soybean
DNA using sblim15 as a probe............................................................133
3.19 Southern hybridization analysis of DNA from various plants
hybridized to sblim15 as a probe..........................................................135
3.20 Northern hybridization analysis of sblim15 transcript.................137
3.21 Northern hybridization analysis of sblim15 transcript
after subjecting tissue to DNA damage agents...............................139
3.22 Predicted amino acid sequence of the smaller transcript based on data from other Lim15 genes.................................140
INTRODUCTION

Recombination is one of the most important factors in the creation of diversity in living beings. The basic principles of genetics were discovered in plant systems. Despite the paramount information contributed to the field of genetics from plant systems, experimental uses of homologous recombination in plants, such as gene targeting, a cornerstone of molecular genetics, are not yet sufficiently advanced to be practical. These manipulations are more routine in mammalian, lower eukaryotes and bacterial systems even though not much more is known about recombination in these systems either. Thus, several questions that need pondering arise: Do plants share a similar recombination machinery with other organisms? What role does homologous recombination play in the life cycle of plants and how is recombination regulated?

Recombination can be loosely divided into two types, homologous recombination and illegitimate recombination. Homologous recombination occurs when the recombining partners have some regions of sequence similarity between them, while illegitimate recombination is homology independent. Both types of recombination occur extrachromosomally, intrachromosomally or between extrachromosomal plasmid DNA and chromosomal DNA (gene...
targeting). A sizable amount of information about recombination substrates and about the products of recombination has accumulated over the past few years. It is the initiation step and the processing of these substrates that has eluded the scientific community. A survey of the models proposed for homologous recombination in other organisms suggests that the process of recombination is a tightly regulated and catalyzed by cellular enzymes (Holliday 1964, Lin et al., 1984, Smith 1988, Szostak et al., 1983).

A considerable effort was spearheaded in the late 1970's to isolate recombination enzymes from plants (Hotta and Sterns 1974, Hotta and Sterns 1978, Hotta et al., 1979, Hotta et al., 1987). The problem with simply purifying the enzymes is that even if enzyme activity is found in plants, it might not be involved in recombination, exonuclease, exonuclease and ligase-like activities are needed for other aspects of DNA metabolism as well. Another approach to characterizing recombination is through genetics, primarily to isolate mutants, and to show conclusively by ectopic expression of recombinase enzymes in these mutants by their respective gene/protein and its involvement in recombination. This approach has been underutilized in plants due to the dual function of recombination enzymes for both recombination and repair. The use of genes from prokaryote's organisms as probes to isolate similar genes from higher organisms is another approach that is being utilized to clone cDNA(s) for recombinase like genes. This approach has enabled isolation of a few cDNA(s) that are homologous to bacterial RecA protein (Pang et al., 1993, Cerutti et al.,
Over-expression of the main recombinase enzyme should enhance the rate of recombination in the plant cells (Shinohara et al., 1993, Reiss et al., 1996) thereby allowing manipulations of recombination frequencies.

In my dissertation work, I have studied three very different but related aspects of recombination in soybean. In chapter one, I examined various types of homologous and illegitimate recombination events that were occurring after co-transformation of twelve different plasmids as a single cocktail into soybean embryogenic cell lines (Hadi et al., 1996). In chapter two, the effects of simultaneous over-expression of a recombinase on the enhanced reconstitution of two overlapping deletions of the GUS reporter gene were studied. The last chapter describes the isolation and initial characterization of a cDNA from soybean that has very high homology to recombinase like-genes.
CHAPTER I
TRANSFORMATION OF 12 DIFFERENT PLASMIDS INTO
SOYBEAN VIA PARTICLE BOMBARDMENT

Abstract

Particle bombardment offers a simple method for the introduction of DNA into plant cells. Multiple DNA fragments may be introduced on a single plasmid or on separate plasmids (co-transformation). To investigate some of the properties and limits of co-transformation, 12 different plasmids were introduced into embryogenic suspension culture tissue of soybean [Glycine max (L.) Merrill] via particle bombardment. The DNAs used for co-transformation included 10 plasmids containing RFLP markers for maize and 2 plasmids separately encoding hygromycin-resistance and β-glucuronidase. Two weeks following bombardment with the 12 different plasmids, suspension culture tissue was placed under hygromycin selection. Hygromycin-resistant clones were isolated after an additional five to six weeks. Southern hybridization analysis of 26 hygromycin-resistant embryogenic clones verified the presence of introduced plasmid DNAs. All of the co-transforming plasmids were present in most of the transgenic soybean clones and there was no preferential uptake and integration of any of the plasmids. The copy number of individual plasmids was approximately equal within clones but highly variable between clones. While some clones contained as few as zero to three copies of each plasmid, other clones contained as many as ten to fifteen copies of each of the 12 different plasmids.
Introduction

Production of transgenic plants has become routine for many plant species, permitting modifications to basic metabolism and introduction of characteristics that could not be incorporated using conventional breeding techniques. Although different procedures exist for the introduction of foreign DNA, particle bombardment has become the method of choice for transformation of many plant tissues. With particle bombardment, the difficulties of using fragile protoplasts and host-range limitations associated with Agrobacterium can be avoided.

Although success in particle bombardment-mediated transformation of plants has increased tremendously in recent years, there is limited information available on the fate of introduced DNA. In order for the DNA to be integrated into the genome, the introduced DNA must be physically modified. These modifications are important in understanding the organization, integration and expression of the introduced DNA.

Southern hybridization analysis (Southern 1975) of DNA from stably transformed plants produced by naked DNA-mediated transformation reveals complex hybridization patterns (Bates et al., 1990). These hybridization patterns give clues as to the nature of plasmid DNA processing following introduction into the cell. Although integration at single sites in the genome is most common (Christou et al., 1989), multiple integration sites have also been observed (Potrykus et al., 1987). Southern hybridization analysis of DNA from transgenic tobacco obtained by electroporation of plant protoplasts (Riggs et al., 1986) and transgenic cotton and soybean (Finer et al., 1990, 1991) obtained via particle bombardment indicates that the introduced plasmid forms head-to-tail
concatemers. This suggests that the plasmids had undergone homologous recombination during the transformation process. In addition to homologous recombination, plasmid DNA(s) could also recombine via illegitimate recombination or, alternatively, the plasmid(s) could be linearized and ligated to other plasmid fragments during the integration process. The high efficiency of co-transformation and the observation of a genetically-linked integration of calf thymus DNA used as a carrier provides evidence for this type of recombination process in electroporated plant protoplasts (Peerbolte et al., 1985).

In order to gain an understanding of plasmid DNA integration and determine some of the limitations of particle bombardment in plants, 12 different plasmids (as a single cocktail) were introduced into embryogenic soybean tissues via particle bombardment. One of these plasmids contained a scorable β-glucuronidase gene, another contained a selectable hygromycin-resistance gene and ten other plasmids contained different RFLP markers from maize. Southern hybridization analyses of stably transformed tissue revealed the following: 1) All 12 of the plasmids could be taken up and incorporated, 2) There was no preferential uptake or integration of any one of the plasmids, 3) Plasmid amplification may have occurred in some clones, 4) Concatemer formation, indicative of homologous recombination, was observed to a limited extent 5) Ligation of plasmid fragments was also occurring at a high rate.
Materials and Methods

Plasmid DNAs

The plasmid pUCGUS (Fig. 1.1) (Finer et al., 1990), which encodes β-glucuronidase (GUS) and pCIB 709 (Fig. 1.2) (Rothstein 1987), which encodes resistance to the antibiotic hygromycin-B were used as the scorable and selectable marker genes respectively. The 5’ regulatory element for both genes was the CaMV35S promoter. Plasmids bnl14.28, and umc 29, 34, 38, 39, 82, 84, 107, 115, and 119 (obtained from David Hoisington, University of Missouri, Columbia, MO) contained RFLP markers for maize. Plasmid DNAs were prepared using the alkaline lysis method (Appendix 14) and were purified by centrifugation in cesium chloride-ethidium bromide gradients as described by Sambrook et al., (1975).

Soybean Transformation

Embryogenic suspension cultures of soybean [Glycine max (L.) Merrill.] cv 'Fayette' were initiated and maintained as described previously (Finer et al., 1988). Cultures were bombarded according to Finer et al., (1991). Briefly, 1 g of embryogenic suspension culture was transferred to a 3.5 cm Petri dish. The excess medium was removed with a pipet tip and the tissue was allowed to air dry in a laminar flow hood for ten to fifteen minutes. Immediately prior to bombardment, the tissue was covered with a 500 μm pore size nylon screen (Tetko Inc. Elmsford, N.Y.).

The twelve plasmids were mixed together in equal amounts (equal mass) to obtain a final total DNA concentration of 1 μg/μl in TE. As the plasmids were 4-5 kb, equal mass was closely equivalent to equimolar. Plasmid DNAs were
precipitated onto tungsten M10 particles (1.1 μm) using a calcium chloride precipitation method (Appendix 15) (Finer et al., 1990). Each sample was bombarded once using the DuPont Biolistics™ Particle Delivery System (model BPG).

Selection for Transformed Tissues

One to 2 hr following bombardment, embryogenic tissue was resuspended in FN maintenance medium (Finer and Nagasawa 1988). After 2 weeks, embryogenic tissue was then transferred to fresh FN medium containing 50 μg/ml hygromycin-B (Calbiochem, LaJolla, CA). Tissues were placed in fresh hygromycin-containing maintenance medium every week for 3 additional weeks.

Six to eight weeks post bombardment, brown clumps of nontransformed embryogenic tissue containing green lobes of hygromycin-resistant tissue were removed and separately cultured in fresh hygromycin-containing maintenance medium. Tissues were periodically removed from these cultures for histochemical GUS assays (Jefferson 1987), DNA extraction and Southern hybridization analysis (Southern 1975).

Molecular Analyses

DNA was extracted from transgenic embryogenic tissue using the CTAB procedure (Appendix 1) (Saghei-Maroof et al., 1984). DNA concentration was determined using a TKO 100 minifluorometer (Labarca et al., 1980) and equal amounts of genomic DNA were digested with the appropriate restriction enzymes. DNAs were then electrophoresed through either a 0.4% or 0.8% agarose gel following digestion with either one or two restriction enzymes
respectively. The gel was treated and the DNA was transferred to nylon membranes as described previously (Finer et al., 1991).

DNA probes consisted of the one kb coding region of the hygromycin-resistance gene, the coding region for GUS and the maize DNA inserts from the bnl and umc clones. DNAs were random primer labeled (Appendix 3 and 4) (Feinberg et al., 1983) and hybridized with membranes containing soybean genomic DNA for 24 to 48 hours. Following hybridization, membranes were washed with 2X SSC/0.1% SDS and then with 0.1X SSC/0.1% SDS at 65°C (Appendix 5). Membranes were then placed on Kodak XAR-5 film with intensifying screens at -70°C to visualize the hybridization pattern.
Results

Over 75 soybean clones that grew in the presence of 50 µg/ml of hygromycin-B were obtained. Twenty six of these clones were selected at random for further analysis; the remainder were discarded due to the large efforts required to separately maintain this amount of tissue. Southern hybridization analysis of Hind III-digested DNA from these transgenic clones (Hind III recognizes only one site on each of the 12 introduced plasmids, the site was not present within the DNA fragments used as probes) revealed unique and complex banding patterns (Fig. 1.3). DNA from all of the clones hybridized with the coding region for hygromycin-resistance gene indicating efficient selection for transformed tissue. Hybridization of the maize RFLP markers to Hind III-digested maize leaf tissue DNA resulted in 1 to 4 single copy fragments while there was no signal from hybridization of those RFLP probes with genomic DNA from non-transformed soybean tissue. A hybridization signal was present at unit-plasmid-length (Fig. 1.3, arrows) in some of the clones.

The copy number of the introduced plasmids was estimated based on the number of bands on the autoradiographs (Table 1.1). Copy number was determined from the original film as some signal definition was lost in the photographic copies (Fig. 1.3). The copy number for individual plasmids varied greatly from zero in some clones to sixteen for plasmid umc 38 in clone 30. All of the clones contained at least some introduced plasmid DNA. Clones 32, 33, 35 and 50 were low copy number clones and contained an average of 2 or fewer copies of each plasmid. Clones 9, 24, 57, 59, 63 and 64 were high copy number clones and these clones contained an average of 10 or more copies of each introduced plasmid. Most of the clones that contained high copies of one plasmid had high copy numbers for all other plasmids, while the low copy
number clones generally had low copy numbers for all plasmids. Four clones (Clones 8, 12, 30 and 65) that varied more widely in copy number of introduced plasmid DNAs, were variable copy clones, while the remaining clones were intermediate copy number clones.

All the clones were tested for GUS activity once at eight weeks after transformation and again at sixteen weeks after transformation. Only six of the 26 clones showed GUS activity at both time points (Table 1.1). Four of the clones that were GUS-positive at the eight week period tested negative at the sixteen week test. However, all clones that were initially GUS-negative remained GUS-negative at the sixteen week point. Southern hybridization analysis indicated the presence of an apparently intact GUS expression unit in all but one clone that at some point expressed GUS (Table 1.1). In addition, some of the clones that never expressed GUS also contained the proper size restriction length fragment associated with the intact GUS expression unit.
**Discussion**

The unique hybridization patterns (Fig. 1.3) displayed by all of the clones indicated that the clones were unique. Each clone, derived from a single transformed cell (Finer et al., 1991), was recovered prior to clone duplication from the original piece of transformed tissue. The timing of clone selection from the original bombarded tissue was important in the recovery of non-duplicated clones.

The observation of unit plasmid length hybridization signals (Fig. 1.3, arrows, e.g. Clone 50) gives some evidence for concatenation of the introduced plasmid DNAs. Clearly, fragments other than unit plasmid length were more common than unit length. None of the clones displayed the hybridization pattern which would result primarily or exclusively from the formation of head-to-tail concatemers. Although plasmid concatenation, which is indicative of homologous recombination either before or after integration (Folgers et al., 1982) was observed, most of the recombination events (as indicated by the complex banding pattern) appear to be illegitimate. Homologous recombination between plasmids can only occur if a region of homology exists between those plasmids. Since all of the plasmids used in this study were pUC derived, they contain approximately 2680 bp of common sequence, where homologous recombination could take place. Plasmid concatenation has been observed with electroporated tobacco cells (Riggs et al., 1986) and bombarded cotton and soybean embryogenic cultures (Finer et al., 1990, 1991). In these studies, as well as most others involving DNA introductions, only one or two different plasmids are typically used. Homologous recombination between identical plasmids may be more common than with similar plasmids. The use of 12
different plasmids, as reported here, makes analysis of recombination events more difficult but additional information can be obtained.

The complex hybridization patterns observed in all of the clones indicate that the plasmids primarily recombined with each other at random. There was low variability in plasmid copy number within clones but higher variability among clones. Six clones were high copy number clones while 4 were low copy number clones. Four clones (Clones 8, 12, 30 and 65) displayed more variable copy numbers while the majority of clones were intermediate and more consistent in plasmid copy number. The consistency of plasmid copy number indicates that there was no preferential uptake of any of the plasmids. The presence of intense hybridization signals at positions other than unit-length indicates that plasmid DNA was amplified in some clones. Amplification of introduced plasmid DNAs was observed in clones 9, 24, 34, 49, and 63. Amplification of plasmid DNA results in either partial or full amplification of the hybridization signal. This is noticeable as some of the hybridization signals are intense while others are either not present or are present at low intensity. Amplification of resistance genes in response to both antibiotic (Czernilofsky et al., 1986) and herbicide (Wang et al., 1991) selection has been reported. Selection of transformed clones using high levels of hygromycin-B may have contributed to amplification of DNAs in some of the clones.

When the copy number and size of the introduced plasmids are considered, approximately 600 kb of plasmid DNA was introduced into the soybean genome for high copy clones. The amount of introduced plasmid DNA in this report is considerably higher than reported earlier for other bombardment and naked DNA-mediated transformation systems. Around three to five plasmid copies were introduced with a liposome mediated transfer to protoplasts
Transgenic soybean obtained via particle bombardment contained approximately five copies of a single plasmid (McCabe et al., 1988). While in transgenic tobacco obtained via microinjection, some cell lines contained less than one copy of the plasmid (Crossway et al., 1980). The large amount of introduced DNA in this report may result from either the use of a large number of different plasmids or an enhanced "transformation competence" (the ability to incorporate foreign DNA) of these soybean cells.

In general, particle bombardment results in low numbers of integration events in soybean (Christou et al., 1989). The number of integration events (separate sites of integration) was not determined in the present study. Although plants were recovered from some of the cultures obtained in this study (unpublished), they showed severely reduced fertility and progeny analysis could not be used to determine the number of integration events. The reduced fertility was probably the result of the extended cell culture period and was not related to the introduction of DNA. Plants recovered from non transformed cultures showed the same phenotype as the transformed plants.

The presence of the intact GUS coding unit did not correlate with the expression of the GUS gene. Of the 13 clones that contained the intact expression unit for GUS, 4 clones never showed histochemical GUS activity and 4 clones displayed GUS activity only after 8 weeks (and not at 16 weeks). GUS assays were performed on embryogenic cultures that were fairly uniform morphologically and were grown under identical conditions. In clones that contained the intact expression unit but did not stably express GUS, there was no relationship between copy number and expression. Co-suppression, which is a suppression of gene expression resulting from introduction of multiple copies of the same gene (Napoli et al., 1990) was not clearly observed in this study.
Co-suppression has been suggested to occur in transformed soybean tissue (Finer et al., 1991), where multiple copies of the intact GUS gene were present but the enzyme was not expressed. The tandemization of DNA giving rise to more than one promoter per integration site could lead to transcriptional interference (Czernilofsky et al., 1986) resulting in absence or lack of GUS activity.

In most cases of naked DNA-mediated transformations, only one or two different plasmid DNAs are utilized. Multiple copy integrations, resulting from the introduction of one or two different plasmids, can lead to formation of large concatemers, where the plasmid DNAs can lie directly adjacent to one another. The proximity of these introduced plasmid DNAs to one another or their position within the genomic DNA may influence expression of the genes carried on this DNA (position effect). In the case of multiple plasmid co-transformations, the introduced plasmid DNAs are separated by each other, thus creating a type of "buffer" region for gene expression. If the introduced genes are adjacent to or associated with regions of introduced DNA that do not reduce or possibly enhance gene expression, it should be possible to modify or stabilize transgene expression thereby reducing position effect. The use of RFLP markers, which are typically low copy number and unmethylated, for co-transformation experiments may actually enhance gene expression, transformation efficiencies and serve as an additional tool for detecting the introduced DNA in the progeny. Scaffold Attachment Regions (Breyne et al., 1992) and Transformation Booster Sequences (TBS) (Meyers et al., 1988), which enhance transformation rates and gene expression, may act in somewhat similar ways.

Studies of co-transformation can provide much information on the process of recombination. Co-transformation can also be used to introduce large
numbers of genes on different plasmids without the labor intensive and inefficient process of repeated transformations. If these plasmids recombine to form large chains of mixed plasmid DNAs, multiple co-transformations may also be useful for construction of subchromosomal regions where the genes of interest could be flanked by expanses of defined DNAs.
Fig. 1.1 Restriction map of pUCGUS. The GUS gene is being driven by the 35S promoter and has the NOS terminator sequence. pUCGUS was used as the scorable marker.
Fig 1.2 Restriction map of pCIB709. The antibiotic resistance gene for hygromycin-B is being driven by the 35S promoter and the NOS polyA signal. pCIB709 was used to select transformed tissue.
Figure 1.3. Representative samples of autoradiographs of Southern hybridization analysis. *Hind* III-digested genomic DNA (10 µg) from non-transformed soybean (wtsb), corn, and 26 transformed soybean clones hybridized with DNAs from umc34 (top panel), umc38 (middle panel) and pUCGUS (bottom panel). The arrows designate unit-length of the plasmid DNA which contained the region being used as a probe. The "+" sign designates clear unit-length fragments for Clone 50.
<table>
<thead>
<tr>
<th>Clone Number</th>
<th>UMC 29</th>
<th>UMC 34</th>
<th>UMC 38</th>
<th>UMC 39</th>
<th>UMC 82</th>
<th>UMC 84</th>
<th>UMC 107</th>
<th>UMC 115</th>
<th>UMC 119</th>
<th>BNL 14.28 pCIB</th>
<th>pUC GUS</th>
<th>GUS Activity 8 wks</th>
<th>16 wks</th>
<th>Intact GUS</th>
</tr>
</thead>
<tbody>
<tr>
<td>08</td>
<td>3</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>11</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>09</td>
<td>11</td>
<td>11</td>
<td>14</td>
<td>11</td>
<td>13</td>
<td>14</td>
<td>11</td>
<td>10</td>
<td>14</td>
<td>13</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>6</td>
<td>9</td>
<td>0</td>
<td>5</td>
<td>9</td>
<td>10</td>
<td>5</td>
<td>12</td>
<td>5</td>
<td>4</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>012</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>7</td>
<td>10</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>9</td>
<td>5</td>
<td>9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>8</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td>9</td>
<td>3</td>
<td>4</td>
<td>9</td>
<td>5</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>024</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>5</td>
<td>8</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>030</td>
<td>7</td>
<td>7</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>13</td>
<td>7</td>
<td>14</td>
<td>8</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>032</td>
<td>4</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>033</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>7</td>
<td>5</td>
<td>6</td>
<td>4</td>
<td>6</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>9</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>5</td>
<td>7</td>
<td>3</td>
<td>9</td>
<td>6</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>035</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>5</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>46</td>
<td>13</td>
<td>11</td>
<td>10</td>
<td>9</td>
<td>4</td>
<td>10</td>
<td>5</td>
<td>9</td>
<td>11</td>
<td>9</td>
<td>8</td>
<td>2</td>
<td>-</td>
<td>Y</td>
</tr>
<tr>
<td>049</td>
<td>4</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>6</td>
<td>12</td>
<td>7</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>5</td>
<td>4</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>050</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>55</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>057</td>
<td>10</td>
<td>13</td>
<td>12</td>
<td>12</td>
<td>13</td>
<td>12</td>
<td>9</td>
<td>12</td>
<td>11</td>
<td>15</td>
<td>10</td>
<td>13</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>059</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>9</td>
<td>11</td>
<td>11</td>
<td>12</td>
<td>13</td>
<td>13</td>
<td>13</td>
<td>7</td>
<td>7</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>62</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>063</td>
<td>10</td>
<td>8</td>
<td>6</td>
<td>12</td>
<td>13</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>11</td>
<td>13</td>
<td>13</td>
<td>7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>064</td>
<td>8</td>
<td>8</td>
<td>10</td>
<td>12</td>
<td>8</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>065</td>
<td>3</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>4</td>
<td>11</td>
<td>4</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>4</td>
<td>6</td>
<td>+</td>
<td>Y</td>
</tr>
</tbody>
</table>

Table 1.1. Plasmid copy number, GUS activity and Intactness of the GUS gene in transformed soybean tissue ( indicates a high copy number clone; indicates a low copy number clone; indicates a variable copy number clone and clones not labeled are intermediate copy number).
Bibliography


CHAPTER 2
ECTOPIC EXPRESSION OF A LILY RECOMBINASE IN
SOYBEAN SOMATIC EMBRYOS AND ITS EFFECTS ON
RECOMBINATION RATES

Abstract

An extrachromosomal recombination assay was used to evaluate the effects of overexpression of a plant recombinase on homologous recombination in soybean. To achieve this goal, a lily recombinase (Iilim15) was co-transformed with two other plasmids containing overlapping deletions of the β-glucuronidase (GUS) gene into somatic embryos of soybean (Glycine max L.) via particle bombardment. The experimental strategy relied on the supposition that, two separate DNA molecules (neither of which could express GUS independently) would recombine to produce a functional recombinant molecule. Constitutive expression of a recombinase would further enhance the recombination rates. Recombination events were scored by the appearance of GUS expressing foci. Unfortunately, GUS expression from reconstitution of the complementary deletions of GUS gene was not observed.
Introduction

Since the first reports on recovery of transgenic plants (Horsch et al., 1985, Fraley et al., 1987 and Schell 1987), genetic transformation techniques for plant cells have become fairly routine for many plants. The discovery that *Agrobacterium tumefaciens* could transfer some of its genes to plant cells was the catalyst for studies in transformation and plant molecular biology. Construction of hybrid selectable markers and the establishment of reliable selection systems for the introduced gene(s) has allowed continued rapid progress in this area (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983a and Herrera-Estrella et al., 1983b). *Agrobacterium* is a plant pathogen which transfers its DNA to host cells after invading the host. As a pathogen, its host range is limited and it can invoke a wound response. Due to these and other limitations of gene transfer using *Agrobacterium*, naked DNA transfer systems have become more feasible for transformation of some plants. *Agrobacterium*-mediated and naked DNA-mediated DNA delivery systems are very different (Potrykus 1990a and Potrykus 1990b) and it is important to consider these differences before the most suitable transformation approach is selected.

Some of the methods available for naked DNA delivery are macroinjection (de la Pena et al., 1987), microinjection (Mathias 1987; Toyoda et al., 1990), pollen and embryo imbibition (Hess 1987; Topfer et al., 1990, Topfer et al., 1989), electroporation of intact tissue (Dekeyser et al., 1990), laser micro beam injection (Weber et al., 1990), pollen tube pathway (Lou and Wu 1988), agroinfection (Grimsley et al., 1988; Grimsley et al., 1987), and particle bombardment, also referred to as the biolistic process (Sanford 1988; Klein et al., 1989). Of all the methods available for direct DNA delivery, the biolistic
process has been the most reproducible and successful in recovering transgenic crop plants. This technique allowed the recovery of the first fertile transgenic maize plants (Fromm et al., 1990; Gordan-Kamm et al., 1990). Transient gene expression and stable transformation has been reported in wheat (Vasil et al., 1992; Lonsdale et al., 1990; Wang et al., 1988; Oard et al., 1990 and Daniell et al., 1991), barley (Creissen et al., 1990; Mendel et al., 1989) and rice (Christou et al., 1991; Oard et al., 1990; Wang et al., 1988). Biolistics has also been effectively used to transform plants that are also responsive to Agrobacterium (Populus, McCown et al., 1991; cotton, Finer and McMullen 1990; papaya, Fitzh et al., 1990; soybean, Finer and McMullen 1991; McCabe et al., 1988; Christou et al., 1989).

Following introduction of naked DNA into plant cells, expression of that DNA can be rapidly evaluated. These “transient assays” are routinely used to access gene function. Transient assays can be performed as early as four hours after the introduction of DNA which suggests that the introduced gene can be expressed prior to integration into the chromosomal DNA. Once DNA becomes integrated, stable transformation is achieved. There must be factors in the target plant cells that determine the fate of the introduced DNA.

In order to determine the fate of foreign DNA in plant cells, protoplast systems have been the best characterized primarily because of the high efficiency of DNA introduction. However, protoplasts are difficult to manipulate and regeneration of plants from protoplasts of most crop plants is extremely difficult. Regardless of whether protoplast or particle bombardment is utilized for DNA introduction, the fate of the DNA is similar. The DNA penetrates the cell wall/membrane (depending on the technique used) travels through the cytoplasm, penetrates the nuclear envelope and integrates into the
chromosomal DNA. This could not be a chance journey alone. There are several cellular factors involved in this journey. DNA introduced into the plant cells appears to integrate into the chromosomal DNA essentially at random. In mammalian systems, this "illegitimate" recombination occurs 100-100,000 times more often than homologous recombination. In plants, when a protoplast system is used for transformation, up to 20 copies of the introduced gene can integrate at a single locus in the genome (Potrykus et al., 1985). With particle bombardment, integration into chromosomal DNA is usually random and from 1 to 50 copies of the plasmid DNA can integrate into the same site (Hadi et al., 1996). If the process of foreign DNA integration into the genome could be controlled, this would provide us with a valuable tool for studying and controlling gene regulation. To achieve this goal, it is important to gain some basic knowledge of the mechanism of recombination.

Genomes are constantly recombining in time and space. Recombination and DNA repair provide both the stability required for inheritance and the flexibility required for change. In sexually reproducing diploid organisms, homologous chromosomes segregate independently of each other at meiosis giving rise to different combinations of genes in the gametes. When male and female gametes fuse, an individual is formed that has a genome different from most if not all other members of the species.

In general, there are two mechanisms that facilitate genetic diversity. These are mutations and recombination. Recombination can be site specific, transpositional, illegitimate and homologous. Genetic recombination can result in the alteration of gene expression, the generation of genetic diversity between the cells in a single individual or the repair of DNA damage.
Site specific recombination occurs when a protein binds to a recognition site and catalyzes exchange to another site recognized by the same protein. Sequence homology can exist for site specific recombination but the basis of recognition is not DNA sequences alone. Protein-DNA and protein-protein interactions are crucial and sequence homology is not absolutely required.

Transpositional recombination is similar to site specific recombination because there is protein-DNA and protein-protein interaction but only one of the recombining partners is specifically recognized by the protein that catalyzes the transposition. The protein recognizes the ends of the element accurately and then interacts with a relatively undefined target site.

Illegitimate recombination can be subdivided into end joining and strand slippage. End joining occurs when double strand breaks in introduced DNA take place and ligate together; their joining does not require any homology. In both cases, there is little or no homology between the recombining partners.

Homologous recombination occurs when DNA sequence homology is used for the recognition of recombining partners. Proteins such as RecA may facilitate this recognition but it is the base pair sequence that provides the specificity of recognition.

Homologous recombination has been most thoroughly studied in prokaryotes (Radding 1982, Smith 1988) and in lower eukaryotes such as yeast (Petes et al., 1991) for which both classical and molecular genetics are most advanced. These studies gave rise to the various models of recombination (Melson and Radding 1975, Szostak et al., 1983). Recombination between genes residing on different chromosomes and plasmids can be enhanced as much as 1000 fold by the induction of double stranded breaks in the region of homology (Orr-Weaver et al 1981). This observation gave rise to the double
strand break repair model for recombination in yeast (Szostak et al., 1983). The double strand break repair model states that a double stranded break starts the process of homologous recombination. The break is enlarged by cellular nuclease and repaired from the homologous sequence of the partner plasmid DNA. This leads to a double stranded crossover intermediate. This crossover can be resolved in two ways. Either the flanking markers remain on the same DNA or they are exchanged. This is a conservative process and both molecules are restored. Presentation of this model led to extensive studies of homologous recombination in higher eukaryotes using the following three different approaches: extrachromosomal recombination between transfected DNA molecules (Anderson and Eliason 1986), chromosomal recombination between repeated genes stably integrated in the genome (Orr-Weaver et al., 1981) and recombination between introduced DNA molecules and homologous sequences in the chromosome (gene targeting) (Rossant and Joyner 1989).

Extrachromosomal homologous recombination has been extensively studied in mammalian systems (Anderson et al., 1986, Kucherlapati et al., 1984). It usually involves the monitoring of the reconstitution of a gene encoding a selectable marker through recombination between two complementary defective sequences. The two most commonly used genes for extrachromosomal homologous recombination studies have been the herpes simplex virus thymidine kinase gene (Brenner et al., 1985; Lin et al., 1984; Shapira et al., 1983; Small and Scangos 1983) and the neomycin resistance gene from bacteria (Ayares et al., 1986; Folger et al., 1985). Using these genes, it was demonstrated that the rate of extrachromosomal recombination in mammalian systems was dependent on the physical state (topology) of the plasmid and the extent of homology between the two defective, complementary plasmids (Ayares
et al., 1986). Double stranded breaks in one or both of the plasmid dramatically enhanced the rate at which recombinants were recovered (Brenner et al., 1985; Lin et al., 1984; Anderson and Eliason 1986; Kucherlapati et al., 1984; Seidman 1987; Wake et al., 1985).

Extrachromosomal homologous recombination has not been as extensively investigated in plant systems as in mammalian systems. Bauer et al., (1990) transformed Nicotiana tabacum protoplast with plasmids containing overlapping regions of the neomycin phosphotransferase II genes and showed that, when one or two of the plasmids were linearized, the reconstitution of the gene increased as the length of overlapping homology increased from 6 to 405 bp. Their experimental results were dependent not only on the initial recombination event but also on the subsequent stable integration into chromosomal DNA and expression of the antibiotic resistance gene (position effect). Such effects could be avoided by using the transient assay system developed by Wong and Capecchi (1986) for rat fibroblast. Puchta and Hohn, (1991) using a modification of this system, demonstrated that the mechanism of extrachromosomal recombination in plant cells was similar to the "single strand annealing method" This model requires a double stranded break in each of the DNA molecule. The double stranded breaks are the entry points for single stranded exonucleases which digest one strand. The resulting single stranded overhangs of the recombining partners are complementary. The single stranded ends can also be generated by a helicase. The two strands base pair and the intermediate structure is repaired using the ends of the double stranded DNA as a primer. As both recombining partners suffer degradation of the DNA ends which are not involved in the exchange, this is a non-conservative process (Lin et al., 1984 and Ayares et al., 1986). The plant single strand annealing reaction
differed from that of the animal cell because the repair of double strand breaks in homologous DNA fragments did not seem to be as efficient in plant cells as in animal cells (Lin et al., 1990). Puchta and Hohn (1991), using tobacco protoplasts, concluded that in a single recombination event, flanking markers were exchanged more often than conserved. Double crossover events occurred two to three times less frequently compared to events where an exchange of flanking marker occurs. Therefore, the double strand repair model involving resolution of double Holliday junctions (Holliday 1964) is not the appropriate model to describe extrachromosomal recombination in *N. plumbaginifolia* cells. These results were consistent with an earlier study by Bauer et al., (1990) who put forth the hypothesis that extrachromosomal recombination was not occurring by a resolution of double Holliday junctions.

Studies on intrachromosomal recombination in cultured cells is of primary importance for three reasons. First, this information would be of value in furthering our understanding of recombination events. Second, the information obtained could eventually be used for gene targeting which could lead to studies on replacement of either mutant or wild type genes as well as correction of defective inherited genes. Gene targeting has been observed in *Arabidopsis thaliana* but at a very low frequency (Ursula et al., 1992).

The single stranded annealing model may be appropriate for extrachromosomal recombination in higher eukaryotes but does not explain intrachromosomal recombination. In the case of intrachromosomal recombination, this process would result in the loss of a chromosome and be lethal to the cell. Because of this loss and data from Liskay et al., (1987), the Holliday model (Holliday 1964) (double strand gap repair model) is accepted as the model for intrachromosomal recombination.
To study intrachromosomal recombination in mammalian cells, plasmids are introduced that have mutations at two different places in the herpes thymidine kinase (H-TK) gene as well as a dominant selectable marker that facilitates introduction and stable integration of the mutant gene duplication into host cell. The recombining plasmid is incapable of autonomous replication and maintenance in the host cell. The recombination substrate is an artificially constructed gene duplication that consists of two different mutants of the HTK gene and resides in the chromosome. Recombination events are detected by growth of the cells in a selective medium. It was found that recombination events are random. Intra-chromosomal recombination events are the results of either a single reciprocal exchange (crossover) or a non-reciprocal exchange (gene conversion). The recombination rate decreases linearly when the length of homology is decreased from 1.8 kb to 300 bp after which, a sharp decrease in recombination is seen. Liskay et al., (1986) found that a minimum homology of 300 bp was required for efficient recombination.

The most commonly used markers for intrachromosomal recombination studies in plant cells are the Cauliflower mosaic virus (CaMV) and the npt II gene from the bacterial transposon TN5. CaMV is an 8 kb double stranded DNA plant virus (Gronenbern 1987). A mixture of CaMV viruses, defective at different places are introduced into plants. Viable virus could only be produced after recombination of the defective viruses with each other in the plant cell. This system has distinct advantages. Recombination event can be visually scored by the appearance of symptoms due to virus infection. Even a single recombination event which gives rise to a fully functional virus can be scored, therefore making the detection of recombinant molecules very sensitive.
The \textit{npt II} gene, from the bacterial transposon Tn 5, is routinely used as a dominant selectable marker in plant cell transformation (Paskowski et al., 1984). Different deletion mutations within the \textit{npt II} gene are created; where either the 3' end or the 5' end of the gene are oriented as direct repeats with respect to each other and are separated by the hygromycin phosphotransferase (hpt) gene (Van der Elzen et al., 1985). These deletions are introduced into the plant cells and the hygromycin resistance is used to select transformed cells. Recombination between the \textit{npt II} gene fragments results in the loss of the fragment containing the hpt gene and a recombined functional reconstitution of the \textit{npt II} gene. Using this assay, Peterhans et al., (1990) showed that 53 bp of homology between the deletion derivatives is sufficient to allow intrachromosomal recombination events with a frequency of about $10^{-6}$.

Although extrachromosomal and intrachromosomal recombination are different processes, they have many things in common. To start, the first step in both cases is the introduction of double stranded breaks, degradation of the DNA and annealing of homologous DNA. Intrachromosomal recombination in yeast and extrachromosomal recombination in mammals are both enhanced in actively transcribing genes, probably due to the unwinding of DNA and removal of nucleosomes (Thomas and Rothstein 1989) during transcription. This makes the DNA more accessible to endonuclease and exonuclease action for generation of double stranded and or single stranded nicks (Nickoloff and Raymond 1990). The next few steps that occur during recombination are also very similar. The same cellular factors are probably involved during intra- and extrachromosomal recombination. In addition, terminal events such as repair of gaps and ligation of nicks are directed by similar enzymes in extra- and intrachromosomal recombination. The reason that extrachromosomal
recombination rates are higher than intrachromosomal recombination rates could be that plasmid DNAs are present in higher numbers and it is easier for proteins of the recombination apparatus to interact with plasmid DNA than with chromosomal DNA. Because chromosomal DNA is bound to the nuclear scaffold, it is simply not as accessible as the plasmid DNA and has more histones than the newly introduced plasmid DNA.

Proper analysis of genomic homologous recombination in plants is hampered by the lack of well characterized and easily scoreable markers. Extrachromosomal recombination studies in plants using introduced plasmid DNAs are more common than intrachromosomal recombination studies due to the ease of experimental design and analysis (Puchta and Hohn 1991). A transient extrachromosomal recombination assay requires two days whereas an intrachromosomal recombination assay takes at least two plant generations (Gal et al., 1991; Peterhans et al., 1990). Although the total picture cannot be drawn without the data from intrachromosomal recombination studies, insight into the recombination machinery of plant cells can be gained using extrachromosomal recombination as a model.

Although mitotic and meiotic recombination in the plant cell are the driving force in plant breeding, the study of molecular basis of recombination is still in its infancy. Advances in direct gene transfer techniques allows transformation of DNA into plants to be performed easily and routinely. These advances in DNA transfer technology provides a vehicle to start asking and addressing questions concerning molecular aspects of homologous recombination in plants. Genetic and molecular analysis of transgenic plants from many different plant species indicates that the integrating DNA does not require any homology to the plant genome for successful integration to occur. The DNA appears to integrate
completely at random with respect to copy number and location. As many as twenty copies of the introduced gene are seen integrated at a single locus. These random integration events are seen irrespective of the method of DNA introduction. It would be desirable to be able to control the process of foreign DNA integration by targeting DNA to predetermined locations in the genome. Gene targeting would provide a valuable method for studies on function and regulation of cellular genes. To achieve such a goal, it is essential to broaden our basic knowledge of the mechanism of homologous recombination.

Little is known about the molecular aspects of DNA recombination in plants. Reconstitution of infectious virus particles from co-infection of 2 non-infectious but complementary viral DNA(s) of Cauliflower mosaic virus (CaMV) resulted in recombinant infectious viral particles (Leubeurier et al., 1982), this suggested very early that extrachromosomal recombination does occur in plants cells. The restoration of the gene function conferring resistance to kanamycin following introduction of plasmid constructions containing incomplete but overlapping regions of the selectable neomycin phosphotransferase marker gene (*nptII*) has been used as an assay for recombination (Bauer et al., 1990, Offringer et al., 1990). The marker gene β-glucuronidase (GUS) has also been used by Holger and Puchta (1991) and Lyznick et al., (1991) but all of these studies introduced recombination substrates into plant cells by either PEG-mediated, electroporation or *Agrobacterium*. While PEG-mediated transformation is a gentle method for introduction of foreign DNA, electroporation is a much harsher procedure. The discharge of electric current has been reported to cause double stranded breaks in DNA. Although disrupted DNA would be an ideal substrate for recombination, electroporation induced double strand breaks make interpretations of results complicated. Particle
bombardment has not been evaluated for delivery of extrachromosomal homologous recombination substrate into plant cells. Almost nothing is known about the proteins involved in extrachromosomal recombination in plant cells. It is assumed that extrachromosomal recombination occurs via single strand annealing and that the recombination reaction would require the following four different enzymes. 1) An exonuclease, which would produce over hanging single stranded tails; (this is seen in *Xenopus lavis* oocytes which have a 5'-3' polarity Lehman et al., 1993), 2) a recombinase type enzyme that would anneal complementary strands like RecA, 3) a polymerase that fills the gaps and nicks, and 4) a ligase. Inactivation of *Nicotiana plumbaginifolia* DNA polymerase alpha in protoplasts reduced incorporation of $^{3}$H into protoplast DNA but extrachromosomal recombination efficiency measured via GUS assay were not affected. This suggests that DNA polymerase alpha is not involved in extrachromosomal recombination.

We evaluated particle bombardment for delivery of extrachromosomal recombination substrate into soybean embryogenic suspension cells. Lim15 (Kobayashi et al., 1993) and sblim15 (next chapter) was also co-introduced and its ability to enhance extrachromosomal recombination was monitored.
Material and Methods

Plasmid Construction

The recombinase substrate, the control plasmids and the expression vector for the lily recombinase were all pUC119 based.

Recombination Substrate

To assay for extrachromosomal recombination, 2 different deletions of the plasmid pUCGUS (Fig. 2.1) (Finer et al., 1990) which encodes β-glucuronidase (GUS), were constructed. Restriction and DNA modifying enzymes were purchased from Promega Corp (Madison WI) and New England Biolabs (Beverly MA) and used according to their recommendations. For bacterial cloning, E. coli strain DH5α and Jm109 were used. For the first deletion, the 3' end of the GUS gene was removed leaving the 35S promoter and the first 1098 bases of the GUS coding region. To generate this deletion, pUCGUS was digested with Csp45 l/EcoR I and the larger fragment of DNA containing the 35S promoter, the 5' end of the GUS coding region and pUC 119 was gel purified (Fig. 2.2). The ends were rendered blunt by DNA polymerase I large fragment and ligated to each other. The plasmid was then transformed into Jm109. The second deletion consisted of the last 1487 bp of the GUS gene and the termination signal of the NOS gene. This was obtained by a double digest of pUCGUS with BamH I/SnaB I which cuts out the first 383 bp of the GUS gene along with the 35S promoter as a single fragment (Fig. 2.3) The larger fragment containing the 3' end of the GUS coding region, the NOS terminator and pUC119 was purified by gel electrophoresis. The ends of the larger fragment were blunt ended before ligating them to each other and transformation of the deletion into Jm109.
For expression of Lim15 in plants, a pLim sense construct was made by removing the Lim15 cDNA (Fig. 2.4) (Kobayashi et al., 1993) from pBluescript using a Xho I/Xba I double digest. The purified Lim15 cDNA was blunt ended by DNA polymerase I large fragment and ligated into CaMV-NOS (Fig. 2.5) cut with Smal I. The resulting plasmid was transformed into Dh5α. Plasmid DNA was isolated and the orientation of the insert between the 35S promoter and NOS terminator was confirmed by restriction enzyme digestion analysis (Fig. 2.6). A Lim15 antisense construct was made by removing Lim15 from pBluescript using Kpn I/Sac I and ligating into CaMV-NOS that was also Kpn I/Sac I digested. The Lim15 antisense was also transformed into Dh5α (Fig. 2.7). For subcloning of sblim15 sense orientation in CaMV-NOS; sblim15 was digested from pBluescript by a Kpn I/Smal double digest and gel purified. The Kpn I site was rendered blunt with DNA polymerase I and the sblim15 cDNA was ligated into Smal I digested CaMV-NOS. The resulting plasmid was transformed into Jm109 and the orientation of sblim15 was determined by restriction digestion analysis (Fig. 2.8). All plasmid DNA(S) were prepared using alkaline lysis as described by Sambrook et al., 1975 (Appendix 13).

Plant Transformation and Tissue Culture
Embryogenic suspension cultures of soybean [Glycine max (L.)] Merrill cv Chapman were initiated and maintained as described by Finer et al., (1991). Plasmids were transformed into soybean using the combinations listed in Table 2.1. The 5' deletion and 3' deletion were made linear by a BamH I and Hind III digest respectively (Fig. 2.9). These enzymes digest the plasmid DNA in the region of overlapping homology. The restriction enzymes were heat inactivated before introduction of DNA into the soybean somatic cultures. Each treatment
was repeated three times and the tissue was bombarded once. One gram embryogenic culture tissue was transferred to a 100x15 mm Petri dish. The excess media was removed with a pipet tip and the culture was allowed to air dry under a laminar flow hood for 15 minutes. Tungsten particles (50 mg M10 Sylvania, Towanda, PA) were sterilized in 95% ethanol, washed four times with water and resuspended in 500 µl of water. A total of 5 µg plasmid DNA was mixed with 25 µl of the tungsten particle suspension. The DNA(s) were precipitated onto the particles by adding 25 µl of 2.5 M CaCl2 and 10 µl of 100 mM spermidine. The tube was placed on ice for 5 min to allow precipitation and then 50 µl of the supernatant was removed and discarded. The DNA coated particle slurry was kept on ice until ready for use. The syringe filter was opened and top and bottom placed in a microfuge tube rack. The DNA(s) particle slurry was vortexed and 2 µl were removed and placed in the middle of the syringe filter. The filter unit was reassemble and installed on the filter holding unit. The Petri dish containing the soybean tissue was covered by a baffle (consisting of a 500 µm pore size nylon screen melted to the bottom of a plastic beaker) and placed on the Plexiglas shelf. The Plexiglas door of the particle inflow gun (PIG) (Finer et al., 1991) was closed while pulling a vacuum on the gun chamber. After the vacuum had reached 28 to 30 inches of Hg, the solenoid was activated and the particles were released and propelled towards the tissue. The tissue was removed and incubated covered for 15 min in the laminar flow hood before transfer to 30 ml of FN (Finer and Nagasawa 1988) medium in a 50 ml polypropylene centrifuge tube. The tubes were shaken at 150 rpm at 27°C for 48 h under 30 µEm^-2s^-1 of cool white light.
GUS Assays.

Histochemical GUS assays were performed as described by Jefferson (1987). Tissue was placed in micro titer plates. Histochemical GUS assay mix (2-3 ml) (Appendix 15) was added to each well of the microtiter plate and placed on a shaker at 37°C for 12 hr. The GUS assay mix was pipetted out of the individual wells and the tissue was washed with 95% ethanol. The wells were filled with 2 ml of 95% ethanol and examined under a dissecting scope 12-36 hours later.
Results

Experimental System:
To investigate somatic extrachromosomal recombination in embryogenic cultures of soybean, a recombination substrate consisting of overlapping deletions of GUS gene was used. The experimental strategy relied on the supposition that two separate DNA molecules (neither of which could express GUS independently), having a 715 bp homologous overlap region, would recombine to produce a functional recombinant molecules (Fig. 2.9). The resulting GUS activity would indicate that homologous recombination had taken place between the two plasmids. The expression of Lim15 protein or sblim15 protein would further enhance the recombination frequency by binding to the introduced DNA and finding its homologue. The whole assay system involved the transfer of DNA into the embryogenic suspension cultures, transcription, translation and metabolism of foreign DNA in the transformed suspension culture cells.

GUS Expression
When soybean embryogenic suspension cultures were co-transformed with the deletions, either with or without the Lim15 or sblim15 expression cassette, only background GUS activity was detected. Background activity is a very faint blue that is clearly distinguished from the intense blue, GUS-positive coloration. This activity was observed in the covalently closed circular deletions by themselves and also in the deletions plus the Lim15 or sblim15 sense construct. When introduced into plant cells in both topological forms, full length pUCGUS gave high levels of GUS activity (Table 2.1).
Discussion

Three different approaches can be used to assay for extrachromosomal recombination.

1. Rescue Cloning: For rescue cloning, substrate DNA (containing complementary deletions) is introduced into the higher organism and the cells are allowed to divide for a few cell cycles or added to sonicated cell extract. Total DNA is extracted from the cells and transformed into E. coli. Transformants are selected for the selectable marker that can be restored in higher cells by homologous recombination (rescue cloning). Rescue cloning is tedious and is the most indirect procedure for assaying extrachromosomal recombination. The DNA(s) is not only in contact with the DNA enzyme machinery of the host cell but also that of E. coli. The cloning procedure might change the composition of the recombined molecules. One problem with rescue cloning is that it is difficult to determine if the recombination took place in the host cell or E. coli. Furthermore, due to the topological state of the marker genes, not all markers can be rescue cloned. Therefore this procedure is not used often (Ayers et al., 1986, Desautels et al., 1990).

2. Integration Assay: The integrative assay is the most commonly used procedure for assaying homologous recombination. It utilizes the whole enzymatic machinery available in the host cell. The problem with this technique is that only molecules that are integrated into the genome and are also expressed can be detected. It is therefore dependent on the position effect. In the animal literature, there is a relationship between the topological state of the introduced plasmid and the frequency of integration. It is expected that the integrative assay would give higher recombination rates when using plasmids with different topological states (Orr-Weaver et al., 1981).
3. Enzyme Assay: This third assay is the monitoring of homologous recombination by reconstitution of DNA encoding enzyme activity. This method has a few advantages over the other assays. The enzyme activity assay is quick and a large number of samples can be processed in a short time. The assay measures only the recombination frequency and not the recombination and integration frequency. As both linear and covalently closed circular DNA molecules code for similar levels of enzyme activity in plant cells, they can be compared to each other in the same experiment. There is no artificial selection pressure placed on the cells for recombination. These advantages made this assay a desirable choice for this study. Once DNA recombination had taken place, the RNA transcript would be produced resulting in an amplification step. These transcripts would be translated into protein which would result in a further amplification step. By using a sensitive enzyme assay, it is theoretically possible to measure recombinational reconstitution of a single enzyme-encoding gene. Such an enzyme assay was used to measure extrachromosomal recombination in rat fibroblast cells by Wong and Capecchi (1986). They were able to detect recombinant events in individual cells after injection with two mutant deletions of hamster adenine phosphoribosyl transferase gene by counting single positive cells on autoradiograms.

In order to monitor extrachromosomal recombination, overlapping deletion mutants of pUCGUS were constructed for this study. The 3' deletion consisted of the 35S promoter and the first 1098 bp of the GUS coding region while the 5' deletion consisted of the last 1487 bp of the GUS coding region and the NOS terminator. Therefore the 5' deletion still contained more than half of the GUS coding region with a functional NOS terminator while the 3' deletion contained over half of the GUS coding region and an intact 35S promoter (Fig. 2.2, 2.3 and
2.9). The PIG was used to introduce these deletions into soybean embryogenic cultures. Embryogenic cultures bombarded with the GUS deletions or co-transformed with the GUS deletions in both topological form did not exhibit any GUS activity. pUCGUS showed high levels of GUS expressing foci (Table 1), indicating that the transformation system was working and that cells were transformation competent.

Although double strand breaks are reported to stimulate recombination in animals cells, no stimulation of recombination was observed when the GUS deletions were linearized. Linearization of both of the deletion plasmids did not stimulate the recombination process. This is in contrast to animal systems in which linearization of the molecules increased the recombination frequency at least ten-fold. This difference between soybean and mammalian systems indicates a different substrate specificity or recombination mechanism in plants (Lin et al., 1990, Puchta and Hohn 1991). In general, in prokaryotes, yeast and higher eukaryotes, an increase in the length of overlap in the region of homology of the recombination substrate increases the efficiency of recombination. Bauer et al., (1990) used recombination substrates having overlapping regions of homology ranging from 6 bp to 405 bp of the kanamycin resistance gene. They found that the use of linearized plasmids increased the efficiency of recombination with increasing length of homology up to a point. This increase was seen irrespective of the location of the digestion site (distal or adjacent to the homologous region). They also reported that 53 bp was the minimum requirement for homology for recombination to occur. However no clear-cut correlation could be established between length of overlapping homology of supercoiled plasmids and recombination frequencies in an integrative assay. This is probably due to the fact that integration dependent assays lead to
underestimations of recombination frequency (Kucherlpati et al., 1984). Puchta and Hohn (1991) used a transient enzyme assay to determine the role of length of overlap with covalently closed circular plasmids and were able to detect recombination with a minimum overlap of 222 bp. Their study also showed that there was a significant drop in recombination efficiency when the homologous overlap was less than 456 bp.

Intrachromosomal recombination in mammalian cells is more efficient if the length of overlap is 295 bp or larger (Liskay et al., 1987). This is much more than is needed for *E. coli*, where 20 bp of homology seems to be sufficient to promote recombination (Watt et al., 1985). Mammals as well as plants have huge genomes in contrast to bacteria. Very few sequences are present in higher organisms which really exhibit more than 300 bp of perfect homology (Kricker et al., 1992). This gives rise to the suggestion that, in plants and mammals, the genome is stabilized by a tighter homology requirement for efficient homologous recombination. Based on these results, the overlap in the present study was kept at 715 bp.

Attempts have been made to introduce recombinase of non-plant origin into plant cells to enhance recombination efficiency (Reiss et al., 1996; Puchta and Meyer 1995). Bacterial recA gene cloned into a plant expression vector when introduced into recA' HB101 restores conjugational recombination in bacteria (Puchta and Meyer 1995). When the same plasmid is co-introduced with recombination substrates into *Nicotiana plumbaginifolia* protoplasts, it did not lead to an enhancement of recombination (Puchta and Meyer 1995). The recombination process involves a multiprotein interaction. The *E. coli* RecA may not able to interact with other eukaryotic proteins involved in recombination (Puchta and Meyer 1995). Our attempts to introduce a plant recombinase from
lily or soybean did not enhance the recombination rates. In plants and mammalian cells, extrachromosomal recombination occurs within the first 30 min of DNA introduction (Puchta and Meyer 1995, Folgers et al., 1985, Fishman-lobell et al., 1992). It is possible that the introduced gene for recombinase may not be producing functional enzyme at that time. Stable transformation of Sblim15 into soybean cultures should yield tissue displaying constitutive expression of this recombinase. These transformations are now under way and may permit efficient recombination without a question of timing. Another possibility to explain the lack of activity of lily Lim15 in soybean is that eukaryotic RecA-like proteins form a specie specific "recombinase" and the lily protein is unable to interact with the rest of the soybean proteins. This species specific interaction has been demonstrated by the inability of human and chicken Rad51 proteins to complement cross specie mutations (Shinohara et al., 1995). Since all of the recombination machinery is in the nucleus and there was no known nuclear target sequence 5' to the lily cDNA or the sblim15 cDNA, the proteins may not be able to get to their locus of action in soybean. The Lim15 protein is just at the exclusion limits of the nuclear pore complex (Reiss et al., 1996). Inclusion of a nuclear localization signal to the lily protein may permit nuclear targeting of the recombinase which would be more effective. The finding that extrachromosomal recombination can not be enhanced by co-introduction of lily Lim15 might indicate that extrachromosomal recombination can not be enhanced by an enzyme from another specie. When protoplasts are transformed about 60% of the target tissue will receive foreign DNA while, in a particle bombardment mediated transformation, only about 0.01-0.1% of the target tissue gets DNA in the cell (J. Finer personal communication). As extrachromosomal recombination is a rare event, it is possible that this considerably lower amount
of DNA being introduced in the target tissue was preventing the formation and
detection of recombinant molecules. Finally, it is not certain if this soybean
embryogenic cell culture line is still recombination-competent as it has been in
culture for a long time and has lost some of its properties. Genetic mutations
accumulate in plant cell cultures with increasing time in culture (Scowcraft and
Larkin 1982).
Fig. 2.1 Restriction map of plasmid pUCGUS. Recombination substrate were deletions of pUCGUS.
Fig. 2.2 Deletion of the 3' end of the pUCGUS plasmid. The plasmid has the first 1098 bp of the GUS gene under the transcriptional control of the 35S promoter.
Fig. 2.3 Deletion of the 5' end of GUS. The deletions consists of the last 1487 bp of the GUS gene with the NOS terminator sequence.
Fig. 2.4. Restriction map of the lily Lim15 cDNA which codes for a RecA-like protein in pBluescript. Solid box = cDNA, Diagonal lines = Domain I of predicted protein, Horizontal line = Domain II of protein.
Fig. 2.5 Restriction map of the plant expression vector CaMV-NOS. All of the recombinase genes were cloned in this vector.
Fig. 2.6. Strategy for subcloning lily Lim15 gene in the sense orientation in the plant expression vector CaMV-NOS.
Fig. 2.7 Strategy for subcloning the lily Lim15 cDNA in the antisense direction in CaMV-NOS.
Fig. 2.8 Strategy for subcloning soybean Lim15 gene in the sense orientation in the plant expression vector CaMV-NOS.
Fig. 2.9. Deletions of the GUS gene with the overlap region where recombination could take place. Restriction sites used to linearize the plasmids in the region of homology are shown.
<table>
<thead>
<tr>
<th>Plasmid Combination</th>
<th>Topological State</th>
<th>GUS Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' GUS deletion</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5' GUS deletion</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>3' GUS deletion</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>3' GUS deletion</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS deletions</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS deletions</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, CaMV-NOS</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, CaMV-NOS</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, liLim Sense</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, liLim Sense</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, liLim Anti.</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, liLim Anti.</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, sbLim Sense</td>
<td>Linear</td>
<td></td>
</tr>
<tr>
<td>5', 3' GUS, sbLim Sense</td>
<td>CCC</td>
<td></td>
</tr>
<tr>
<td>pUCGUS</td>
<td>CCC</td>
<td>++++</td>
</tr>
<tr>
<td>pUCGUS</td>
<td>Linear</td>
<td>++++</td>
</tr>
</tbody>
</table>

Table 2.1. Combinations of plasmids transformed into soybean prior to assaying for extrachromosomal recombination.
Bibliography


64


CHAPTER 3
ISOLATION AND CHARACTERIZATION OF A recA-LIKE GENE FROM SOYBEAN

Abstract

RecA protein plays a key role in prokaryotic recombination. It functions in both DNA repair and genetic recombination by catalyzing pairing and strand exchange. The RecA-like proteins DMC1 and Rad51 in yeast and are both required for meiotic recombination and repair of double stand breaks; Rad51 is also required for mitotic recombination. The Lim15-like proteins (recombinase-like proteins first isolated from plants) of eukaryotes are also similar to recA based on sequence similarity. This study describes the isolation and characterization of a genomic and cDNA (sblimIS) encoding a recA-like gene from soybean (Glycine max L.) flower tissue. The primary sequence of the cDNA termed sblim15 was investigated by comparing the primary structure of the open reading frame to other recA-like genes. The predicted protein exhibits strong sequence homology to the lily and Arabidopsis Lim15-like genes. Southern hybridization analysis indicates that the gene is present in very low copy number. Northern hybridization analysis detected two transcripts in all tissue
and a third transcript in field-grown leaf tissue. Transcript levels are not enhanced in response to DNA damaging agents.
Introduction

Genetic recombination during meiosis and the duplication, deletion, inversion and translocation of genes were recognized long before DNA was even identified as the genetic material. Recombination is also involved in DNA repair which allows cells to maintain genetic integrity under adverse conditions. The ability of DNA chromosomes to pair homologously and to exchange DNA lies at the heart of all general recombination models. DNA recombinase-like proteins requires sequence similarities between two DNA(s), the search of molecules, recognition of homology and finally the mutual exchange of DNA strands.

Using UV light, Clark and Margulies (1965) isolated a number of recombination mutants in *Escherichia coli*. Since then, a large number of genes and proteins that play important roles in recombination have been identified (Kowalczykowski and Eggleston 1994). One of the earliest mutants that was identified was *recA* (recombination A). The product of this gene, RecA, has a molecular mass of 38.5 kD. The RecA protein in *E. coli* plays a pivotal role in genetic recombination and, based on this observation, a considerable effort went into biochemical characterization of this protein.

In bacteria, recombination is an essential cellular process catalyzed by cellular enzymes encoded and regulated by the cell. Recombination rates are increased following exposure of both prokaryotic and eukaryotic cells to DNA damaging agents such as x-rays, ionizing radiation, mitomycin C, methyl
methane sulfonate (MMS) and stress (Lebel et al., 1993 and Kohler et al., 1989). This response has been particularly well characterized in *E. coli* as the “SOS response”. The SOS response is composed of the simultaneous derepression of a number of *E. coli* genes by the RecA and LexA protein (conditions that effect DNA replication induce the SOS response) and is responsible for the repair of damaged DNA. The substrate that invokes the SOS response is either single stranded DNA or double stranded nicked or cleaved DNA.

**RecA Protein**

Our most detailed understanding of the mechanism of recombination comes from the study of the RecA gene product of *E. coli*, an enzyme that carries out the fundamental reaction in recombination; the making of base paired hybrids that joins two DNA molecules. The RecA gene product is a very interesting protein with multifaceted roles, some of which have been characterized and some which are under active investigation (Roca and Cox 1990). The *E. coli* RecA protein belongs to a class of single strand DNA binding proteins (proteins that bind to single stranded DNA and remove secondary structure). Mutants defective in RecA do not participate in homologous recombination, post-replication repair or any of the induced SOS functions. The properties of the purified protein provide insight into the central role of this enzyme in nearly all modes of recombination. Purified *E. coli* RecA protein is a
38.5 kD monomer which binds cooperatively and stoichiometrically (one monomer of RecA for every four to six nucleotides) to single stranded DNA forming a nucleoprotein complex. This nucleoprotein complex is the active specie in DNA strand exchange and has a half-life of about thirty minutes. When RecA binds to single stranded DNA, the secondary structure of DNA is modified so that the single stranded DNA is completely coated. Electron micrographs showed that the DNA in the nucleoprotein filament is stretched 150% compared to normal B-form duplex DNA with the axial spacing between base pairs changed from 3.4 Å to 5.1 Å. The nucleoprotein filament is a right handed helical structure with a pitch of 95 Å and 18.6 bp per turn. The average rotation per base pair is 19.4° so the DNA in the nucleoprotein filament becomes significantly relaxed. This activity is important for strand exchange. Other activities of the RecA protein include ATP hydrolysis and cleavage of the repressor protein LEX.A; this co-protease activity of RecA protein is also activated by the binding of RecA to single stranded DNA. RecA-DNA binding to DNA exhibits Michaelis-Menton kinetics with single stranded DNA as the limiting component. ATP is not required for association of the protein with DNA and the addition of ATP or ADP increases the rate of protein dissociation. RecA also binds to duplex DNA but with much less affinity than for single stranded DNA; the association is facilitated by low pH (about pH 6) or the presence of single stranded regions within double stranded DNA, the DNA helix is unwound with an ATPase action however the turnover number is relatively low for an ATPase (Roca and Cox 1990). Binding
of RecA to single stranded DNA is directional proceeding from 5' to 3' direction. As a result, the RecA molecules migrate to the 3' end of the linear DNA and accumulate there.

RecA catalyzes strand exchange between a large variety of homologous DNA molecules. The structural requirements for RecA-mediated recombination are a region of single stranded DNA for assembly of a RecA filament, DNA-DNA homology between the two partners, and a free end within the region of homology for the strands to intertwine (this can be substituted by a topoisomerase in case of a covalently closed molecules). The isolation of reaction intermediates indicates that E. coli RecA protein associates with DNA molecules, recognizes homologous DNAs, exchanges base paired DNA strands and catalyzes extensive branch migration.

Recognition of the activity of RecA homologous recombination led to the search for similar proteins from other sources using the strand exchange assay (West et al., 1983, Lovett and Roberts 1985). In prokaryotes, RecA homologues have been found which can invoke the SOS response in mutant E. coli (Owttrim and Coleman 1989). The SOS response is not invoked by eukaryotic RecA homologues in E. coli and ATP is not required for function; however, homologues can partially complement recombination deficient E. coli.
Other Prokaryotic Nucleases

Nucleases appear to play a central role in recombination (Smith 1988). Nucleases function in both the initiation and the resolution steps. However, due to their multiple roles in the cell, the exact role of nuclease in recombination is not clear. The multifunctional RecBCD and RuvABC enzyme complexes displays exonuclease, endonuclease and helicase activities and are the principle nucleases involved in recombination in E. coli (Chaudhury and Smith 1984, Shinagawa et al., 1988). The RecBCD complex specifically cleaves chi sequence leading to the integration of lambda DNA into E. coli chromosomal DNA during infection. However, reconstitution experiments show that these enzymes can be partially complimented by other enzymes which indicates the existence of alternate recombination pathways.

Specific cleavage of DNA with structures resembling Holliday junctions has been seen in eukaryotes (Mizuuchi et al., 1982), yeast (West and Korner 1985) and human cell extract (Waldman and Liskay 1988). This specific cleavage indicates that the RuvABC enzymes recognize the forked structure but do not recognize the adjacent sequence or the junction. The enzymes introduce a pair of nicks near the base of the four way branch resolving the molecule into two linear pieces. The RecBCD and RuvABC enzymes (Kuzminov 1993) are thought to be involved in resolution of Holliday structures in E. coli. This activity has been duplicated in vitro (Smith 1988, Radding 1982).
Eukaryotic homologs of the recA protein: Rads

Evidence is accumulating that all organisms have a functional homologue of the prokaryotic RecA protein, suggesting that the basic mechanism of recombination may be conserved in all organisms. RecA-like proteins are involved in UV- and radiation-induced DNA repair in *Saccharomyces* (Basile et al., 1992). Mutants that are unable to repair DNA after radiation (radiation sensitivity) are assigned "Rad" names followed by a locus number. The first 50 numbers are given to those loci whose mutants primarily show sensitivity to UV or both to UV and ionizing radiation. Loci that primarily control ionizing radiation sensitivity with minor effects on UV response are numbered from Rad50 upwards. Radiation sensitive mutants in yeast can be loosely classified into three groups based on epistasis analysis and on mutant phenotype. The groups were originally defined on the basis of double mutant interaction. If a yeast strain carrying mutations in two or more genes conferring radiation sensitivity showed no further increase in sensitivity beyond that shown by the single mutant, then the mutant belonged to the same group. Increased sensitivity in double mutants implied that the two mutations conferred sensitivity via different mechanisms. The mutation belongs to different groups since no increase in sensitivity should be seen by eliminating an additional step if a repair pathway was already blocked. Three epistasis groups have been identified so far (Fig 3.1). To date, 24 genes have been formally designated with the Rad loci number in the UV-sensitive series. The UV-sensitive series make up two of the three epistasis
groups. Only eight loci (Rad50 to Rad57) comprise of the third epistasis group, which play a major role in x-ray repair. Genes in the third epistasis group which is called the Rad52 epistasis group are responsible for the mitotic and meiotic recombination in yeast (Game 1993).

**Genes in the Rad52 Epistasis group**

**Rad50 Gene**

The 3.3 kb Rad50 gene encodes a 153 kD protein that has a nucleotide binding domain at its amino terminal. It has two heptad repeat sequences which are separated by a spacer that is repeat-free (Alani et al., 1989). Such sequence arrangement can form an alpha helical structure where the repeat units coil around each other to form a regular helical structure termed an alpha helical coil, similar to myosin and keratin (McLachlan and Korn 1982, McLachlan and Korn 1983, Cohen and Parry 1986). The purified protein binds double-stranded DNA in an ATP-dependent reaction (Raymond and Kleckner 1993) but does not require ATP hydrolysis to do so. There is no evidence to suggest that the protein binds to specific sequences or to DNA ends. At high ionic strength, Rad50 forms a homodimer. Physical properties of the protein change when the ionic strength is lowered gradually (Raymond and Kleckner 1993) and bending of the heptad-free hinge region may be an important determinant of this transition. A transient
increase in the mRNA levels of Rad50 are seen during meiosis; however, the protein levels remain constant (Raymond and Kleckner 1993). Yeast strains having point mutations in the Rad50 gene are very sensitive to x-ray and MMS and are unable to repair double strand breaks. In contrast to mutations in other genes from the same epistasis group, spontaneous mitotic recombination and chromosomal integration of linearized plasmids by homologous recombination is not reduced in Rad50 mutants (Alani et al., 1990, Schiestl et al., 1994). The meiotic defects of this gene have been studied in detail. A mutation in the consensus ATP binding domain results in significant sensitivity to MMS and causes blockage of meiosis at an early stage (Alani et al., 1990). In this mutant, the synaptonemal complex does not form which gives rise to a tripartite structure in which condensed paired homologous chromosomes are connected in meiotic prophase (Alani et al., 1990). Another mutation in Rad50 (rad50s) which is a point mutation has been characterized in detail. This mutant (rad50s) does not cause sensitivity to MMS and allows meiosis to proceed to an intermediate stage (Alani et al., 1990) compared to deletion mutants of Rad50 where meiosis is interrupted. In this point mutant, persistent strand breaks at recombination hot spots are generated but not processed. The ability of these rad50 mutants to carry out meiotic recombination has been analyzed in "return to growth" experiments (Esposito and Esposito 1974), where meiosis in a sporulating medium is interrupted at different times by transfer to vegetative medium. Gene conversion events among heteroalleles (indicating a commitment to meiotic
recombination) is scored. Neither the rad50 point mutation nor the null mutation show meiosis specific enhancement of recombination during incubation in sporulation medium (Alani et al., 1990). This gives rise to the question of how a protein is able to repair double strand breaks in mitotic cells and acts in the induction and correct processing of double strand breaks in meiotic cells. It is suggested that Rad50 protein binds to certain hypersensitive sites on chromatin that are destined to be double stranded breaks during meiosis and, when the strand breaks are formed, Rad50 serves as a nucleation site for the recombination machinery (Raymond and Kleckner 1993).

**Rad51 Gene and its Homologs**

The Rad51 gene in *Saccharomyces cerevisiae* produces a 1.6 kb transcript which encodes a 400 amino acid long protein having a molecular mass of 43 kD. The protein has considerable homology to bacterial RecA protein (Basile et al., 1992). A region extending from residues 3 to 227 in RecA and 126 to 362 in Rad51 has 26.5% identity and 67% conservative homology. This amounts to 59% and 64% of Rad51 and RecA respectively (Basile et al., 1992). This region of RecA is responsible for oligomer formation and recombination (Ogawa et al., 1992) which includes the walker A and B box nucleotide binding domains. Site directed mutation in this region of the Rad51 protein has shown this to be an important region for repair and recombination (Shinohara et al., 1992). Rad51 mutants in yeast are very sensitive to x-ray and MMS. They are
defective in repair of double strand breaks (Contopoulou et al., 1987) and in the formation of viable spores during meiosis (Petes et al., 1991). Rad51 mutants are viable but spore formation is reduced to less than 10% of the wild type level with very low viability (Shinohara et al., 1992). Rad51 mRNA levels are increased during meiosis and the mitotic cell cycle, with high levels present at G1/S boundary (Basile et al., 1992, Shinohara et al., 1992). Upstream of the Rad51 gene promoter is a region that contains two restriction sites for the enzyme Mlu. These Mlu recognition sequences are characteristic of genes involved in DNA metabolism that are coordinately expressed at higher level at G1/S boundary. Further upstream of the Mlu recognition sequence of Rad51 is a 29 bp segment called the damage response sequence (DRS) (Basile et al., 1992). This sequence has been shown to be sufficient to impart x-ray inducible expression on a heterologous reporter gene (Cole and Mortimer 1989). Rad54 shares 11 out of 13 bases with part of this 29 base pair DRS region which is also responsible for inducibility in Rad54 (Cole and Mortimer 1989). Rad51 is also induced by DNA damaging agents in mitotic cells (Basile et al., 1992, Shinohara et al., 1992). Unlike E. coli, there is no common regulatory region in all the Rad-like genes; however, common sequences in each epistasis group has been proposed (Siede et al., 1989).

Yeast Rad51 protein is able to bind double stranded and single stranded DNA in an ATP-dependent manner. This protein also binds single stranded DNA in an ATP-independent manner and has a single stranded DNA dependent
ATPase activity. Rad51 binds Rad52 as seen by retention of RadD51 on Rad52-sepharose column and with a yeast two hybrid genetic system (Field and Song 1989). In the presence of ATP, Rad51 protein forms a helical filament with double stranded DNA, similar to RecA protein. Common features include extensive stretching and unwinding of B-DNA. Detailed comparison of RecA, Rad51 and their homologs including bacteriophage T4 UvsX protein show conservation of residues not only in or around the nucleotide binding domain, but also in other identically located and presumably functionally equivalent regions (the hydrophobic core of the protein and the monomer interface). This analysis has further strengthened the assumption that all organisms have evolved and diversified from a common ancestral protein like RecA, Rad51 catalyzes complete strand exchange between single stranded and double stranded homologous DNA in an ATP and Mg\textsuperscript{2+} dependent reaction \textit{in vitro}. The single stranded DNA binding protein, replication protein A (RPA) was found to be an important accessory factor for this reaction. Based on all this evidence, Rad51 must be considered a real functional homologue of RecA in prokaryotes (Ogawa 1993). Rad55 and Rad57 which are also involved in recombination repair, also share homology to Rad51.

The DMC1 (\textit{disrupted meiotic cDNA #1}) gene from \textit{Saccharomyces cerevisiae} produces a 1002 bp transcript, which is enhanced in meiosis (Bishop et al., 1992). The 1.3 kb cDNA is interrupted by a 91 bp intron. Among the ten yeast genes specifically required in meiosis, recombination and chromosome
pairing, DMC1 is the third to have an intron (Engebrecht et al., 1991, Menees et al., 1992). This abundance of intron-containing meiosis-specific genes in yeast contrasts with the vegetatively expressed genes, in which introns are rare (Bishop et al., 1992). The DMC1 polypeptide consists of 334 amino acids having a molecular mass of 39.4 kD. The homology between DMC1 and RecA is considerable. The strongest homology between the two proteins occurs over a 238 residue region, which includes the structural core of the RecA protein (Story et al., 1992). DMC1 protein is also homologous to RAD51 protein, with 46% amino acid identity and 68% overall homology. DMC1 was isolated in a screen for genes specifically expressed during meiosis (Bishop et al., 1992). The phenotype of DMC1 and Rad51 mutants are very similar but the mitotic levels of DMC1 mRNA using a northern blot analysis are hardly detectable, even though the gene has all the consensus sequences known for splicing of yeast transcripts in mitotically dividing cells. Deletion mutants do not confer a mitotic phenotype; that is MMS and x-ray resistance is not affected.

Homologues of Rad51 have been cloned from the following lower and higher eukaryotes: Schizosaccharomyces pombe (Muris et al., 1993 and Jang et al., 1994), Neurospora crassa (Cheng et al., 1993), Ustilago maydis (Rubin et al., 1993), Arabidopsis thaliana (Sato et al., 1995), Lilium longiflorum (Kobayashi et al., 1994a, 1994b), Gallus gallus (Bezzubova et al., 1993), mouse (Sato et al., 1995, Shinohara et al., 1993, Morita et al., 1993 and Habu et al., 1996), human (Sato et al., 1995, Shinohara et al., 1993, Yoshimura et al., 1993 and Habu et
al., 1996), *Drosophila melanogaster* (McKee et al., 1996 and Akaboshi et al 1994), *Xenopus laevis* (Maeshima et al., 1995) and *Candida albicans* (Diener and Fink 1996). The predicted mouse and human protein differ by only four amino acids. The human and mouse proteins share 83% homology with *S. cerevisiae* and *S. pombe* Rad51 proteins, 64% homology to DMC1 and 55% to *E coli* RecA protein. Over expression of the mouse gene in yeast can complement the MMS sensitivity of a *S. cerevisiae* rad51 deletion mutant (Morita et al., 1993). The mouse gene is the only Rad51 gene which can complement a yeast mutation; no other species is able to complement a cross species mutation, probably due to species specific multimer formation (Shinohara et al., 1993 and Ogawa et al., 1995).

**Rad52 Gene**

The Rad52 gene codes for 504 amino acid protein with a molecular mass of 52.4 kD (Adzuma et al., 1984). Rad52 can interact with Rad51 *in vitro* (Shinohara et al., 1992) and seems to be conserved among lower and higher eukaryotes (Bezzubova et al., 1993, Muris et al., 1994). A region of 126 amino acids in the amino terminal in Rad52-like proteins is conserved (Bezzubova et al., 1993). Degenerate primers designed from this end were used to clone a chicken Rad52 homolog by the polymerase chain reaction (Bezzubova et al., 1993, Muris et al., 1994). Sequence comparison of the yeast, chicken, mouse and human proteins reveals a strongly conserved region between positions 40
and 178 indicating that this part of the protein is under strong evolutionary pressure (Bezzubova et al., 1993). This region might be required for interaction with Rad51. Human, mouse and chicken Rad52 genes are transcribed in testis and thymus tissue (Muris et al., 1994, Bezzubova et al., 1993). Unlike Rad51, yeast Rad52 is not expressed in response to DNA damaging agents (Cole et al., 1989). Mutants for Rad52 are characterized by their defects in mitotic and meiotic recombination. Rad52 mutants are unable to repair double stranded breaks induced by ionizing radiation and can not undergo post-replicative repair following UV irradiation of cells defective in nucleotide excision repair. Rad52 is necessary for integration of introduced linearized plasmid and for mating-type switching. If mating switch is attempted in a rad52 deletion background, it results in cell death (Weiffenbach and Haber 1981).

**Rad53 Gene**

The Rad53 gene (also known as SAD1, MEC2 or SPK1) encodes an essential serine/threonine protein kinase (Zheng et al., 1993, Allen et al., 1994). The role of Rad53 along with MEC1 (which controls phosphorylation of Rad53 protein) is thought to be the arrest of cell cycle at S/G2 in response to DNA damage and unreplicated DNA (Weinert et al., 1994)

**Rad54 Gene**
The Rad54 gene codes for a 898 amino acid protein. The deduced protein sequence of the Rad54 gene has strong homology to DNA helicases (Emery et al., 1991). There is also considerable homology of Rad54 to SNF2 which is required for transcriptional activation of a number of diversely regulated yeast genes (Sclafani et al., 1984). The protein has six nucleotide binding sites and a nuclear targeting sequence (Emery et al., 1991). Rad54 is induced in response to x-ray, MMS and UV radiation. Transcription in response to DNA damage is shown to be dependent on a 29 bp AT rich region found upstream of the start codon (Cole and Mortimer 1989). This AT rich region is apparently the binding site for a factor involved in the direct or indirect sensing of DNA damage. The 5' region of the gene also contains a Mlu restriction enzyme site which are present in genes that are cell cycle regulated (McIntosh et al., 1991). Transcripts of human and mouse homologs of Rad54 are seen in all tissues but are increased in thymus spleen and testis (Kanaar et al., 1996). Since there is growing evidence that DNA repair is coupled to transcription, it is possible that Rad54 gene induction may be result from both occurrence and also repair of DNA lesions.

**Rad55 Gene**

The Rad55 gene codes for a 405 amino acid protein with a molecular mass of 46kD (Lovett 1994). This protein has sequence similarity to Rad51, DMC1 and Rad57. Although, the strongest homology is in the walker A and B
box region, they are also similar in adjoining areas and share most of the conserved residues of prokaryotic RecA protein (Roca and Cox 1990), known to be required for ATP binding and hydrolysis. Mutants of Rad55 are sensitive to x-ray and deficient in x-ray induced mitotic recombination as well as being mitotically lethal (Lovett and Mortimer 1987). A striking feature of the rad55 null mutant is that they exhibit phenotype conditional with temperature, mating type and osmotic strength of the growth medium (Lovett and Mortimer 1987). Conditional mutants are usually missence mutants where changes in temperate and salt concentration may correct defective folding or interaction with other proteins. The basis of conditionally of these mutants in not known nor is the biochemical function of Rad55 gene in recombination clear.

**Rad56 Gene**

Rad56 is an undercharacterized locus. Available mutants for Rad56 are moderately sensitive to x-ray compared to wild type and exhibit no change at cold temperature (Game 1993).

**Rad57 Gene**

The Rad57 protein consists of 460 amino acid residues with a molecular mass of 52.8 kD. The cDNA for Rad57 has homology to Rad51 and bacterial RecA but lacks some of the conserved residues thought to be important for RecA function (Kans and Mortimer 1991). Rad57 interacts with Rad55 as shown
by the yeast two hybrid fusion system (Fields and Song 1989). The protein participates in the repair of x-ray induced damage to DNA and in meiosis (Game and Mortimer 1974, Game 1983). Mutants are more sensitive to x-ray at 23°C than at 36°C, and are unable to undergo mating type switch. Mutants for rad55 and rad57 can be suppressed by over expression of Rad51 and Rad52. A possible explanation for the ability of Rad51 and Rad52 to partially overcome the defects of rad55 and rad57 mutants is that the addition of Rad51 and or Rad52 stimulates an alternative DNA repair mechanism from that of Rad55 and Rad57. Repair of double stranded breaks in S. cerevisiae may be performed by a multiprotein complex (Lovett and Mortimer 1987, Milne and Weaver 1993) and both Rad55 and Rad57 maybe part of this complex termed a recombinase (Hays et al., 1995). Phenotype of deletion mutants in these genes indicate that they are involved in DNA double strand break repair at low temperature, since they are x-ray sensitive at 23°C and but not at 36°C (Game 1993, Ho and Mortimer 1975 and Lovett and Mortimer 1987). This could be due to a different mechanism for recombination repair or that Rad55 and Rad57 are needed to stabilize this multimeric complex at low temperature (Game 1993 and Lovett and Mortimer 1987). Because Rad55 and Rad57 appear to be partially dispensable for double stranded break repair and recombination under certain conditions, it is possible that Rad55 and Rad57 are needed in an auxiliary way, perhaps to stabilize the complex which is unstable at low temperatures. Excess Rad51 and Rad57 may
overcome a lack of functional Rad55 and Rad52 by driving or stabilizing complex formation by "mass action" (Hays et al., 1995).
RecA-like Gene products in plants

The first published report of a RecA-like product in plants was that of Hotta et al., (1985). They purified and characterized a meiotic and somatic RecA-like protein from lily (*Lilium longiflorum*). Meiotic Rec (m-rec) had a molecular weight of 43 kD, a temperature optimum of 25°C and behaved much like RecA for the activities tested. Meiotic Rec protein peaked at early pachytene. Somatic Rec (s-rec) had a molecular mass of 70 kD and had a temperature optimum higher than m-rec. No further characterization of these proteins ever appeared in the literature. More recently, a gene from pea showing DNA strand transfer activity was isolated and characterized. It codes for a peptide immunologically related to RecA (Cerutti and Jagendorf 1993, Cerutti et al., 1992). It is nuclear coded and chloroplast targeted. A cDNA from *Arabidopsis thaliana* was isolated by partial complementation of a recA mutant in *E. coli* (Pang et al., 1992). This cDNA also has a plastidial targeting sequence but the protein is different from pea.

A recombinase-like gene from lily was isolated by screening for messages preferentially expressed during meiotic prophase of microsporogenesis in *Lilium longiflorum* (Kobayashi et al., 1994). This gene was first called a Rad51-like gene and then later a DMC1-like gene. Finally it was placed in a class by itself. Lim15 (*Lily induced message #15*) codes for a protein that has a molecular weight of 38 kD consisting of 349 amino acids. The protein has high homology to Rad51 and DMC1 protein of yeast (Kobayashi et al., 1994). The homology
extends throughout the protein except at the amino end. The coding region can be divided into two domains (Fig. 3.2). There is similarity between Lim15 and Rad57, especially in the nucleotide binding domain (Kans et al., 1991). The lim15 gene is expressed in prophase I of meiotic cells (Kobayashi et al. 1993, 1994) and is seen localized on lily chromosomes at leptotene and early zygotene stage (Terasawa et al., 1995). Another protein in lily microsporocytes that cross reacts with an antibody produced against yeast Rad51 protein is also seen bound to the Lim15 protein at the same foci. Antibody to the yeast Rad51 protein detected this cross reacting protein in pachytene chromosomes although the number of anti-Rad51 cross reacting foci decreases as meiosis progresses (Terasawa et al., 1995). This suggests that these two proteins may have overlapping functions or function coordinately. A Lim15 homologue has been isolated from Arabidopsis thaliana (Sato et al., 1995). The cDNA from Arabidopsis was isolated by reverse transcriptase PCR of polyA mRNA from flower tissue. The cDNA has 1026 bp and codes for a 37.3 kD protein (Sato et al., 1995).

In this present study, a search for a RecA-like gene in the genome of soybean [Glycine max (L.) Merrill] was conducted to determine the presence of these genes in a field crop plant. Isolation and overexpression of this gene would further the understanding of the process of recombination in soybean and allow targeted introduction of foreign genes to become more efficient. Using Lim15 cDNA as a heterologous probe, a genomic fragment from soybean post-
emergence seedling tissue was isolated and used to assay for induction of Lim15-like transcripts in various tissues of soybean plant. A flower cDNA library was then screened to isolate a cDNA. The isolation of this cDNA has shown that the molecular mechanisms of recombination may be more conserved in all prokaryotes and eukaryotes than previously thought. Further studies on these genes may provide important information in understanding the molecular mechanism underlying recombination in plants.
Material and Methods

Genomic Library Screening

A genomic library of *Glycine max* cv "Resnik" in EMBL3, SP6/T7 was obtained from Dr Desh P. Verma. (The Ohio State University Columbus OH) *E coli* K802 was infected with phage and plated on 150 mm Petri plates at a density of 50,000 pfu/plate. Plates were incubated at 37°C for 8-12 hours until the plaques were confluent and kept at 4°C overnight before two replica filters were prepared from each plate (Appendix 9). DNA was fixed and denatured onto the nylon filters by autoclaving at 100°C for 1 min with fast exhaust. Prehybridization was carried out in 5X SSC, 5X Denharts, 0.2% SDS and 250 µg/ml¹ denatured salmon sperm DNA for 8 hrs at 55°C. Filters were hybridized to radiolabelled Lim15 cDNA (Kobayashi et al., 1994) generated by random priming (Appendix 3 and 4) (Feinberg and Vogelstein 1983). Filters were hybridized for 48 hrs at 55°C (Appendix 3, 4, 9), and then washed four times for five min each with 2X SSC, 0.1% SDS at 55°C. Plaques showing positive hybridization signal were further purified by four rounds of plaque purification.

Subcloning of Genomic Fragments and Sequencing

Phage DNA was prepared according to Schuller and Zalenski with some modifications (Appendix 10), digested with *Xho I* to release insert DNA and gel purified (Appendix 13). The gel purified fragment was subcloned into the *Xho I*
site of pZero™ (Invitrogen, Ca). The Xho I fragment was restriction mapped and
shot gun subcloned as Xho I/EcoR I fragment into pBluescript™. Subclones that
hybridized to Lim15 were identified by colony hybridization (Appendix 12) and
two of them were partially sequenced (Appendix 19).

cDNA Library construction

Total RNA was isolated from soybean flower tissue and x-ray irradiated
soybean embryogenic tissue (Appendix 6). mRNA was purified from total RNA
(Appendix 7). Seven micrograms of mRNA was used to construct unidirectional
cDNA libraries in Uni-ZAP™XR vector according to the instructions provided
with the cDNA synthesis kit (Stratagene Corp. LaJolla CA). First strand of the
cDNA was primed using oligo(dt). Prior to cDNA cloning, EcoR I adapters were
ligated to double stranded cDNAs and the cDNAs were size fractionated through
a Sephacryl S-500 spin column (Stratagene Corp. LaJolla CA). The Uni-
ZAP™XR recombinants were packaged in vitro using Gigapack III Gold
packaging extract (Stratagene Corp. LaJolla CA).

PCR amplification of exon13 region

To amplify a 136 bp region corresponding to exon 13 of the Arabidopsis
thaliana Lim15 gene, two oligonucleotide primers were synthesized. Exon-13-1
5'TGATCCAGGAGGTGCGT3' and Exon13-2 5'GGCGTCAAACACTCTGG3'.
Genomic DNA from the soybean library was used as a template for amplification.
PCR was carried out in 50 μl volume containing 100 pg of DNA, 280 pmol of each primer, 5 μl of 10X Amplitaq buffer (Perkin-Elmer Cetus, CA), 200 mM of each dNTP and 2.5 U Taq. polymerase (Perkin-Elmer Cetus, CA). Reaction conditions were: initial denaturation at 96°C for 5 min, followed by 94°C for 60 s, 57°C for 60 s, and 70°C for 90 s for a total of 35 cycles followed by a final extension at 70°C for 10 m. After the reaction, products were gel purified (Appendix 13).

**cDNA library Screening**

*E. coli* XL1-Blue MRF' (Jerpseth et al., 1992) host strain was infected with the phage from flower and x-ray irradiated cDNA libraries at 37°C mixed with 10 ml of NZY top agarose and plated on 150 mm NZY agarose (Sambrook et al., 1989) Petri plates at a density of 50,000 pfu/plate. Plates were incubated at 37°C for 8-12 hours until the plaques were confluent; plates were kept at 4°C overnight before two replica filters were prepared from each plate (Appendix 9). DNA was fixed and denatured onto the filters by autoclaving at 100°C for 1 min with fast exhaust. Prehybridization was carried out in 5X SSC, 5X Denharts, 0.2% SDS and 250 μg/ml1 denatured salmon sperm DNA for 8 hrs at 65°C (Appendix 9). Filters were hybridized to the PCR amplified fragment of exon 13 as a probe generated by random priming (Feinberg and Vogelstein 1983) for 48 hrs at 65°C (Appendix 3, 4, 9). Filters were washed four times for five min each with 2X SSC, 0.1% SDS at 65°C; followed by four washes for five min each with
0.1X SSC, 0.1% SDS at 65°C. Plaques identified with autoradiography were further purified by four rounds of plaque purification.

**In vivo excision of the pBluescript phagemid from the Uni-ZAP™XR vector and Sequencing**

Plaques giving positive signal were cored from the agarose plate and transferred to sterile tubes containing 500 µl of SM buffer (Sambrook et al., 1989) and 20 µl of chloroform. The tubes were vortexed at room temperature for 4 hrs. SM buffer containing 1X 10^5 phage particles were mixed with 200 µl of *E. coli* strain XL1-Blue MRF' (Jerpseth et al., 1992) OD<sub>600</sub> of 1.0 and 1X10^6 pfu of ExAssist helper phage (Stratagene Corp, LaJolla CA). Phage particles were allowed to adhere for 20 min at 37°C. LB broth (3 ml) (Sambrook et al., 1989) was added and the tubes incubated for 5 hours at 37°C with constant shaking. Tubes were incubated at 65°C for 20 min and then centrifuged for 15 min at 1000 Xg. The supernatant containing the excised pBluescript™ phagemid packaged as a filamentous phage particle was saved in a sterile tube. To plate the phagemid, 200 µl of *E. coli* strain SOLR (Stratagene Corp. LaJolla, CA) at a density of OD<sub>600</sub> of 1.0 were mixed with 10 µl of the supernatant, incubated for 15 min, plated on LB-ampicillin agarose plates and incubated overnight at 37°C. Plasmids were sequenced from the 3’ end using the dideoxy chain termination method (Appendix 19) (Sambrook et al., 1989). Sequence data was analyzed using the GCG software (1996) (Genetics Computer Group, Madison WI) and...
Blast program (National Institute of Health). Full length cDNA was sequenced by the DNA sequencing and synthesis facility at Iowa State University using a Perkin Elmer ABI 377 automated DNA sequencer.

Southern Hybridization Analysis

Genomic DNA was isolated from leaf tissue of soybean cv "Chapman" according to Saghai-Maroof et al., 1984 (Appendix 1). Genomic DNA was digested with restriction enzymes, electrophoresed and transferred onto a nylon membrane (Genescreen Plus NEN Research, Boston MA) (Appendix 5). The membranes were prehybridized in 5X SSC, 5X Denharts, 0.2% SDS and 250 μg/ml1 denatured salmon sperm DNA overnight and then hybridized for 24 hrs with random primer labeled DNA probes (Feinberg and Vogelstein 1983) (Appendix 3 and 4). Membranes were washed either at low stringency (2x SSC, 0.1% SDS at 65 °C) or high stringency (0.1x SSC, 0.1% SDS at 65 °C) and then exposed to Kodak film with intensifying screens for 3-4 days (Appendix 5).

Northern Hybridization Analysis

Total RNA was isolated from various tissue (Appendix 6). To induce DNA damage prior to total RNA extraction, soybean somatic embryos were subjected to 1.5% ethyl methane sulfonate (EMS) and 1.5% methyl methane sulfonate (MMS) for 2 h at room temperature in FN medium (Finer and Nagasawa 1988). Heat shock was performed by placing the somatic embryo culture tissue in 30 ml
of FN medium at 50°C for 2 h. Dark treatment was administered by placing the culture flasks in dark for 12 h. The culture was also subjected to 10 Krads of x-ray from a cobalt$^{60}$ source. The mRNA was purified from total RNA using a batch oligo(dt) cellulose method (Appendix 7). Fifty μg of total RNA or 5 μg of mRNA was separated on a 1.4% agarose-2.2 M formaldehyde gel and blotted onto a nylon membrane (Genescreen Plus NEN Research, Boston MA) (Appendix 8). The membrane was prehybridized for 6 hrs at 60°C, hybridized to various regions of sblim15 cDNA for 48 hrs at 47°C, washed in 2XSSC, 5% SDS at room temperature for 15 min followed by 0.1X SSC, 5% SDS at 55°C for 30 min (Appendix 8).
Results

Genomic Library Screening

The soybean cv “Resnik” genomic library had a titer of $5 \times 10^{10}$ pfu/ml. To identify a genomic fragment of soybean homologous to Lim15 of lily, a total of $6 \times 10^8$ pfu were screened using the lily Lim15 cDNA as a probe. Seven plaques were identified that gave a strong positive signal in replicate filters. Among these seven plaques, three plaques had lost the Xho I restriction enzyme site used to clone the genomic fragment into the EMBL3 phage vector. Other sites in the multiple cloning sites had also been lost. These three plaques were discarded due to problems associated with cloning artifacts. Another three plaques were identical based on the size of the insert (12.4 kb) and the restriction enzyme digestion pattern. The seventh plaque (15.7 kb) was unique. Isolated plaques containing the 12.4 kb and 15.7 kb inserts were named clone1 (C1) and clone2 (C2) respectively. C1 hybridized to Lim15 cDNA(s) domain I and II, both at low and high stringency (Fig. 3.2).

Subcloning of Genomic Fragments and Sequencing

Clone1 had an insert of 12.4 kb while Clone2 had an insert of 15.7 kb. Insert from Clone1 was subcloned into the Xho I site of pZero™ (Invitrogen, San Diego, CA). The insert from Clone1 was also shotgun subcloned by digesting the insert with EcoR I and ligating into EcoR I/Xho I-digested pBluescript. Insert containing plasmids were selected using blue/white selection. To identify white
colonies that hybridize to Lim15, a colony lift hybridization was performed (Appendix 12). Two plasmids were identified that hybridized to Lim15 and these were named (EcoR I/Xho I-1 and 2). These plasmids were partially sequenced from the EcoR I and the Xho I site. In one of the plasmids (Ecor I/Xho I-1), 180 bp downstream of the EcoR I site, a region of 159 bp was found to have 78% homology to a region from domain II of Lim15 (915-1047) and 75% homology to exon13 of Arabidopsis thaliana (Fig 3.3, Fig. 3.4) EcoR I/Xho I-1 was sequenced downstream and upstream for a total length of 1000 bp but no other sequence homology was found. A 497 bp fragment encompassing 159 bp region of homology was subcloned as a EcoR I/Spe I fragment into pBluescript (Fig. 3.4).

cDNA Library Construction

The cDNAs libraries were constructed from flower tissue and x-ray irradiated soybean somatic embryogenic tissue. Flower cDNA (150 ng) and x-ray irradiated somatic embryo cDNA (100 ng) was ligated into Uni-ZAP™ XR vector. After in vitro packaging the libraries were titered. Flower library had a titer of 9.3X10^8 pfu/μl while x-ray irradiated tissue library had a titer of 2.2X10^8 pfu/μl.

cDNA Library screening

To identify a soybean homologue of Lim15, two oligonucleotide primers were designed to amplify out a 136 bp region of the soybean genomic fragment exhibiting high homology to Lim15 genes of lily and Arabidopsis. This fragment
was labeled with $^{32}$P dCTP and $3 \times 10^6$ pfu of the flower library was screened. The initial screen identified 5 plaques that gave a positive signal. On subsequent rounds of purification, only one plaque showed a strong hybridization signal to the amplified fragment. This 522 bp cDNA fragment had a very high sequence homology to domain II of Lim15; 94% to lily and Arabidopsis, and over 70% to human, mouse, *Xenopus laevis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Candida albicans* Rad51-like genes. Analysis of the sequence data indicated that this 522 bp cDNA was not a full length cDNA (Fig. 3.5). Although an open-reading frame was detected, the 5' end of the gene was missing. The flower library was then rescreened to attempt to isolate a full length cDNA. $1.2 \times 10^7$ pfu were screened using this partial cDNA as a probe and 9 plaques that gave strong hybridization signals were identified. These were carried through four rounds of purification and the 5 plaques that gave a strong hybridization signal were designated F1, F2, F3, F4, and F5.

**Characterization of the Isolated cDNAs**

To determine if these cDNA(s) were similar to each other, pBluescript phagemid were *in vivo* excised from all Uni-ZapXR™ vectors. pBluescript was digested with *EcoR I/Xho I* to release the cDNA, electrophoresed on a 1% agarose gel and the DNA was transferred to a nylon membrane. When membranes were cross hybridized to each isolated cDNA and to themselves, all the cDNA(s) hybridized to each other at the same intensity except F1 which did
so at a lower intensity. All five of the cDNA were then partially sequenced from
the 3' end (Appendix 19). Sequence data indicated that cDNA(s) F2, F3, F4, F5
were identical and showed homology to the partial cDNA used as a probe to
isolate these cDNA(s). Only the sequence of F1 was different.

Sequence Analysis of F5 and Predicted Protein

Double stranded full length nucleotide sequence of F5 was determined.
The nucleotide sequence is 1362 bp long. It has an open reading frame that is
1038 bp, a 71 bp 5' untranslated region and a 253 bp 3' untranslated region. A
polyadenylation signal of AATATTT is present at 1168 bp in the 3' untranslated
region; 187 bp up stream of the polyA tail (Fig. 3.6). The DNA sequence was
submitted to genbank using the bankit software and was assigned accession
number U-66836. Comparison of the cDNA to the other eukaryotic recA-like
gene and E. coli recA using the Pileup program shows that all the genes are
related to each other and the F5 cDNA is grouped in the Lim15-like group. The
Rad51-like genes are in another group (Fig. 3.7). The Pileup program places the
Lim15-like gene much closer to F5 when the analysis is done on the Lim15-like
genes alone (Fig. 3.8). The distance matrix, which calculates the pairwise
comparison, shows homology between 47-100% between the eukaryotic cDNAs
(Table 3.1). The prokaryotic recA has homology in the range of 24-29.8% to the
eukaryotic genes. Homology between the Lim15 group is in the 40-93% range
(Table 3.2). Using the Growtree program, phylogenetic analysis shows that
among all the recombinases, F5 is more related to Lim15-like genes (Fig. 3.9). Among the Lim15 genes, F5 is evolutionarily related to plant Lim15 genes (Fig. 3.10).

The predicted protein encoded by the cDNA is rich in non-polar amino acids, has an isoelectric point of 5.39, a charge of -7 and an extinction coefficient of 10480. The protein has 346 amino acids with a molecular weight of 37.47 kD, a start codon AUG at position 71 and a stop codon TAA at position 1106 (Fig. 3.6). Based on homology with Lim15, Domain I of the predicted protein consists of 68 amino acids and is coded by nucleotides between the region of 172 to 376. Domain II is coded by nucleotides between 377 to 1048 and consists of 224 amino acids. The protein has two nucleotide binding sites, both of which are in domain II; a walker A box with the consensus amino acid residues GEFRSGKT between 467 to 494 bp and a walker B box with consensus amino acid sequence of LLIVD between 743 to 758 bp. Pileup program places the predicted protein of F5, the Lim15-like protein and yeast DMC1 in a group together, while Rad51-like proteins are in another group (Fig. 3.12). Analysis of only Lim15-like proteins places the predicted protein of F5 closer to atm15 (Fig. 3.13). Distances matrix (GCG Madison WI) calculated for the entire proteins shows the homology of the eukaryotic protein to be in the 95-37%, while the homology to the RecA protein is in the range of 29-32% (Table 3.3). Distances calculates the homology among the Lim15 group to be between 60-96%. Growtree matrix places the F5 predicted protein in the Lim15 protein group and shows a common evolutionary
ancestor (Fig. 3.14). Among the Lim15 group, F5 is more related to atlim15 which most probably arose from a common ancestor (Fig. 3.15).

Clone F5, based on sequence similarity of the cDNA and the predicted protein, encodes a Lim15 homologue in soybean. Therefore, it will be referred to as soybean lim15 cDNA (sblim15). Sequence alignment of the Lim15-like proteins, DMC1 and recA protein along with the consensus is given in Fig. 3.16.

Genomic southern blot analysis of Sblim15

Leaf DNA of soybean was digested with various enzymes and hybridized with sblim15 cDNA coding region both at high and low stringency (Fig. 3.17 and 3.18). Few bands were detected at low and high stringency. When membranes were hybridized with the coding region, the 5' untranslated or the 3' untranslated regions of sblim15 the same banding patterns were observed. Of all the enzymes used to digest the genomic DNA, only EcoR I, BamH I, Hind III, Xba I, Sac I and Sau3A I digest the cDNA. Spe I does not digest the cDNA and apparently digests in one of the introns. Pst I, EcoR V, Xho I, Dra I and Kpn I does not digest the cDNA.

Conservation of Sblim15 in other species

Hybridization of Sblim15 cDNA with genomic DNA from tomato, tobacco, corn, wheat and buckeye resulted in a low number (2-3) hybridization signal in all species (Fig 3.19). This indicates that Lim15 might be an essential gene and
under strong evolutionary pressure. Since Lim15-like genes are conserved from lower eukaryotes to higher eukaryotes, they must be involved in some function that is essential for all organisms.

Expression patterns of Sblim15 in different soybean tissue

To determine tissue specific distribution of sblim15, northern blot analysis using the sblim15 cDNA coding region as probe showed the presence of two RNA species of 1.4 kb and 1.55 kb in all tissue (Fig. 3.20). To determine if the two species corresponded to the same transcripts, blots were probed with 5' untranslated and 3' untranslated region. The same two RNA species were seen. Furthermore, northern blot analysis of mRNA and total RNA indicates that both transcripts are polyadenylated. The transcripts are present in abundance in leaf and flower tissue. Another transcript of 2.5 kb was seen in field grown tissue. RNA transcripts were not enhanced in response to DNA damaging agents (Fig. 3.21)
Discussion

A genomic fragment was identified from a soybean post emergence seedling library that hybridized to Lim15 cDNA Domain I and II (Fig. 3.2). Shotgun subcloning and partial sequencing of one of the subclones identified a region that had a high sequence homology to the 3' end of Domain II of lily and exon13 of the Arabidopsis Lim15-like gene (Fig. 3.3). Some of the clones identified in the initial screenings were discarded due to loss of sites that were used to generate the library. Such cloning artifacts have been seen with lambda libraries. The 12.4 kb genomic fragment probably contains the full length soybean homolog of Lim15 based on the following findings: The region of homology was found in a subclone that was 3.8 kb long and the size of exon13 (136 bp) was the same size as in Arabidopsis. This leaves a 8.6 kb fragment which is upstream of this region of homology. The whole Arabidopsis genomic clone was in a fragment that is 5.09 kb where 1.3 kb are up stream of the AUG codon and 0.73 kb are down stream of the termination codon (Sato et al., 1995) (Fig. 3.3).

High titer cDNA libraries were obtained. Screening the libraries with the 497 bp EcoR I/Spe I fragment identified cDNA(s) that, upon sequencing, showed no homology to any Rad51-like or Lim15-like genes. Amplification of the exon13 region (Fig 3.3) from the EcoR I/Spe I subclone and use of this PCR product as a probe to screen the cDNA library allowed identification of a partial cDNA which was used to screen the flower library to obtain a full length cDNA. Similar
problems of obtaining full length cDNA were reported by Habu et al., (1996) and Bishop et al., (1992) for isolating the human, mouse and yeast genes for DMC1. Habu et al., (1996) used the 5' race technique to obtain a full length cDNA. There could be a secondary structure in the 5' end of the gene that may make full length cloning of this cDNA problematic. A number of direct and indirect repeats are present in the sblim15 gene which may contribute to problems with folding. However it is unclear where this secondary structure could be. A total of 1.2X10^6 pfu were screened to isolate this cDNA.

From the total 6 cDNA(s) isolated, sequence analysis indicated that 5 were almost identical and one was different. The cDNA(s) were sequenced from the 3' end and the sequences were identical to each other upstream of the polyA signal except for F1. Variations in transcripts at the extreme 3' end have been reported even from the same gene. This has been attributed to a weak polyA signal (Jang et al., 1996) which could also be present in sblim15. All the cDNA(s) hybridized to each other at the same intensity except F1 which hybridized at a weaker intensity. F1 might have some regions that are common to sblim15. F5 was one of the largest cDNA isolated and both strands were fully sequenced. It is 1362 bp long and has an open reading frame of 1038 bp which codes for a protein of 37.47 kD. The protein initiates at the first AUG and terminates at TAA. A polyA signal of AATATT is present 66 bp downstream of the stop codon. There is 87 bp of 3' untranslated region before the addition of the polyA tail. Domain I has 68 amino acids and this part of the protein has a
lower homology to Rad51-like proteins, while Domain II has very high homology to other Rad51 and Lim15-like genes. From sequence comparisons and crystallographic data analysis, it has been shown that an appreciable proportion of proteins that bind ATP or GTP share a number of more or less conserved sequence motifs (Walker et al., 1982). The most prominent of these is a glycine rich region which forms a flexible loop between a beta-strand and an alpha-helix (Fry et al., 1986). This loop interacts with one of the phosphate groups of the nucleotide. This sequence motif is generally referred to as the "A" sequence motif (Walker et al., 1982) or the "P" loop (Sarasle et al., 1990). The consensus sequence of the motif is GEFRSGKT which is present verbatim in sblim15. This motif is characteristic of RecA-like, Rad51-like, DMC1-like and all Lim15-like proteins. The glycine residue at the start of the loop is thought to begin ATP phosphate binding loop. Lys72 point mutation to Arg72 in E. coli results in a decrease in the catalytic activity of the protein indicating that it is involved in catalytic activity (Rehrauer and Kowalczykowski 1993). Thr is supposed to interact with Mg\(^{2+}\) and beta phosphate of the ADP-ATP (Logan and Knight 1993). Asp at 165 is thought to stabilize loop containing Gly167 by H-bond to the backbone of the Gly167 and Thr169 (Kowalczykowski 1991).

The beta nucleotide binding domain or the Walker B box is between amino acids 222 and 228. It is LLIVD and is also present verbatim. This structural relationship between Lim15, UvsX, DMC1 and Rad51, which are all related to RecA, suggests that they are all functionally similar. Presumably, the
existence of these several related genes reflects the type of functional
specialization that are well known for other types of proteins such as
topoisomerases, helicases and polymerases (Story et al., 1993). Different
proteins may have additional features not common to the family such as the
ability of bacterial RecA(s) to promote cleavage of certain repressor proteins and
UmuD.

Soybean sblim15 protein was compared to other Rad51, DMC1 and
Lim15-like proteins using the Pileup program of GCG. Pileup divides these
proteins into two groups; Rad51 and Lim15 (Fig. 3.12). In the Lim15 group,
sblim15 is closely related to the Arabidopsis protein and the lily protein (Fig.
3.13). Evolutionary analysis of the proteins is done by the Growtree program of
GCG (Fig. 3.14), where Lim15 and DMC1 are in one group and all the Rad-like
proteins are in another group.

Soybean genomic DNA was digested with several restriction enzymes,
probed with the coding region of sblim15 and washed at low and high stringency.
Two to four fragments were detected in each lane. The size and intensity of the
fragment suggests that it is a very low copy number gene (Fig. 3.17 and 3.18).
Digestion of genomic DNA from tomato, tobacco, corn and buckeye hybridization
to the sblim15 coding region and washing at moderately high stringency gave 2-3
bands where one band in each specie is more intense than the rest (Fig 3.19).
This pattern indicates that Lim15-like genes are essential genes which are
conserved in most of the species and may function in a similar manner.
The expression pattern of sblim15 was assayed by northern blot analysis. The expression is not consistent with DMC1, Rad51 and Lim15-like genes except for a two case (human and Arabidopsis Lim15). In all cases, Lim15 transcripts were detected only in meiotic tissue while, in human tissue, Lim15 transcript was detected in all tissue. In yeast, DMC1 transcript was detected in meiosis by northern hybridization analysis and was barely detected in mitosis (Bishop et al., 1992). Rad51 transcripts are specifically enhanced in response to DNA damaging agents and are also detected in mitosis and meiosis (Shinohara et al., 1993 and Basile et al., 1992). Lim15 transcript was detected by northern blot analysis in lily at prophase only (Kobayashi et al., 1994). In human and mouse, Habu et al. (1996) were able to detect two transcripts by northern and reverse transcriptase PCR. A 2.2 kb and a 1.9 kb message were detected in ovary and testis of mouse and human. On further characterization, Habu et al., (1996) found that these two transcripts were from the same gene and the shorter transcript is missing an exon. The deleted exon codes for the region between the two NTP binding domains which has important residues that are thought to be responsible for NTP interaction. This altered transcript might have a different function or may function independent of ATP. Kowalczykowski and Krupp (1995) and Posselli and Stasiak (1990) observed that the E. coli RecA did not require ATP to function and still had a strand exchange activity. No direct evidence of altered transcription in plants has been reported but, based on the mouse and human data, the altered protein from sblim15 would have 60 less amino acids.
(Fig. 3.22). Sato et al., (1995) reported detection of Lim15 transcript in human by reverse transcriptase PCR. A full length transcript was detected in testis, ovary and spleen. The reverse transcriptase PCR band was very strong in testis and not so strong in spleen and ovary. A smaller transcript which is missing 165 bp region between the two NTP binding domains was seen in all tissues. These partial disagreement in tissue specific transcription between Habu et al., (1996) and Sato et al., (1995) could be due to the sensitivity of their respective experimental system. Habu et al., (1996) used total cDNA synthesized using oligo(dt)$_{18}$ as template for their reverse transcriptase PCR reactions. On the other hand, Sato et al., (1995) preamplified the cDNA with Lim15 specific primers flanking the Lim15 coding region thereby considerably enriching for Lim15 cDNA for the second reverse transcriptase PCR reaction using primers that were internal to the coding region. This enrichment of transcript and subsequent detection of the message in all tissue indicates that the Lim15 gene is transcribed at very low levels in all tissue and the detection might depend on the technique used to detect the message. In mouse, Sato et al., (1995) were able to detect only one transcript in testis. This discrepancy can be explained by the choice of specific primers and template in the reverse transcriptase PCR reaction. The forward primer used for reverse transcriptase of mouse cDNA is in a region that is lost in the smaller human transcript (Sato et al., 1995, Habu et al., 1996). This primer combination would only amplify the larger transcript. In
addition, the template was not preferentially enriched for Lim15 transcript as in the human study (Sato et al., 1995).

In soybean, using northern blot analysis, two transcripts are seen in all tissue (Fig. 3.20). Both transcripts are poly-adenylated as they both can be detected using polyA and total RNA for northern hybridization analysis. Although the two bands could be an altered form of the same polyadenylated cDNA, the relationship between the two remains to be determined (Sato et al., 1995 and Habu et al., 1996). Jang et al., (1996) reported differential expression of Rad51 gene from Schizosaccharomyces pombe; where the same gene transcribes three different sized messages ranging from 1.3 to 1.9 kb depending on the stage of the cell cycle or the kind of stress imposed on the cell. Although Sato et al., (1995) did not report tissue-specific transcription, subsequent studies indicate that the Arabidopsis gene is transcribed in all tissue (S. Tabata personal communication). Northern hybridization analysis using total RNA isolated from field grown mixed pollinated leaf tissue yielded a third band of 2.5 kb. (Fig. 3.20). This could either be an additional transcript or an intermediate in RNA processing similar to the 3 kb transcript from Rad51-like gene seen only in ovaries and testis of Xenopus lavis (Maeshima et al., 1995). Using reverse transcriptase PCR in Xenopus lavis, expression of the Rad51 gene was observed in all tissue (Maeshima et al., 1995).

Sblim15 gene expression levels was evaluated after exposure soybean tissue to DNA damaging agents. No increase in transcript level of sblim15 was
observed from any of the treatments evaluated (3.21). Yamamato et al., (1996) reported the presence of Rad51 protein in every proliferating mammalian cell. The expression of Rad51 is dependent on the proliferative state of the cell (Yamamato et al., 1996 and Taki et al., 1996) which suggests that it is involved in recombination repair. Tsuzuki et al., (1996) introduced a mutation in the Rad51 gene of mouse and transmitted the mutation through the germ line. Mice that are heterozygous for the mutation are viable but in the homozygous state, the mutation results in a defective preimplantation. Sequence similarity and functional similarity of Rad51 proteins would lead to the assumption that the inactivation of Rad51 would lead to a germ line defective phenotype. However, Tsuzuki et al., (1996) observed that embryos homozygous for the rad51 null mutation rarely survived past the four cell stage. This indicates that in mouse and other higher eukaryotes, recombinase-like genes may have an essential role in mitosis. Tsuzuki et al., (1996) attempted but were unable to obtain homozygous developed embryos. There are several factors that could be attributed to the inability to obtain a homozygous embryonic stem cell (Tsuzuki et al., 1996).

It is clear that sblim15 gene plays an important function in vegetative cell growth as shown by the antisense inhibition of Rad51 gene (Taki et al., 1996). Enzymes involved in recombination may also function in repair. Why is Rad51 not critical for survival in yeast but essential in mouse? Tsuzuki et al., (1996) suggest that the Rad51-like gene products have acquired an essential novel role
in mouse. Noriok et al., (1995) reported that, in *Myxococcus xanthus*, which has 2 homologs of the bacterial recA gene, one of them is essential and the other is not. The cell cycle regulated expression of Rad51 is indicative of its function other than DNA recombination (Basile et al., 1992). DMC1 in yeast and its homologs in eukaryotes might also have another function in addition to their recombinase activity. This observation is based on the presence of an intron in yeast DMC1 gene, which is not common for yeast genes. Altered transcripts of the Lim15 have been detected in human and mouse (Sato et al., 1995, Habu et al., 1996). *Candida albicans* has a Lim15 homologue but it has never been known to undergo meiosis. The *Candida albicans* Lim15 promoter is functional in *Candida albicans* so it either undergoes cryptic meiosis or the Lim15 gene product has a yet unknown function (Diener and Fink 1996).

Sblim15 may also be involved in some function other than recombination such as a step in DNA replication. The protein might function to segregate newly replicated strands or have some role in the DNA replication machinery. The functional properties of sblim15 would have to be studied by constructing a plant where the gene can be inactivated at specific stages of development or in particular tissue. The identification of a gene involved in recombination in soybean is encouraging because it may permit manipulation of cellular genes in a predefined way (gene targeting). It is very desirable to control the process of foreign DNA integration by targeting DNA to predetermined genomic positions. Gene targeting would provide a valuable tool for studies of the function and
mechanisms of regulation of cellular genes. Complementation of bacterial and yeast mutants with the sblim15 gene may help identify functional roles of this gene. Clearly this research is a starting point in the exploration of a very interesting and important area of plant molecular biology.
Fig. 3.1 Epistasis groups for yeast RAD genes.
Fig. 3.2 Structure of the Lim15 gene and protein from lily.
Fig. 3.3 Position of Exon13 region on the soybean genomic fragment identified from a soybean genomic library. Positions of the primers used to amplify the region homologous to Exon13 are shown.
ACCGAACTCA NTTCCAAATTG ATTGTTTTGA AGCTGTTGTT 40
TACAACAAGC AGCAACATTA TCATTGATC ATTAGTTG 80
GCTTTTATGA GAAGACCAGA GGCATAATGT TTAATCTACG 120
TTACATTGTG AGTTTGGTT GCAGTCATAT CTGATCCAGG 160
AGGTGGCTGTG TCGTAACCTG ATCCAAAGAA ACCAGCAGGA 200
GGGCATGTGCG TAGTCCATGG CAGCACCAGT AAGGTTGATG 240
TTCAGGAAAG GGAAGGGGA ACAGCGCATT TGCAGAGTGT 280
TTGACGCCCC CAACCTGCCA GAGGCTGAAG CAATATCCTT 320
CTACCTGAGT GTTTGGACCT TCAAAATAAT CTTTGGTTG 360
AACAAAATAT GCCTGTAGCT AACTCAATGT TAGTGTTTCTT 400
GAAATCCGCC CCAACTCAGT AGTTACGAAA TTATGGGTTG 440
TAAAACATCA TGAAATTAGC TTACACATAC TTTAAATAA 480
CCAGCCACAC TAGT

Fig 3.4 Sequence of the EcoR I/Spe I subclone fragment. Regions showing high homology to Exon13 of Arabidopsis genomic clone for Lim15 and to the 3' of lily Lim15 cDNA are underlined.
Fig 3.5. Sequence of partial cDNA isolated from the flower library using PCR amplified Exon13 region as a probe. cDNA has very high homology to Lim15 and RAD51-like genes.
Fig. 3.6 Nucleotide sequence of the full length cDNA for sblim15 gene. Start codon, stop codon and polyA signal are highlighted.
Fig. 3.7 Pileup cluster diagram of DNA sequences from all recombinase-like genes. The program divides the sequences into two groups (abbreviations are explained in Appendix B)
Fig. 3.8 Pileup cluster diagram of DNA from all Lim15-like genes (abbreviations are explained in the Appendix B).
Fig. 3.9 Growtree analysis performed on DNA sequence from all recombinase-like genes (abbreviations are explained in the Appendix B).
Fig. 3.10 Growtree analysis performed on the DNA sequences of Lim15-like genes (abbreviations are explained in the Appendix B).
<table>
<thead>
<tr>
<th>Sequence</th>
<th>Description</th>
<th>Start Site</th>
<th>Domain Highlighted</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLATLKSEES SGQLQIVERE DIDDDDELPE AIDKLIAGGI</td>
<td>Predicted protein for sbim15 gene. Start codon and nucleotide binding domains are highlighted.</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>NAGDVKKLQD AGIYTONGLM MHTKKNLTGI KGLSEAKVDK</td>
<td></td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>ICEAAEKLVN FGYITGSDLAL LKRKSVIRIT TGSQALDELL</td>
<td></td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>GGGVETSAIT EAFGEFRSGK TQLAHTLCVS TQLPTNMRRG</td>
<td></td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>NGKVAYIDTE GTFRPDRIVP IAERFGMDPG AvLDNIIYAR</td>
<td></td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>AYTYEHQYNL LLGLAAKMSE EPFRLLIVDS VIALFRVDFS</td>
<td></td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>GRGELADROQQ KIAQMLSRLI KIAEEFNVAV YMTNQVISDP</td>
<td></td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>GGGVFVTDPK KPAGGHVLAH AATVRLMFRK GKEQIRICKV</td>
<td></td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>FDAPNLPEAE AVFQITAGGI ADAKD</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3.12 Pileup cluster diagram of the predicted protein sequences from all recombinase like genes. The program divides the sequences into two groups (abbreviations are explained in Appendix B).
Fig. 3.13 Pileup cluster diagram of protein sequences from all Lim15-like genes (abbreviations are explained in the Appendix B).
Fig. 3.14 Growtree analysis on the predicted protein sequences of all recombinase-like gene (abbreviations are explained in the Appendix B).
Fig. 3.15. Growtree analysis of the predicted proteins of the Lim15-like genes (abbreviations are explained in the Appendix B).
Fig 3.16. Aligned protein sequence of the Lim15-like proteins, DMC1 and RecA proteins. Conserved nucleotide binding domains are underlined ('-' on the consensus sequence line denotes no consensus; '.' in the protein sequence denotes gapping to produce optimal alignment of sequence).
Fig 3.16. Continued.
Fig. 3.17 Southern hybridization analysis of soybean genomic DNA hybridized to sblim15 cDNA at high stringency.
Fig. 3.18 Southern hybridization analysis of soybean genomic DNA hybridized to sblim15 cDNA at low stringency.
Fig. 3.19 Hybridization of sblim15 cDNA to genomic DNAs from various plant species.
Fig 3.20 Northern blot analysis of transcripts for sblim15. Total RNA from various tissue hybridized to sblim15 cDNA. (s. embryos = somatic embryos, Yng. leaf = young leaf, a. leaf = adult leaf, f = field tissue, gh = green house tissue)
root
stem
petiole
s. embryos
yng. leaf (f)
a. leaf (gh)
a. leaf (f)
closed flower (f)
open flower (f)
Fig. 3.21 Northern blot analysis of transcripts after subjecting soybean somatic embryogenic tissue to various DNA damaging agents and hybridizing to sblim15 cDNA.
<table>
<thead>
<tr>
<th>s. embryos</th>
<th>somatic embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dark</td>
</tr>
<tr>
<td></td>
<td>h. shock</td>
</tr>
<tr>
<td></td>
<td>ems</td>
</tr>
<tr>
<td></td>
<td>mms</td>
</tr>
<tr>
<td></td>
<td>x-ray</td>
</tr>
</tbody>
</table>

| 9.4 | 7.4 | 4.4 | 2.3 | 1.3 | 0.2 |

**fig 3-21**
Fig 3.22. Predicted protein for sblim15 gene. Start codon and nucleotide binding domains are highlighted. Predicted amino acids that would not be included in the altered transcript based on data from other Lim15-like genes are underlined.
Table 3.1 Pair wise comparison of DNA sequence of RecA-like genes. Percent nucleic acid identities across pair wise alignment was calculated using the distances program of Genetics Computer Group are shown (abbreviations are defined in Appendix B).
<table>
<thead>
<tr>
<th></th>
<th>Hulim15</th>
<th>Molim15</th>
<th>Sblim15</th>
<th>Atlim15</th>
<th>Lilim15</th>
<th>DMC1</th>
<th>RecA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hulim15</td>
<td>100</td>
<td>90.23</td>
<td>58.19</td>
<td>53.96</td>
<td>52.29</td>
<td>31.19</td>
<td>30.88</td>
</tr>
<tr>
<td>Molim15</td>
<td></td>
<td>100</td>
<td>57.84</td>
<td>53.81</td>
<td>52.15</td>
<td>29.95</td>
<td>31.52</td>
</tr>
<tr>
<td>Sblim15</td>
<td></td>
<td></td>
<td>100</td>
<td>76.24</td>
<td>72.93</td>
<td>32.59</td>
<td>30.54</td>
</tr>
<tr>
<td>Atlim15</td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>66.74</td>
<td>30.49</td>
<td>29.93</td>
</tr>
<tr>
<td>Lilim15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>30.49</td>
<td>29.27</td>
</tr>
<tr>
<td>DMC1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td>28.4</td>
</tr>
<tr>
<td>RecA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

Table 3.2 Pairwise comparison of sequences of RecA-like genes. Percent DNA homology across pairwise alignment was calculated using the Distances program (abbreviations are defined in Appendix B).
Table 3.3 Pairwise comparison of sequence of RecA-like proteins. Percent amino acid identities across pairwise alignment was calculated using the distances program of the Genetics Computer Group are shown (abbreviations are defined in Appendix B).
Table 3.4 Pairwise comparison of sequences of RecA-like proteins. Percent amino acid identities across pairwise alignment was calculated using the Distances program (abbreviations are defined in Appendix B).
Bibliography


148


McKee B.D., Ren X.J. and Hong C.S. 1996. A recA like gene in *D melanogaster* that is expressed at high levels in female but not in male meiotic tissue. Chromosoma. 104:479-488.


Program manual for Wisconsin package. ver 8 sept. 1994. GCG 575 Science Dr. Madison WI.


General Discussion

In my dissertation work various aspects of recombination in soybean were studieds.

Co-transformation of twelve different plasmids as a single cocktail showed that extensive extrachromosomal recombination occurs in soybean somatic embryos (cv 'Fayette'). There appears to be no preferential uptake of any of the twelve plasmids and concatemer formation indicative of homologous recombination was also observed. In light of this observation, the next study was designed to evaluate the enhancement of extrachromosomal homologous recombination by over-expression of a recombinase gene. Two overlapping deletions of the reporter gene GUS were constructed and co-introduced into a soybean embryogenic cell line (cv 'Chapman') either with or without a recombinase gene, which was cloned in either the sense or antisense orientation in a plant expression vector. The recombination events would occur extrachromosomally and be scored with the appearance of a functional GUS enzyme. If an enhancement in GUS expressing foci in the cell lines co-transformed with the sense recombinase construct was observed, this would
conclusively show that the recombinase enzyme was involved in some step of the recombination pathway. Unfortunately, GUS expressing foci indicative of recombination events were not detected by GUS staining. Due to the extremely low endogenous levels of recombination events in the soybean cell line, it was not possible to determine whether or not Lim15-like proteins are involved in recombination. Similar studies in transgenic cell lines containing a nuclear targeted gene under the regulatory control of a constitutive promoter might shed some light on the function of Lim15-like genes in the recombination pathway.

The isolation of a cDNA that encodes a Lim15-like protein from soybean which is a bonified homolog of the bacterial RecA protein, yeast Rad51 protein and yeast DMC1 protein was very encouraging. The presence of this cDNA shows that at least some of the enzymes involved in recombination are conserved from prokaryotes to far more complex higher eukaryotes. The expression pattern of this gene indicated that, in addition to its function as a recombinase, it might also be involved in some other aspect of DNA metabolism. Identifying the genes involved in recombination in plants is still challenging. The isolation and characterization of yet more genes involved in recombination will help us to eventually understand and manipulate the process of recombination.
LIST OF REFERENCES


Christou P., Tameria L.F. and Kofron M. 1991. Production of transgenic rice (Oryza sativa L.) plants from agronomically important indica and japonica
varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Biotechnology. 9:957-962.


161


163


McKee B.D., Ren X.J. and Hong C.S. 1996. A recA like gene in *D. melanogaster* that is expressed at high levels in female but not in male meiotic tissue. Chromosoma. 104:479-488.


Program manual for Wisconsin package. ver 8 sept. 1994. GCG 575 Science Dr. Madison WI.


ATP requiring enzymes and a common nucleotide binding fold. EMBO J. 1:945-951.


APPENDIX A

Protocols and Solutions

1. Genomic DNA Extraction from Plant Tissue (Saghi-Maroof et al., 1984)

Transfer 300-400 mg of powdered tissue to a 16 ml polypropylene tube.

Add 8 ml of CTAB (Hexadecyltrimethyl ammonium bromide) extraction buffer.

Mix by inverting tube. Incubate 30-60 min at 60°C with occasional mixing.

Remove samples in a rack from bath and let cool for 10 min.

Add 4.5 ml of Chloroform/Octanol (24:1) and rock gently to mix.

Spin in table top centrifuge for 10 min at 2500 rpm (1100 Xg).

Carefully remove aqueous layer and decant into a 16 ml tube containing 5.33 ml of Isopropanol, mix gently by inversion.

Immediately remove precipitated DNA with a glass hook and transfer to a 5 ml tube containing 2-3 ml 76% ethanol, 0.2 M sodium acetate for 20 minutes.

Dip DNA on hook briefly in 1.5 ml of 76% ethanol, 10 mM ammonium acetate.

Dissolve DNA in 400 μl of TE over night.

Next day, centrifuge out any undissolved solids in microfuge at high speed for 10 min.

CTAB Extraction Buffer

<table>
<thead>
<tr>
<th>Chemical</th>
<th>for 500 ml</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAB</td>
<td>10 g</td>
<td>2 %</td>
</tr>
<tr>
<td>Tris HCl 1M (pH 8.0)</td>
<td>50 ml</td>
<td>100 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>40.9 g</td>
<td>1.4 M</td>
</tr>
<tr>
<td>EDTA 0.5 M</td>
<td>20 ml</td>
<td>20 mM</td>
</tr>
<tr>
<td>H2O</td>
<td>430 ml</td>
<td></td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>5 ml</td>
<td>1 %</td>
</tr>
</tbody>
</table>
2. Dry Blot (Kempler et al., 1991)

After electrophoresis, depurinate gels in two volumes of 0.25 N HCl until the bromophenol blue marker dye turns yellow.

Rinse gels with ddH₂O and denature gels in two volumes of 0.4 M NaOH (2 x 15 min).

Rinse gels with ddH₂O and equilibrate in two volumes of 0.25 M Tris acetate, 0.1 M NaCl, pH 8.0 for 15 min.

Rinse gels with ddH₂O and equilibrate gels in two volumes of 0.025 M Tris acetate, 0.1 M NaCl, pH 8.0 for 15 min.

After spreading out a sheet of saran wrap, the gel is laid on a sheet of Whatmann 3MM paper soaked in 0.025 M Tris acetate, 0.1 M NaCl, pH 8.0.

Wet a membrane in 0.025 M Tris acetate, 0.1 M NaCl, pH 8.0 and place on top of gel. Roll out the bubbles with a pipette. Cover the membrane with a sheet of Whatmann 3MM paper soaked in 0.025 M Tris Acetate, 0.1 M NaCl, pH 8.0, roll out the bubbles. Complete the stack with 4 sheets of dry Whatmann 3MM paper and a 4 cm stack of paper towels. Add weight of 1 kg (sigma catalog).

Blot for 1-4 hours. Wash membrane in 2X SSC briefly. Fix the DNA to the membrane according to manufacturer’s recommendations.
3. Random Primed DNA Probe (Feinberg and Vogelstein, 1983)

Mix H$_2$O and 30 ng of DNA to a final volume of 10 µl.

Boil for 5 min and immediately chill on ice.

Spin tube to collect solution at the bottom and add 11.5 µl L.S. mix.

Add 1 µl BSA (10 µg/µl stock).

Move behind Plexiglas shield and add dCT$^{32}$P (2-5 µl).

Add one unit klenow fragment and incubate at room temperature for 2-4 hours (could also go overnight).

| L.S MIX Stock/ml | (add in the order below)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>118 µl</td>
</tr>
<tr>
<td>Hepes pH 6.6</td>
<td>250 µl</td>
</tr>
<tr>
<td>Random Primers (P$(dn)<em>6$, 50 A$</em>{260}$ U dissolve in 1 ml TE pH 8.0)</td>
<td>252 µl</td>
</tr>
<tr>
<td>dT$	ext{M}$</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

| dT$	ext{M}$ Stock/µl | (add in the order below)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TM</td>
<td>997 µl</td>
</tr>
<tr>
<td>dATP</td>
<td>1 µl</td>
</tr>
<tr>
<td>dGTP</td>
<td>1 µl</td>
</tr>
<tr>
<td>dTTP</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

| TM Buffer Stock/Add | (add in the order below)
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris pH 8.0</td>
<td>250 µl</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>25 µl</td>
</tr>
<tr>
<td>BME</td>
<td>3.49 µl</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>721.5 µl</td>
</tr>
</tbody>
</table>
4. Removal of Unincorporated Nucleotides from Klenow Reaction

Equilibrate a Sephadex G50 nick column with 5 fillings of TE.

Bring the volume of Klenow reaction to 100 µl with TE.

Add the diluted Klenow reaction to the bead layer of G-50 column.

Add 400 µl of TE to the column and allow to pass through the column.

Add 400 µl of TE and collect the elute in a microfuge tube.

Read 1 µl of elute in the scintillation counter.
5. Southern Blot Hybridization

Prehybridization and Hybridization Solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final</th>
<th>Stock</th>
<th>200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>5 X</td>
<td>20 X</td>
<td>50 ml</td>
</tr>
<tr>
<td>Dnetharts Solution</td>
<td>5 X</td>
<td>100 X</td>
<td>10 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5 %</td>
<td>10 %</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td>130 ml</td>
</tr>
</tbody>
</table>

Boil ssDNA for 5 minutes and add to H2O first.

Prehybridize for 6 to 8 hours at 65°C.

Hybridize for 24-48 hours at 10°C below Tm.

Washing Blots

Heat 2 L of 2X SSC and 0.1% SDS to hybridization temperature.

Double glove. Cut one corner of the seal a meal bag and drain the hybridization solution into the liquid waste container.

Remove blots and place in a cake pan containing 400-500 ml of 2X SSC and 0.1% SDS. Swirl membranes in the solution and discard wash solution.

Wash membranes in 2X SSC, 0.1% SDS in 400-500 ml batches changing every 5-10 min in the water bath (set at hybridization temperature).

Follow with 2 l of 0.1X SSC, 0.1% SDS (this varies with stringency).

Dry membrane briefly and expose to film at -80°C.
5. Southern Blot Hybridization (continued)

DNA Probe Stripping

Wash membrane in 0.4 M NaOH for 30 min.

Neutralize for 30 min in

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 7.5)</td>
<td>1 M</td>
<td>200.0 ml</td>
</tr>
<tr>
<td>SSC</td>
<td>20 X</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>292.5 ml</td>
</tr>
</tbody>
</table>
6. Total RNA Extraction from Plant Tissue

Place 500 mg of fresh or frozen tissue in a 15 ml tube.

Add 1 ml grinding buffer.

Add 1 ml of water saturated phenol.

Polytron 1 min at high speed (keep on ice).

Add 1 ml of chloroform.

Vortex 1 min at high speed.

Centrifuge 10 min at 9750 Xg in swinging bucket rotor.

Carefully remove aqueous phase into a clean 15 ml tube.

Add 2 ml chloroform, and vortex 1 min at high speed.

Centrifuge as before for 5 min.

Make 2M in LiCl (8M), 2M in Urea (8M), and 1mM EDTA (500 mM).

add to 1 ml supernatant 500 μl LiCl, 500 μl urea, and 4μl EDTA in 2 ml tube.

add to 750 μl supernatant 375 μl LiCl, 375 μl urea and 2 μl EDTA in a 1.5 ml tube.

Precipitate overnight at 4°C.

Centrifuge 30 min in cold microfuge.

Resuspend pellet in 300 to 500 μl of pellet resuspension solution.

Pellet debris in microfuge for 5 min at 4°C.

Transfer supernatant to a clean tube.

Add 30 μl of 3M NaOAC and 750 μl of EtOH. precipitate 2 hrs at -20°C.

Resuspend pellet in 300 μl of 0.1X TE.

Repeat EtOH precipitation.

Resuspend RNA in 20-50 μl of 0.1X TE.
6. Total RNA Extraction from Plant Tissue (continued)

Grinding buffer

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Final</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 8.0)</td>
<td>1 M</td>
<td>50 mM</td>
<td>5 ml</td>
</tr>
<tr>
<td>Na pAmino Salicylic Acid</td>
<td></td>
<td>4 %</td>
<td>4 g</td>
</tr>
<tr>
<td>Na Naphthalene 1,5 disulphonic Acid</td>
<td></td>
<td>1 %</td>
<td>1 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td>95 ml</td>
</tr>
</tbody>
</table>

Keep in a dark bottle at room temperature.

Pellet Wash Solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Final</th>
<th>100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 7.5)</td>
<td>1 M</td>
<td>40 mM</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Na Acetate</td>
<td>3 M</td>
<td>20 mM</td>
<td>0.67 ml</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>0.5 M</td>
<td>5 mM</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>1 %</td>
<td>1 g</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td></td>
<td>94.33</td>
</tr>
</tbody>
</table>
7. Poly A RNA Isolation

Dissolve total RNA in TE or water.

Hydrate oligo (dt) cellulose by washing three times in NaTES at room temp. Final concentration of oligo (dt) should be 100 mg/ml.

Heat in a 1.5 ml microfuge tube 1 mg of total RNA in 600 µl of TES to 65°C for 5 min. Chill on ice.

Add 1/10 volume 5 M NaCl.

Add 600 µl of oligo (dt) slurry. Mix at room temperature.

Incubate 10 min at 37°C inverting every 3 min.

Spin 12000 xg at room temp for 5 min.

Wash Pellet with 1 ml NaTES

Wash pellet with 1 ml ice cold water

Resuspend pellet in 400 µl dddi water and heat for 5 min at 55°C.

Spin and save the supernatant. Repeat twice and combine supernatants.

Ppt samples with 2 M ammonium acetate and 2 volumes of 100% ethanol.

Typical yields are 20-25 µg.

NaTES

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5 M</td>
<td>0.5 M</td>
</tr>
<tr>
<td>Tris-HCl (pH 7.5)</td>
<td>1 M</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>0.5 M</td>
<td>1 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>

TES

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 7.5)</td>
<td>1 M</td>
<td>10 mM</td>
</tr>
<tr>
<td>EDTA (pH 8.0)</td>
<td>0.5 M</td>
<td>1 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
<td>0.1 %</td>
</tr>
</tbody>
</table>
8. RNA BLOT ANALYSIS

For a 100 ml 1.4% agarose/formaldehyde gel mix:
- 1.4 g agarose
- 88 ml dd H₂O
- 50 µl DEPC

Incubate 30 min RT while stirring
Autoclave with stirbar
Cool to 55°C

Add
- 10 ml 10X gel buffer
- 3 ml formaldehyde

Cast gel for 30 min.

Resuspend 35 µg of total RNA or 5 µg poly A⁺ in 4-4.5 µl of H₂O
Add
- 10 µl deionized formamide
- 3.6 µl formaldehyde
- 2 µl 10X gel buffer
- 1 µl EtBr (1 mg/ml)

Heat to 55 °C for 15 min
Add 2.2 µl 10X dye buffer
Load and run in 1X gel buffer <15V per cm for 9 cm in the hood.

Capillary Transfer

Wash gel in dd H₂O for 5 min x 3.
Soak a piece of membrane in ddH₂O and then 10X SSC.
Place sponges in a tray of 10X SSC, hydrate completely. Cover with 2 layers of Whatmann 3MM.
Place gel on filter paper. Frame gel with saran wrap.
Lay membrane on top of gel. Remove bubbles.
Place 2 saturated Whatmann 3MM filters cut to size on the membrane.
Cover with 2 dry Whatmann 3MM filters cut to size on the wet paper.
Stack 10 cm of paper towels plus weight (1kg).
Transfer overnight.

Remove towels and Whatmann filters. Mark wells
Wash membrane in 2X SSC for 5 min.
Bake at 100°C for 2 hours.
8. RNA BLOT ANALYSIS (continued)

Prehybridization Mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate pH 6.5</td>
<td>0.5M</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>0.1 M</td>
</tr>
<tr>
<td>SSC</td>
<td>5X</td>
</tr>
<tr>
<td>SDS</td>
<td>5%</td>
</tr>
</tbody>
</table>

Hybridization Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate pH 6.5</td>
<td>0.5M</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>0.1 M</td>
</tr>
<tr>
<td>SSC</td>
<td>5X</td>
</tr>
<tr>
<td>SDS</td>
<td>5%</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>10%</td>
</tr>
<tr>
<td>Formamide</td>
<td>50%</td>
</tr>
<tr>
<td>YtRNA</td>
<td>50 μg/ml</td>
</tr>
<tr>
<td>ssDNA</td>
<td>50 μg/ml</td>
</tr>
</tbody>
</table>

Hybridize at 47°C with shaking.

Wash membrane for 15 min in several changes of 1X SSC, 5% SDS at RT.
Wash membrane for 30 min in 500 ml of 0.1X SSC, 5% SDS at 50°C.
Wash membrane with 0.1X SSC at 50°C for 5 min.
Dry membrane briefly, place on film.

10X Gel Buffer

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS pH 7.0</td>
<td>200mM</td>
</tr>
<tr>
<td>NaOAc</td>
<td>50 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>10mM</td>
</tr>
</tbody>
</table>

10X dye Buffer

0.5 % Xylene cyanol in DEPC H₂O.
9. LAMBDA PLAQUE LIFTS

Pour 150 mm LB agar plates and dry well.

Start 5-10 ml LB culture of *E. coli* indicator strain, shake 37°C overnight.

Add 50,000 phage to 600 μl of *E. coli* overnight culture.

Incubate 37°C for 20 min to adsorb phage.

Add 9 ml of melted top agarose (<50°C) mix and immediately pour on plate.

Store at room temperature for at least 20 min.

Incubate 37°C overnight.

Place plates at 4°C for at least 2 hour.

Place filter on plate and key with 22 gauge needle + ink.

Keep first filter on just until wet.

Keep second filter on for 1 min.

Keep third filter on for 2 min.

Denature, neutralize and fix DNA on filters according to manufacturers recommendations.

Prehybridize using 3 ml per 150 mm filter.

Hybridize overnight in the same solutions amended with 250 μg/ml boiled and chilled ssDNA.

Prehybridization and Hybridization Solution

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final</th>
<th>Stock</th>
<th>200 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSC</td>
<td>5 X</td>
<td>20 X</td>
<td>50 ml</td>
</tr>
<tr>
<td>Denharts Solution</td>
<td>5 X</td>
<td>100 X</td>
<td>10 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5 %</td>
<td>10 %</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td></td>
<td>130 ml</td>
</tr>
</tbody>
</table>
10. Large Scale λ Phage DNA Isolation

Mix 50 μl of undiluted phage suspension with 100 μl of fresh overnight culture of indicator strain. Incubate 15 min at 37°C.

Inoculate phage broth with this mixture. Shake ON at 37°C.

Centrifuge for 15 min at 7700 × g.

Add 6 g NaCl (1 M final) per 100 ml and swirl gently to mix.

Pour supernatant into a clean flask and add 20 μl DNase (1.5 mg/ml), and 375 μl RNase1 (10 mg/ml). Incubate 37°C for 30 min.

Add 9 g PEG 8000 and swirl until dissolved. Chill on ice for 2 hours.

Centrifuge for 30 min at 10000 × g and discard supernatant.

Resuspend in 7 ml phage extraction buffer and extract with equal volume of CHCl₃.

Add 1/10 volume 5% SDS, 1/40 volume 0.2 M EDTA and 6.25 μl Proteinase K (20 mg/ml [so μg/ml final]). Incubate for 15 min at 60°C.

Add equal volume of Phenol:CHCl₃ and vortex; incubate for 20 min at RT.

Transfer to a 30 ml corex tube and spin for 10 min 4°C at 13800 × g.

Collect aqueous layer, add 1 ml 2 M NaCl (0.2 M final) and 15 ml EtOH; incubate ON at -20°C.

Pellet DNA by centrifugation for 15 min 4°C at 10000 × g.

Resuspend pellet in 2 ml H₂O, add 4 ml EtOH to precipitate DNA. Repeat twice.

Resuspend final pellet in 500 μl T 1/10E.
10. Large Scale λ Phage DNA Isolation (continued)

**Phage Broth**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Bacto tryptone</td>
<td>8 g</td>
</tr>
<tr>
<td>MgCl₂6H₂O</td>
<td>2 g</td>
</tr>
</tbody>
</table>

**Phage Extraction Buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Final</th>
<th>250 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl pH 7.5</td>
<td>1 M</td>
<td>50 mM</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>10 mM</td>
<td>0.3 g</td>
<td></td>
</tr>
</tbody>
</table>
11. *In Vivo* Excision of pBluescript phagemid from Uni-Zap-XR Vector

Core the plaque of interest from the agar plate and transfer to a 500 μl SM buffer and 20μl of chloroform.

Vortex for 4 hrs at room temp.

In a 15 ml falcon tube mix 250 μl of phage stock with 200 μl of XL1-Blue MRF' and 1X10⁶ pfu of Exassistent helper phage.

Incubate at 37°C for 20 min.

Add 3 ml of LB broth and continue incubation with shaking for 3 hrs.

Heat the tubes at 65°C for 15 min and then spin at 1000Xg for 15 min.

Decant the supernatant into a sterile tube.

Add 200 μl of SOLR cells OD₆₀₀ 1.0 to 10 μl of supernatant.

Incubate at 37°C for 20 min.

Plate on LB-ampicillin plates and incubate overnight at 37°C.
12. Colony Hybridization

Start bacteria on LB agar plates with appropriate antibiotics.

Grow for 8 hours, then streak the plate with probe-containing bacteria and grow another 2 hours.

Chill plate for 2 hours.

Label gene screen filter with indelible marker.

Place filter on the plate and key with 22 gauge needle + ink.

Peel off the filter.

Place filter on a sheet of 3M paper (DNA side up) 0.5 M NaOH saturated with denaturation solution till colonies are glossy.

Neutralize filter for 5 min in 1 M Tris pH 7.5.

Transfer filter to salt solution for 5 min; 0.5 M Tris, 1.5 M NaCl

Shake filter in chloroform for 5 sec.

Bake at 60°C for 20 min.
13. Paper Slurry DNA Recovery

Paper Slurry
Cut a 50 cm² piece of Whatmann 3MM paper into pieces about 5 mm x 5 mm. Add 40 ml of TE and shake vigorously by hand for 5 min (can be kept for 6 months in a refrigerator).

0.25 % Linear polyacrylamide

Add together:
- 50 mg acrylamide
- 20 µl 2.0 M Tris-HCl, pH 8.0
- 6.5 µl 3.0 M Sodium Acetate
- 2 µl 0.5 M EDTA, pH 8.0

Adjust volume to 1 ml with dH₂O.
Add:
- 20 µl 10 % (w/v) Ammonium persulfate
- 2 µl Temed

Incubate at room temperature for 30 min.
Add 2.5 ml cold 95 % Ethanol.
Centrifuge for 2 min at 1000 xg.
Discard the supernatant and dissolve the pellet in 20 ml H₂O.
Acts as a carrier and can be kept for 1 year at 4°C.

Procedure

Make a sieve tube by punching a hole in the bottom of a 0.5 ml microfuge tube with a 22 gauge needle and placing 100-200 µl of paper slurry into it.

Place the sieve tube in a 1.5 ml microfuge tube and spin briefly to remove excess buffer.

Place the agarose block containing the DNA in the sieve tube and centrifuge at 16000 xg for 10 min at 4°C.

Add 7.5 µl (approx 20 µg) 0.25% linear acrylamide
1/10 volume 3 M sodium acetate
2.5 volumes cold 95% ethanol
Incubate at -20°C for 15 min to overnight.
Centrifuge at 16000 xg for 15 min at 4°C.
Discard supernatant and wash pellet with 70% Ethanol.
Dry and resuspend in appropriate amount of TE.
14. RAPID ALKALINE PLASMID PREP

Grow a 50 ml overnight culture of cells in TB at 37°C.

Centrifuge cells at 5000 xg for 5 min, pour off supernatant.

Resuspend cells in 2 ml solution I and transfer to 15 ml tube.

Add 4 ml of fresh solution II, mix. Incubate for 5 min at RT.

Add 3 ml of ice cold KOAc. Invert sharply several times. Incubate 10 min on ice.

Centrifuge at 12000 xg for 20 min.

Transfer 8 ml of supernatant and warm to RT, add 4.8 ml of RT isopropyl alcohol.

Incubate RT for 15 min.

Centrifuge at 12000 xg for 20 min.

Pour off supernatant and wash pellet first with 5 ml of 70%, then with 100% ETOH. Dry pellet.

Resuspend pellet in 1.5 ml TE.

Extract twice with 1:1 phenol:CHCl₃ Extract twice with CHCl₃

Precipitate RNA/protein with 1.5 ml cold NH₄OAc (pH 5.2).

Incubate on ice for 10 min. Centrifuge at 12000 xg for 20 min.

Transfer supernatant to 15 ml corex tube.

Precipitate DNA with 7.5 ml cold 95% ETOH.

Centrifuge to collect DNA.

Dry and resuspend pellet in 1.7 ml of TE. Add 2 µl of RNase cocktail (Promega Corp. Madison, WI).

Incubate 37°C for 15 min.

Add 635 µl 5M NaCl and 940 µl 30% PEG 6000. Mix well incubate O/N at 4°C.

Centrifuge 12000 xg for 30 min.

Drain pellet well. Wash with 70%, and then 100% ETOH.

Dry under vacuum. Resuspend pellet in 200 µl TE.
### Solution I

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Stock</th>
<th>Add for 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20%</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>Tris-HCl pH 8.0</td>
<td>1 M</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>EDTA pH 8.0</td>
<td>500 mM</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td>191 ml</td>
</tr>
</tbody>
</table>

### Solution II

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>STOCK</th>
<th>Add for 50 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH (make fresh)</td>
<td>3 M</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>10 %</td>
<td>5 ml</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td>41.70 ml</td>
</tr>
</tbody>
</table>

### SOLUTION III

<table>
<thead>
<tr>
<th>CHEMICAL</th>
<th>STOCK</th>
<th>Add for 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium Acetate</td>
<td>5 M</td>
<td>60 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td></td>
<td>11.5 ml</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td>28.5 ml</td>
</tr>
</tbody>
</table>
15. Operation of Particle InFlow Gun

Particle Preparation
Weigh 50 mg of tungsten (M10 Sylvania Towanda PA) into a sterile microfuge tube. Sterilize the particles in 500 µl of 95% ethanol for 20 min. Wash the particles 4 times with sterile water by repeated centrifugation and vortexing. Resuspend in a final volume of 500 µl of sterile water.

Tissue Preparation
A variety of petri dishes can be used. For soybean clumps of tissue is placed in a 3.5 cm dish and the tissue is air dried in a laminar flow hood for 15 min.

Bombardment
Adjust the helium pressure in the line to the solenoid to between 60 to 80 PSI. Turn the vacuum pump on and close the vacuum vent line.

Add 25 µl of particles to 5 µl of DNA (1 µg/ml). Precipitate the DNA by adding 25 µl of 2.5 M CaCl₂ and then immediately add 10 µl of 100 mM spermadine. Vortex and incubate on ice for 5 min. Remove 50 µl of supernatant and discard. Keep coated particles on ice till ready to use.

Open the syringe filter and place the top and bottom in a microfuge rack. Finger vortex coated particles and remove 2 µl; place in the middle of the syringe filter and reassemble the filter unit. Place the Petri dish containing the dehydrated soybean tissue covered by a baffle (consisting of a screen melted to the bottom of a beaker) on the adjustable Plexiglas shelf. Install the filter containing the coated particles in the filter holding unit.

Close the Plexiglas door of the particle in flow gun while pulling a vacuum on the gun chamber. After vacuum has reached about 28 to 30 inches Hg, activate the solenoid and release the particles.

Close the vacuum line and slowly open the vent valve. It should take approximately 30 seconds for the chamber to vent and the door to open. Remove the tissue and incubate 15 min in the laminar flow hood covered. Transfer to appropriate media.
16. Histochemical GUS Assay

Place tissue in a multiwell plate. Remove as much media as possible.

Pipette GUS assay mix so as to almost submerge tissue.

Incubate overnight at 37°C with gentle agitation.

Count the number of blue foci under a dissecting microscope.

GUS Assay Mix

<table>
<thead>
<tr>
<th>Chemical</th>
<th>100 ml assay mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.37 g</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>100 µl</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>4.8 g</td>
</tr>
<tr>
<td>K₃Fe(CN)₆</td>
<td>66 mg</td>
</tr>
</tbody>
</table>

Bring up to 95 ml and adjust pH to 7.0 with 10 N NaOH

Add 25 mg X-GLU in 1 ml DMSO

Bring up to 100 ml
17. **Fluorometric GUS Assay** (Jefferson et al., 1987)

Grind plant tissue in extraction buffer on ice. Centrifuge extract for 5 min in a microfuge at full speed.

Extract can be kept at -80°C for up to 2 months.

Measure fluorescence according to fluorometer instructions.

**Extraction Buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄ (pH 7.0)</td>
<td>50 mM</td>
</tr>
<tr>
<td>Na₂EDTA (pH 8.0)</td>
<td>10 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1 %</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.1 %</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>10 mM</td>
</tr>
</tbody>
</table>
18. PCR Assay for Putative Transformants

Place the tissue (leaf or callus) in a 1.5 ml microfuge tube.

Add a pinch of acid washed autoclaved sand and 100 μl (200 μl if using leaf) of extraction buffer.

Grind with disposable microfuge pestle till the solution is milky.

Add another 300 μl of extraction buffer and finger vortex.

Centrifuge for 5 min at full speed, and transfer 300 μl of supernatant to a clean tube with a pipette.

Extract with equal volume of chloroform.

Transfer supernatant to a clean tube.

Add 300 μl of room temperature isopropanol and incubate at room temp. for 2 min.

Centrifuge as before for 5 min and wash pellet with 70%, 90% ethanol; speed vac.

Dissolve pellet in 100 μl TE.

Use 10 μl as template in a 50 μl PCR reaction.

**Extraction Buffer**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl (pH 5.7)</td>
<td>200 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>250 mM</td>
</tr>
<tr>
<td>EDTA</td>
<td>25 mM</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
</tr>
</tbody>
</table>
19. Sequencing with Sequenase

Annealing of Primer to Template

1 µl primer (0.5 pmole/µl)
2 µl 5X reaction buffer
7 µl DNA template

Heat to 65°C for 2 m in a heating block and remove heating block with the samples. Allow to cool slowly to 30°C over at least 30 min. Place on ice. Use within 4 hours.

Labelling

Dilute dGTP labelling reaction mix five-fold.
Dilute Sequenase 1:8 in ice cold enzyme dilution buffer.

Add to the annealing reaction tube

1 µl DTT (0.1 M)
2 µ diluted labelling mix
0.5 µl 35S dATP
2 µl diluted Sequenase (add last)

Centrifuge briefly and incubate 3-5 min at room temperature

Termination

Label four tubes A, C, G and T.

Add 2.5 µl of appropriate dNTP to each tube and warm to 37°C.

Add 3.5 µl of labelling reaction into each tube, mix and incubate at 37°C for 3-5 min.

Add 4 µl of stop dye.
19. Sequencing with Sequenase (continued)

Preparation of a 6% Acrylamide Gel.

Mix in a 200 ml flask

- 12 g acrylamide
- 0.6 g bis-acrylamide
- 84 g urea
- 18 ml 10X TBE
- 100 mg ammonium persulfate
- water to a final volume of 200 ml.

When ready to pour add 50 µl of TEMED. Degas by pulling solution in a syringe and pour gel.
After pouring insert comb upside down into gel 1-2 mm and clamp the comb to the plates.

Running Sequencing gel

After gel polymerizes, remove the comb and clamp it into the electrophoresis unit and pre-run for 40 min.

Boil samples for 5 min. Chill on ice and load 3 µl per lane.

Run gel to the desired length for optimal resolution.

When Run is Over

Pry open the plate-gel sandwich.

Fix gel on plate in 10% Acetic acid, 10% Methanol for 30 min.

Transfer gel to Whatmann paper and dry for 4 hours.

Exposure to film for at least 2 days.
APPENDIX B

List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Atlim15d</td>
<td><em>Arabidopsis thaliana</em> Lim15-like gene</td>
</tr>
<tr>
<td>Atlim15p</td>
<td><em>Arabidopsis thaliana</em> Lim15-like protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>ChRad51d</td>
<td><em>Gallus gallus</em> Rad51-like gene</td>
</tr>
<tr>
<td>ChRad51p</td>
<td><em>Gallus gallus</em> Rad51-like protein</td>
</tr>
<tr>
<td>CTAB</td>
<td>Hexadecyltrimethyl ammonium bromide</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>dddi</td>
<td>double distilled de-ionized</td>
</tr>
<tr>
<td>DMC1d</td>
<td><em>Saccharomyces cerevisiae</em> DMC-1 gene</td>
</tr>
<tr>
<td>DMC1p</td>
<td><em>Saccharomyces cerevisiae</em> DMC-1 protein</td>
</tr>
<tr>
<td>DrRad51d</td>
<td><em>Drosophila melanogaster</em> Rad51-like gene</td>
</tr>
<tr>
<td>DrRad51p</td>
<td><em>Drosophila melanogaster</em> Rad51-like protein</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiotheirtol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra acetate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>EtBr</td>
<td>ethidium bromide</td>
</tr>
<tr>
<td>FrogRad51d</td>
<td><em>Xenopus laevis</em> Rad51-like gene</td>
</tr>
<tr>
<td>FrogRad51p</td>
<td><em>Xenopus laevis</em> Rad51-like protein</td>
</tr>
<tr>
<td>FFFRad51d</td>
<td>Fruit fly female Rad51-like gene</td>
</tr>
<tr>
<td>FFFRad51p</td>
<td>Fruit fly female Rad51-like protein</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>Hulim15d</td>
<td>Human Lim15-like gene</td>
</tr>
<tr>
<td>Hulim15p</td>
<td>Human Lim15-like protein</td>
</tr>
<tr>
<td>HuRad51d</td>
<td>Human Rad51-like gene</td>
</tr>
<tr>
<td>HuRad51p</td>
<td>Human Rad51-like protein</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>Lilim15d</td>
<td>Lilly Lim15-like gene</td>
</tr>
<tr>
<td>Lilim15p</td>
<td>Lilly Lim15-like protein</td>
</tr>
<tr>
<td>Molim15d</td>
<td>Mouse Lim15-like gene</td>
</tr>
<tr>
<td>Molim15p</td>
<td>Mouse Lim15-like protein</td>
</tr>
<tr>
<td>MoRad51d</td>
<td>Mouse Rad51-like gene</td>
</tr>
<tr>
<td>MoRad51p</td>
<td>Mouse Rad51-like protein</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>RecAd</td>
<td><em>Escherichia coli</em> recA gene</td>
</tr>
<tr>
<td>RecAp</td>
<td><em>Escherichia coli</em> recA protein</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>Sblim15d</td>
<td>Soybean Lim15-like gene</td>
</tr>
<tr>
<td>Sblim15p</td>
<td>Soybean Lim15-like protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>ScRad51d</td>
<td><em>Saccharomyces cerevisiae</em> Rad51-like gene</td>
</tr>
<tr>
<td>ScRad51p</td>
<td><em>Saccharomyces cerevisiae</em> Rad51-like protein</td>
</tr>
<tr>
<td>SpRad51d</td>
<td><em>Saccharomyces pombe</em> Rad51-like gene</td>
</tr>
<tr>
<td>SpRad51p</td>
<td><em>Saccharomyces pombe</em> Rad51-like protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride, sodium citrate</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Salmon sperm DNA</td>
</tr>
<tr>
<td>SM</td>
<td>Sodium chloride, magnesium sulphate</td>
</tr>
<tr>
<td>TE</td>
<td>Tris, EDTA</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris base</td>
</tr>
<tr>
<td>ToRad51d</td>
<td>Tomato Rad51-like gene</td>
</tr>
<tr>
<td>ToRad51p</td>
<td>Tomato Rad51-like protein</td>
</tr>
<tr>
<td>ytRNA</td>
<td>Yeast TRNA</td>
</tr>
</tbody>
</table>