BARD

FINAL REPORT

PROJECT NO. US-2948-97

**Nonhomologous DNA End-Joining in Plants:
Genes and Mechanisms**

C.F. Weil, A. Levy, A.B. Britt

2001

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BARD Project Number: US-2948-97

Project Title: Nonhomologous DNA end-joining in plants: genes and mechanisms

Investigators

Institutions

Principal Investigator (PI): Clifford F Weil

University of Idaho

**Co-Principal Investigator (Co-PI): Avraham Levy
Anne B. Britt**

**Weizmann Institute
UC Davis**

Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Arabidopsis; DNA repair;

Abbreviations commonly used in the report, in alphabetical order:

**DNA = deoxyribonucleic acid; DSB = double strand break; HR = homologous recombination;
RNA = ribonucleic acid; UV = ultraviolet**

Budget: IS: \$

US: \$300,000

Total: \$

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

BARD Research Grant
Signature Page

BARD Number: US-2948-97

Proposal Title: Nonhomologous DNA end joining in plants

Investigators

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	0	1	1	2
Submitted, in review, in preparation	0	4	0	4
Invited review papers	0	1	1	2
Book chapters	0	2	0	2
Books	0	0	0	0
Master theses	0	0	1	1
Ph.D. theses	0	1	0	1
Abstracts	0	5	0	5
Not refereed (proceedings, reports, etc.)	0	0	0	0

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Dr. Yoav Barak -- partial funding

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	0	1	3	4
Longer Visits (Sabbaticals)	0	0	0	0

Description Cooperation:

See attached pages for details. Briefly, cooperation consisted primarily of exchanging biological materials (mutants, constructs, etc.) and information among all the PIs. Communication was by e-mail, telephone and at meetings in the US.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted	0	0	0	0
Issued (allowed)	0	0	0	0
Licensed	0	0	0	0

Subject: Re: BARD US-2948-97 Final Report

Date: Wed, 5 Sep 2001 09:44:11 -0500

From: Clifford Weil <cweil@purdue.edu>

To: Haim Katz <haim@bard-isus.com>

Dear Haim--

Here you go. Let me know if this does what you need it to. If not, I can re-write it as necessary.

Best wishes,

Cliff Weil

ABSTRACT

Repair of DNA breaks is an essential function in plant cells as well as a crucial step in addition of modified DNA to plant cells. In addition, our inability to introduce modified DNA to its appropriate locus in the plant genome remains an important hurdle in genetically engineering crop species. We have taken a combined forward and reverse genetics approach to examining DNA double strand break repair in plants, focusing primarily on nonhomologous DNA end-joining. The forward approach utilizes a gamma-plantlet assay (miniature plants that are metabolically active but do not undergo cell division, due to cell cycle arrest) and has resulted in identification of five Arabidopsis mutants, including a new one defective in the homolog of the yeast RAD10 gene. The reverse genetics approach has identified knockouts of the Arabidopsis homologs for Ku80, DNA ligase 4 and Rad54 (one gene in what proves to be a gene family involved in DNA repair as well as chromatin remodeling and gene silencing)). All these mutants have phenotypic defects in DNA repair but are otherwise healthy and fertile. Additional PCR based screens are in progress to find knockouts of Ku70, Rad50, and Mrell, among others. Two DNA end-joining assays have been developed to further our screens and our ability to test candidate genes. One of these involves recovering linearized plasmids that have been added to and then rejoined in plant cells; plasmids are either recovered directly or transformed into E. coli and recovered. The products recovered from various mutant lines are then compared. The other assay involves using plant transposon excision to create DNA breaks in yeast cells and then uses the yeast cell as a system to examine those genes involved in the repair and to screen plant genes that might be involved as well. This award supported three graduate students, one in Israel and two in the U.S., as well as a technician in the U.S., and is ultimately expected to result directly in five publications and one Masters thesis.

ACHIEVEMENTS

Using a forward genetics approach and the gamma-plantlet assay (miniature plants that are metabolically active but do not undergo cell division, due to cell cycle arrest), we identified five Arabidopsis lines that failed to form true leaves at unusually low doses of ionizing radiation. One was defective in a previously identified gene (uvh1/AtRADI). Two, like uvh1, were also sensitive to the effects of UV radiation and one of these lines (based on mapping) is probably defective in the Arabidopsis homolog of the yeast RAD10 gene, a protein required for RAD1 function. The other, and a fourth mutation that is not UV-sensitive, are still being mapped. A fifth line is sterile. The sensitivity of all these lines to chemical mutagens is also being characterized. This work was by Eli Hefner, a grad student at UC Davis (manuscript in prep.).

In a reverse genetics approach; we used a PCR-based assay to isolate Arabidopsis lines defective in homologs of genes required for nonhomologous end joining of double strand breaks. One of these is defective in a gene thought to be required for initiation of this pathway

(Atku80) and a second line is defective for a gene required for its completion (the ligase AtDNL4). Both mutants are extremely sensitive to the gamma-plantlet-inducing effects of gamma radiation and are entirely normal (fertile and healthy) in the absence of exogenous DNA damaging agents. This is in marked contrast to the phenotype of mice knockouts in these genes, particularly DNL4^{-/-} mice, which die a p53-dependent death early in embryogenesis. The Arabidopsis homolog of Ku70, which acts in a dimer with Ku80, was also identified and we are currently screening for lines carrying knockouts of this gene, as well as knockouts of AtRAD50 and AtMRE11. In yeast and in humans there are differing third partners in the Rad50/Mre11 complex; however, there are no homologs to either of these third proteins in Arabidopsis. Our results to date suggest that Arabidopsis may be a novel, multicellular model for studying repair of double strand breaks. This work was by Joanna Freisner a grad student at UC Davis, by the PI at the Univ. of Idaho, and by Larry Petrov, a technician. Three manuscripts are in prep., one for rapid submission.

One of our working hypotheses was that plants defective in end-joining of DNA breaks may be more efficient in homologous repair, supported by recent analysis of an AtRAD50 mutant affected in end-joining and at the same time enhanced for homologous recombination. As an additional product of our bioinformatics screening for genes similar to DNA repair and recombination genes in yeast and mammalian cells (reviewed by Gorbunova and Levy 1999) we focused on a family of Arabidopsis genes similar to the yeast RAD54 and RAD5 genes. The roles of these genes are not well understood, however they are members of a superfamily of genes that encode an ATPase/helicase domain and are involved in various functions such as homologous and non-homologous repair, chromatin remodeling, gene silencing, and transcription. We characterized the structure and expression of one gene, AtRAD54-1, and by the end of the project, with most of the Arabidopsis genome sequenced, we were able to perform a phylogenetic analysis of this gene family. Published in the Masters thesis of Ilia Rochlin, a grad student at the Weizmann Institute.

Finally we have developed one rapid end-joining assay for utilization in plants (Gorbunova et al. 2000) and a second end-joining assay using a plant transposon in yeast cells (Weil and Kunze, 2000). The plant assay is based on leaf bombardment with a linearized plasmid. Upon end joining, i.e. circularization of the plasmid, a luciferase reporter gene is activated and can be monitored by low-light imaging or with a luminometer. Another version of this assay is based on circularization of a linear plasmid in plant cells, followed by transformation into *E. coli* to analyze the novel junctions. The yeast cell assay involves end-joining repair of DNA following Ac/Ds excision. This assay serves as a screening tool for yeast genes that affect the repair so that we can target searches for Arabidopsis homologs of these genes, and 2) as a system for screening plant genes that may affect end-joining repair.

Finally, we have analyzed an hyper-recombinogenic mutant in tobacco, based on its strong somatic crossover activity seen as twin sectors in tobacco leaves. This mutant was tested for radiosensitivity, HR and end-joining. It is resistant to gamma but not to UV radiation, shows increased somatic crossover between homologs, increased extrachromosomal recombination but no effect on either intrachromosomal recombination or end-joining (Gorbunova et al. 2000).

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C. Weil, University of Idaho

We are crossing putative repair defective *Arabidopsis* mutants sent to us by the Britt Lab to lines carrying an *Ac* transposon. These include a T-DNA knockout of the Arabidopsis Ku80 homolog and three UV-resistant lines identified by Eli Hefner, a graduate student in the Britt Lab supported by this award. We are currently engineering an RNAi mutant for the Arabidopsis homolog of Ku70. No significant effects have been observed yet, but these experiments need to be carried to F3 generations before any result will be clear.

We developed and improved an *Ac/Ds* transposition system in yeast cells for use as a screening tool to identify plant NHEJ genes involved in transposition. After successfully demonstrating that a *Ds* can be mobilized by *Ac* transposase in yeast, we developed an autonomous *Ac* for yeast cells for which activity is inducible (J. Yu and CFW, manuscript in preparation). In addition, we have used a series of yeast DNA repair defective mutations to analyze their effects on transposition and the NHEJ of transposon excision sites. To minimize the effects of persistent, broken DNA in cells defective for repair (which often triggers cell death), we assayed transposition from a dispensable, extrachromosomal plasmid as well as from yeast chromosomes. Thus far, we have analyzed mutations in *YKU70*, *YKU80*, *MRE11*, *XRS2*, and *RAD50*. With the notable exception of *XRS2*, for which there is no plant homolog known, mutation of any of these genes prevents end-joining of a transposon footprint in haploid yeast cells; experiments to compare the results when a template for HR is available are under way now. We are also collaborating with Jim Haber at Brandeis to look at particular Mre11 mutants his lab has constructed so that we can better understand details about the role of Mre11 in the repair process accompanying transposition. We have also obtained the complete collection of yeast open reading frame deletion mutants, however we need to improve our use of either *LEU2* or *URA3* as an excision marker before we begin the large-scale experiments testing these with our *Ac*. Mutations that show any change in transposition frequency or product will identify genes to target heavily for reverse genetic screens in *Arabidopsis*, maize and tomato. We have constructed the appropriately marked plasmids and insertions to begin these screens on a larger scale. Efforts to recover plant homologs of *Mre11*, *Rad50*, *Ku70* and *Ku80* are already in progress as described in the renewed award proposal. Data suggesting that the *Ds* reinsertion events occur by a mechanism different than the rejoining of the excision site can provide additional information about ways that DNA can insert into plant chromosomes.

Efforts to characterize the *Ac* transposase using the system developed under this award have produced several mutants with increases in activity. These increases are seen in plant cells as well (R. Adolphs, CFW, R. Kunze, in preparation). Finally, constructs developed under this award have been shared with colleagues who have now demonstrated that it works in the filamentous rice blast fungus *Magnaporthe* as well. Efforts to develop a *Magnaporthe* mutagenesis system are currently under way.

Resources from this award partially supported a technician, Latchezar Petrov, and the research efforts of four undergraduate students: Ashley Reed, Brian Wood, Lindsay Barry and Judy Nitcy. Presentations of this work acknowledging BARD and its support have been made at the Maize Genetics Conference, International Botanical Congress, 2000 Keystone Symposium, the Yeast Genetic Conference, and invited presentations at the Univ. of Florida, the Univ. of Missouri, Iowa State Univ., Univ. of Wyoming, Purdue Univ. and Gonzaga Univ.

Publications during this award acknowledging BARD support are:

Weil, C. F. and R. Kunze (2000). Transposition of maize *Ac/Ds* transposable elements in the yeast *Saccharomyces cerevisiae*. *Nature Genet.* 26: 187-190.

Kunze, R and C. F. Weil (2001). *Ac/Ds* and *En/Spm* element families in plants. Invited chapter for *Mobile DNA II*, Boeke, J., N. Craig, M. Gellert and N. Grindley eds., ASM Press, Washington, DC, in press

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A. Levy, Weizmann Institute

DNA double strand break repair is done either repair by end joining or by homologous recombination (HR). Our working hypothesis was that plants defective in end-joining of DNA breaks may be more efficient in repair via HR. This hypothesis has been supported by a recent analysis of an *AtRad50* mutant that is affected in end-joining and at the same time shows a enhanced, somatic, homologous recombination. In the first stage of this project, we carried out a bioinformatics analysis of genes that are similar to genes in yeast and mammals and involved in DNA repair and recombination. This work was published as part of a review (Gorbunova and Levy 1999) on DNA DSB repair in plants. We then chose to focus on a family of genes similar to the yeast *RAD54* and *RAD5* genes. The role of these genes is not well understood; however they are members of a superfamily of genes that code for an ATPase/DNA helicase domain and are involved in various types of DNA metabolic functions, including homologous and non-homologous repair, chromatin remodeling, gene silencing and transcription. At the time only one gene from this family was sequenced in Arabidopsis; we called it *AtRAD54-1* and characterized its gene structure (genomic and cDNA) and expression. By the end of the project a high proportion of the Arabidopsis genome had been sequenced and a phylogenetic analysis of this important family was done. All this work is published in the master thesis of Ilia Rochlin, a copy of which was enclosed in our previous report. The work on the *RAD54*-like genes of Arabidopsis is the basis for our renewed grant and plants either null for or overexpressing these genes will be used for gene targeting and end-joining assays. Finally, we developed a rapid end-joining assay for utilization in plants based on leaf bombardment with a linearized plasmid. Upon end-joining, i.e. circularization of the plasmid, a luciferase reporter gene is activated and can be monitored by low-light imaging or by a luminometer. This assay was described in Gorbunova et al. 2000. Another version of this assay is also based on circularization of a linear plasmid, followed by transformation into *E. coli* to analyze the sequence of the novel junctions. Finally, in the course of our work we have analyzed an hyperrecombinogenic mutant of tobacco based on its increased somatic crossover activity (seen as twinned sectors on leaves). This mutant was tested for radiosensitivity, HR and end-joining. It turned out to be resistant to gamma, but not UV radiation, had enhanced somatic crossover between homologs, and increased extrachromosomal, but not intrachromosomal recombination. The mutation had no effect on end-joining. Work on this mutant was published in Gorbunova et al. 2000.

Publications including BARD acknowledgement:

Gorbunova, V and Levy A.A.1999. How plants make ends meet: DNA double-strand break repair. *Trends in Plant Sciences* 4:263-269.

Gorbunova, B., Avivi-Ragolski, N., Shalev, G., Kovalchuk, I., Abbo, S., Hohn B and Levy, A.A. 2000. A new hyperrecombinogenic mutant of *Nicotiana tabacum*. *Plant J.* 24: 601-611.

Theses based on BARD-supported funding:

Ilia Rochlin, *Arabidopsis thaliana* *RAD54/ATRX*-like gene: A novel member of the *SWI2/SNF2* family, July 1999, Feinberg graduate school, Weizmann Institute, Rehovot, Israel.

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A. Britt, UC Davis

Grad student Eli Hefner: Hunting for mutants sensitive to gamma radiation can identify genes involved in DSB repair. Eli has screened families of siblings for those with enhanced sensitivity to the effects of gamma radiation on cell division. Specifically, he screens for plants that, after exposure to a low (10Krad) dose of gamma, germinate at a normal rate and expand their cotyledons, but fail to produce new leaves. Our lab has shown that this "gamma plantlet" phenotype is produced by cell cycle arrest in G2 induced by persisting DNA damage. DSBs are powerful arrest-inducing lesions in other organisms, and it is likely that this G2 arrest is due to the persistence of DSBs in *Arabidopsis*.

Eli has screened 4500 EMS-mutagenized families and obtained 4 independently isolated gamma sensitive mutants representing at least 3 different complementation groups. One of these mutants is a third allele of the previously described *uvh1* mutation, defective in an *Arabidopsis* homolog of the yeast *RAD1* gene (Fidantsef et al., 2000). 5 additional mutant families await further genetic characterization and all of these mutants are being mapped. In addition, Eli is currently preparing vectors for a luciferase based assay for homologous and nonhomologous repair of DSBs in transiently transformed *Arabidopsis* protoplasts. He is also checking the efficiency of stable T-DNA transformation of his mutant lines via the *in planta* procedure and developing biolistic assays for end joining in leaf tissue.

Graduate student Joanna Friesner: Joanna's efforts have focused on *Arabidopsis* insertion mutations into homologs of known to play a role in NHEJ in humans and yeast. Using the NSF-sponsored *Arabidopsis* knockout facility at the University of Wisconsin, she has already identified insertions in the *AtKu80*, *AtLig4*, *AtMRE11* and *AtFEN-1* genes among the facility's populations and is characterizing the phenotypes of these mutants in more detail. The putative Ku80 mutant is hypersensitive to gamma-irradiation, consistent with a role in DSB repair. She plans to determine whether these plants are defective in meiosis, defective in T-DNA transformation, and defective in end-joining of plasmid substrates obtained from the Levy Lab. The mutant has been sent to the Weil Lab for testing with *Ac* transposition.

Britt AB. (2000) Plant biology. An unbearable beating by light? *Nature* 406:30-31.

Fidantsef AL, Mitchell DL, Britt AB. (2000) The *Arabidopsis* *UVH1* gene is a homolog of the yeast repair endonuclease *RAD1*. *Plant Physiol.* 124:579-586