BARD

FINAL REPORT

PROJECT NO. IS-2726-96

**Endoreduplication in Maize Endosperm: an
Approach for Increasing Crop Productivity**

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(Cover Page)

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Project Title: Endoreduplication in maize endosperm: an approach for increasing crop productivity

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Continuation of (Related to) Previous BARD Project:

☐ Yes

☒ No

Number:

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

Cell cycle, cyclin, wheat dwarf virus, high mobility group protein, retinoblastoma

Abbreviations used in the report, in alphabetical order: CDK, cyclin-dependent kinase;
DAP, days after pollination; HA, hemagglutinin, HMG, high mobility group, HU, hydroxyurea;
ZmRb, *Zea mays* retinoblastoma

Budget: IS: \$150,000

US: \$150,000

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Signature
Principal Investigator

Signature
Research Authority, Principal Institution

Publication Summary (numbers)

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

Patent Summary (numbers)

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

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	1		2
Sabbaticals				
Postdoctorates				

- Cooperation, briefly explain whether synergistic, complementary or supportive.

Complementary and partly synergistic _____

INTRODUCTION

The focus of this research project is to investigate the role of endoreduplication in maize endosperm development and the extent to which this process contributes to high levels of starch and storage protein synthesis. Although endoreduplication has been widely observed in many cells and tissues, especially those with high levels of metabolic activity, the molecular mechanisms through which the cell cycle is altered to produce consecutive cycles of S-phase without an intervening M-phase are unknown.

Our previous research has shown that changes in the expression of several cell cycle regulatory genes coincide with the onset of endoreduplication. During this process, there is a sharp reduction in the activity of the mitotic cyclin-dependent kinase (CDK) and activation of the S-phase CDK. It appears the M-phase CDK is stable, but its activity is blocked by a proteinaceous inhibitor. Coincidentally, the S-phase checkpoint protein, retinoblastoma (ZmRb), becomes phosphorylated, presumably releasing an E2F-type transcriptional regulator which promotes the expression of genes responsible for DNA synthesis.

To investigate the role of these cell cycle proteins in endoreduplication, we have created transgenic maize plants that express various genes in an endosperm-specific manner using a storage protein (γ -zein) promoter. During the first year of the grant, we constructed point mutations of the maize M-phase kinase, p34^{cdc2}. One alteration replaced aspartic acid at position 146 with asparagine (p3630-CdcD146N), while another changed threonine 161 to alanine (p3630-CdcT161A). These mutations abolish the activity of the CDK. We hypothesized that expression of the mutant forms of p34^{cdc2} in endoreduplicating endosperm, compared to a control p34^{cdc2}, would lead to extra cycles

of DNA synthesis. We also fused the gene encoding the regulatory subunit of the M-phase kinase, cyclin B, under the γ -zein promoter. Normally, cyclin B is expected to be destroyed prior to the onset of endoreduplication. By producing high levels of this protein in developing endosperm, we hypothesized that the M-phase would be extended, potentially reducing the number of cycles of endoreduplication. Finally, we genetically engineered the wheat dwarf virus RepA protein for endosperm-specific expression. RepA binds to the maize retinoblastoma protein and presumably releases E2F-like transcription factors that activate DNA synthesis. We anticipated that inactivation of ZmRb by RepA would lead to additional cycles of DNA synthesis.

FINAL SCIENTIFIC REPORT

IS ENDOREDUPLICATION REQUIRED FOR STORAGE PROTEIN SYNTHESIS?

Endoreduplication has been very well documented and quantified in plants and animals, yet its biological significance is unclear. Based on the fact that endoreduplication is common in tissues displaying high metabolic activity, it has been proposed that it might provide a mechanism whereby cells can increase the availability of DNA templates and thus increase the level of gene expression. While it seems likely that the degree of endoreduplication influences levels of gene expression, no mutations of genes controlling endoreduplication in seeds have been described, hence, the validity of this hypothesis has not been tested. We therefore have made attempts to experimentally address the

relationship between endoreduplication and storage protein gene expression by chemically manipulating the level of DNA replication in cultured maize kernels.

Ears at 6 days after pollination (DAP) were collected from the field, cut aseptically into small blocks, each containing 6-8 kernels, and placed on appropriate medium essentially as described by Cully et al. (1984) [*Plant Physiol.* 74, 389-394]. To investigate the relationship between endoreduplication and storage protein synthesis, kernels were treated with hydroxyurea (HU), a blocker of DNA synthesis. Samples were collected at various DAPs, and isolated endosperms were ground into flour in liquid nitrogen using mortar and pestle and stored at -80°C. Total proteins were extracted in borate buffer and zein storage proteins in 70% ethanol, essentially as described [Wallace, et al., (1990) *Plant Physiol.* 92, 191-196].

These experiments were carried out during the first and the second year of the proposal yielding similar results. As demonstrated in Fig. 1, kernels grown under a high concentration of HU (100 mM) displayed a dramatic reduction in storage protein synthesis (Fig. 1B). This reduction appears to be specific for storage proteins inasmuch as no apparent effect on total protein synthesis can be detected (Fig. 1A). Our results provide additional clue that supports a link between endoreduplication and storage protein synthesis.

Fig. 1. DNA replication (endoreduplication) is required for storage protein synthesis. Kernels were grown either in the field (lanes 2-5) or in culture (lanes 6-17) without (hydroxyurea) HU (lanes 6-8) or with the indicated concentrations of HU (25, 50 and 100 mM). At the indicated DAPs, total proteins were extracted in borate buffer and resolved on SDS/PAGE (A), or storage proteins extracted with 70% ethanol (B). Gels were stained with comassie blue. M indicates molecular weight markers (in kD).

MANIPULATION OF ENDOREDUPLICATION IN TRANSGENIC MAIZE

As described in last year's report, we encountered a number of technical difficulties, especially during the first year of this grant, in establishing an efficient transformation and regeneration system for maize. While the procedures are fairly standard, it took time and experience to establish the system and learn to deal with technical and biological problems, such as insect pests in the greenhouse. Consequently, it took us a year longer than anticipated to develop transgenic maize plants containing the genes we wished to evaluate. However, we now have transgenic plants in hand for each of the constructs that were originally described in this research proposal. Plants expressing the wheat dwarf virus RepA sequence and wild type *cdc2* sequences are the most advanced, with plants containing the maize cyclin B gene not far behind. Finally, plants containing the mutant *cdc2* constructs, which we initially could not regenerate, are now growing in the greenhouse, and we should have preliminary data regarding kernel development for these plants in the next few months.

It is standard practice that the phenotype of a transgenic event not be evaluated until the T3 generation, as non-specific phenotypic effects may arise simply as a consequence of genetic defects resulting from tissue culture. Consequently, we have been backcrossing the transgenic plants into a wild type B73 background for three generations before selfing them to create homozygous loci. We are currently growing the T3 generation of the plants expressing the wheat dwarf virus RepA protein and the wild type *cdc2* gene. As explained below, preliminary characterization of endosperm from T2 plants demonstrated that the transgene is being expressed for several of the

transformation events, and we were unable to detect any effect on the cell cycle (endoreduplication). T2 transgenic plants, containing maize cyclin B driven by a gamma-zein promoter, are currently being grown. The following is a summary of the work to date.

WHEAT DWARF VIRUS REPA

Out of the 15 initial transformation events for the RepA gene, we were able to propagate plants from 9 that were PCR-positive for the transgene. Using an antibody against RepA, we found varying levels of RepA protein in endosperms from three of the events. The two events with the highest level of RepA expression develop kernels with opaque, somewhat shrunken endosperms. The lowest expressing event is weakly opaque. We analyzed by flow cytometry endosperm from these three events during the T1 and T2 generations and were unable to observe any difference in the level of endoreduplication between the segregating wild type and transgenic kernels. Thus, while it appears that varying levels of RepA expression has some consequence for endosperm development, it does not appear to alter the degree of endoreduplication. We are planning to characterize these transgenic lines in more detail in the next two generations in order to address questions related to the subcellular localization of the RepA protein and the identification of proteins with which it interacts. We are also planning to profile gene expression in the developing endosperm in order to gain some insight into how RepA alters development.

CDC2 WILD-TYPE SEQUENCE

Although we had difficulty regenerating transgenic plants expressing the Cdc2 mutant constructs (CdcT161A and CdcD146N), we were able to recover transgenic plants (two events) expressing the wild type Cdc2. This recombinant cdc2 is tagged with a hemagglutinin (HA) epitope, which causes it to migrate slower than the native cdc2 on SDS-PAGE. This allowed us to simultaneously detect the two proteins in extracts from single endosperms. The results of immunoblotting showed higher expression of the transgene than the endogenous cdc2 protein. However, we have not yet demonstrated that the recombinant cdc2 is incorporated into a functional CDK. Nevertheless, we have been able to demonstrate by adsorption chromatography that the HA-tagged Cdc2 binds to p13^{suc1} and ZmRb1. Experiments are in progress to determine if the engineered Cdc2 is associated with histone H1 kinase activity. Flow cytometric analysis of developing endosperm from these two events showed no difference in the degree of endoreduplication.

Plants representing transformation events (T-0) of the two cdc2 mutants are currently growing, and we should have some information regarding their ability to propagate in the near future.

MAIZE CYCLIN B

We have recovered plants from five transformation events representing maize cyclin B under the control of the gamma-zein promoter. Because of poor seed recovery, only three of these are currently growing, and they are in the T2 generation. Besides confirming that these are transformed (based on expression of the herbicide resistance

gene), we performed immunoblotting with HA-antibody. This assay did not detect any protein, which could be a consequence of protein turnover or failure to express the gene. We are planning to characterize the developing endosperms of some kernels from the T2 generation in the coming year.

FACTORS INVOLVED IN ACTIVATION OF GENE TRANSCRIPTION DURING MAIZE ENDOSPERM DEVELOPMENT

During the second and the third year of the proposal the Israeli group has extended the project toward identification of regulatory proteins involved in transcriptional activation of storage protein genes that accompanies endoreduplication. We have identified and characterized the function of the high mobility group (HMG)-I/Y protein in alleviating the histone H1-mediated transcriptional repression in maize endosperm. This part of the work is summarized in the attached manuscript [(Zhao, J. and Grafi, G. (2000) J. Biol. Chem. 275, 27494-27499].

SUMMARY

Although there are as yet no formal research publications resulting from the original proposal, we anticipate several will be forthcoming in the next year. Unfortunately, the development of transgenic maize plants is not only technically challenging, but it is also slow and labor intensive. As a consequence, it was impossible to complete the experiments we proposed within the 3-year period of this grant. Nevertheless, we have made a great deal of progress with this research, and we plan to achieve the research objectives of the proposal very soon.

SUMMARY: EXTENSION OF THE PROJECT/THE ISRAELI GROUP.

As mentioned above it took us a year longer than anticipated to develop transgenic maize plants containing the genes we wished to evaluate. Therefore, the Israeli group has extended the project toward understanding the molecular basis for endoreduplication and storage protein synthesis.

we provide evidence that support a link between endoreduplication and storage protein synthesis and characterized a key factor, the HMG-I/Y protein, that is involved in activation of storage protein gene expression. These findings provide us with additional candidate for future manipulating seed and storage organ development in transgenic plants.