ANALYSIS OF MAIZE ENDOSPERM ENDOREDUPLICATION

by

Brian Russell Dilkes

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PLANT SCIENCES

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2003

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Brian R. Dilkes entitled Analysis of maize endosperm endoreduplication

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Brian A. Larkins	4-21-03 Date
Vicki Chandler	4-22-03 Date
Ramin Yadegari	4/22/03 Date
Richard Jorgensen	4/21/03 Date
Marc Orbach	4/21/03 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

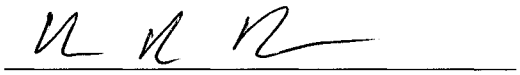
I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Brian A. Larkins	4-21-03 Date

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: 

ACKNOWLEDGMENTS

I would like to acknowledge the Department of Plant Sciences for training me. In particular, the effort and care with which Dr. Brian Larkins has mentored me in the latter half of my stay. Much needed advice and encouragement has also been provided by my committee (past and present): Drs. Hans Bohnert, Vicki Chandler, Richard Jorgensen, Marc Orbach, and Ramin Yadegari. Among the excellent Post-Doctoral researchers I have benefited from training under, Drs. Yeujin Sun and Burkhard Schulz deserve special mention as inspirations and teachers.

The extremely fruitful collaboration between the Larkins Lab and Pioneer Hi-Bred International should also be acknowledged. I have never had a finer pleasure than collaborating with Dr. William Gordon-Kamm and Keith Lowe. My thinking about the cell cycle and plant development have been forever altered as a result.

The work presented herein would not have been possible without the technical assistance and support of Dr. Brenda Hunter and Georgina Lambert. Dr. Robert Leonard provided the Partec flow analyzer and, along with Drs. Larkins, Schulz and Ray, kept me from dropping out. While some may doubt their wisdom, I am very grateful. Dr. Bruce Walsh for discussing quantitative genetic concepts with me, at length; without his guidance Chapter 2 would not have happened. The Department of Sociology and Jason and Christy Clark Miller provided statistical software resources as well as many useful discussions. Additionally, Dr. Curt Hannah of the University of Florida, Dr. Steven Spiker of North Carolina State University, Drs. Jim Murray and Sandra Healy of Cambridge University, Dr. Joe Colasanti of the University of California at Berkeley and Drs. Yuko Ohashi and Sunichi Kosugi of the National Institute of Agrobiological supplied antisera used in Chapter 3.

Special acknowledgement is reserved for all the members of the Larkins laboratory, past and present, who contributed via critical reading of manuscripts, discussion, collaboration, and example.

DEDICATION

This dissertation is dedicated to:

The American citizens interned during the Second World War, particularly those who taught me the dignity of plants.

Susan Grether for taking a boy to a scientific conference and Marshall W. Nirenberg for having the diplomacy to answer his question (really two questions) with "That's the thing about science; there are plenty of good questions with no answers."

Librarians and lawyers everywhere. God bless them, every one.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

During maize endosperm development, the cell cycle in the majority of cells switches from a mitotic to an endoreduplication cell cycle. This results in cells of varying ploidies within the tissue, and is presumed to be a factor in its rapid growth. Investigating the inheritance of variation in endoreduplication in maize endosperm will begin to elucidate the genetic mechanisms controlling it. It has been hypothesized that retinoblastoma-related proteins (RRBs) negatively regulate the G1/S transition during both mitotic and endoreduplication cell cycles. Testing this hypothesis in both mitotic cells and endoreduplicating endosperm cells will further our understanding of the molecular mechanisms regulating endoreduplication.

Flow cytometry was used to assess the variability of endoreduplication in endosperms of maize inbred lines. High levels of endoreduplication were observed in popcorns relative to Midwestern dent corns. To study the genetic regulation of endoreduplication, four inbreds were crossed to B73 and developing endosperms from parental, reciprocal F1, and backcross generations were subjected to flow cytometric analysis. Maternal zygotic effects, often considered a form of parental imprinting, and maternal sporophytic effects were detected. To test the feasibility of introgressing a high endoreduplication phenotype into a Midwestern dent inbred line, a backcross population was generated using B73 as the reciprocal parent and the popcorn Sg18. The heritabilities calculated from an analysis of the backcross population generally agree with the values calculated in the larger crossing experiments.

The Wheat Dwarf Virus RepA protein binds RRBs and is predicted to activate the cell cycle. RepA and the maize RRB, ZmRb1, were tested for cell cycle regulatory activity in tobacco BY-2 cells and determined to be an activator and repressor, respectively. The effect of RepA on endoreduplication was evaluated in both mitotically-active maize callus cultures and developing endosperms. Flow cytometric measurements of nuclear ploidy showed that RepA expression was sufficient to convert a mitotic into an endoreduplication cell cycle in calli but had no discernable effect on endopolyploidy in developing endosperm by 18-days after pollination (DAP).

CHAPTER 1

LITERATURE REVIEW

General introduction

Introduction to the cell cycle

Plant architecture is ultimately determined by growth (increases in size) differentiation (increases in cellular diversity), and morphogenesis (the spatial manifestation of these). Growth is accomplished by a combination of increases in cell number and cell size (Gilbert, 2000), and an increase in cell number is affected by modulation of the cell cycle. For the purposes of this review, the reference cell cycle to which all others will be compared is that in angiosperm meristems. This meristematic cell cycle involves the progression through a series of states that allow the complete replication of the genetic material into two copies. These two copies of the genome must be subsequently partitioned, along with cytoplasm, into two new cells by a mitotic division. The two new daughter cells, which take the place of the original mother cell, can then re-enter this process and "cycle" through again to produce more cells. Generally, this cell cycle is discussed as comprising four phases. In the first phase, gap one (G1), cells have a basal DNA content or C value, where a single C unit corresponds to the haploid DNA content of the organism; during G1, cells prepare to replicate their DNA. The second step is S-phase or synthesis phase, in which the DNA of the cell is faithfully and completely replicated. Replicated chromatids remain associated, so they

can be separated later into two complete chromosomes. In the next phase, gap two (G2), the cell prepares to divide the separated genetic material and cytoplasm, and marks the plane of cell division with a radial microtubule array called the pre-prophase band (PPB). Collectively, these three phases are often called interphase, due to the lack of cytologically distinct structures (in animal and yeast cells) during these periods. The fourth phase, mitosis, is a programmed set of cytological events beginning with the condensation of chromatin and culminating in the separation of replicated DNA into two spatially distinct but fundamentally genetically identical sets of chromosomes compartmentalized in nuclei of the two daughter cells. At the culmination of mitosis in meristems, a new cell wall and plasma membrane is assembled by a specialized set of secretory vesicles and cytoskeletal structures called the phragmoplast.

This mitotic cell cycle I have described, similar to the mitotic cycles of fungi and animals, is the standard cell cycle that most authors refer to as the "cell cycle". Frequently, perhaps unintentionally, it is assumed that this cell cycle is the only mechanism for increasing cell number and increases in cell number are the only way in which the cell cycle affects growth. This assumption is a gross oversimplification and distortion, as many variations on this cell cycle exist in most organisms. Some of these alter cell size or cell division orientation such that modulation of the cell cycle can influence growth, differentiation and morphogenesis by increasing the number, size, type, and distribution of cells within an organism. For example, the very rapid cell cycles in early development of the angiosperm endosperm do not involve the formation of cell walls. Moreover, these nuclear divisions require a mere 6 hours (h), unlike the mitotic

cycles in plant leaves which are typically 15-36h (Olsen et al., 1992; Granier and Tardieu, 1998; Tardieu and Granier, 2000). These endosperm cell cycles do give rise to cells, each with its own cell wall, as multiple rounds of nuclear divisions are followed by a temporally distinct wave of cellularization using a modified phragmoplast (Otegui and Staehelin, 2000; Olsen, 2001). A reciprocal modification of the standard mitotic cell cycle has been described for *Chlamydomonas reinhardtii* (John, 1984; John et al., 1989; Umen and Goodenough, 2001). In this single-celled alga, cells engage in a growth phase in which they enlarge and accumulate cytoplasmic material. As material accumulates, cells enlarge until they reach a critical size. At this time, multiple cycles of DNA synthesis and mitotic division ensue and continue these divisions until a population of haploid cells is generated (John, 1984). Thus, cellular proliferation and growth can be accomplished by temporally and mechanistically distinct cell cycles, e.g. cycles of DNA synthesis followed by cycles of mitotic division.

Genetic and cell biological experiments from animal, plant, and fungal systems have shown that mitotic and other types of cell cycles utilize many of the same regulatory proteins. Mitotic-type cycles can be modified into another type of cell cycle by altering the activity of one or more regulatory factors (van den Heuvel and Harlow, 1993; Moreno and Nurse, 1994; MacAuley et al., 1998; Niculescu et al., 1998; Schnittger et al., 2002). As these many types of cell cycles are somewhat interchangeable, a broader concept of the cell cycle, capable of integrating many variations of cell cycle type, is necessary. I will therefore use the term "cell cycle" as a more general term, referring to any series of events comprised by the stages of the meristematic cell cycle. This definition is inclusive

to any sequence of events that can be explained by alteration of the meristematic cell cycle by the removal, duplication, or addition of a subset of events in the meristematic cell cycle.

The four phases discussed above do not explain the behavior of cells in the standard cell cycle, as shown in a number of genetic and cell biological experiments. In the yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, mutants have been identified that arrest before and during S-phase and mitosis, suggesting that the onset and progression of these phases are under some shared and some distinct genetic controls (Elledge, 1996; Tourret and McKeon, 1996; Weinert, 1997; Foiani et al., 1998; Rhind and Russell, 2000). Also, mutants defective in cell cycle progression have been identified that either arrest during DNA replication or mitosis or continue through the cycle without completing all the steps typically required; mutations of the same genes affect non-mitotic cell cycle events (e.g. (Dutcher and Hartwell, 1983; Surana et al., 1991). While some of the genes encoding these functions are structural (e.g. catalyze a reaction within DNA synthesis), others clearly are signaling molecules that transmit information regarding the completion of "subroutines" (Dutcher and Hartwell, 1983; Tourret and McKeon, 1996; Rhind and Russell, 2000). Results from genetic and cell biological experiments in yeast led to the proposal of checkpoints within each stage, that divide the cell cycle into a series of states (Weinert and Hartwell, 1989; Hartwell, 1992; Hartwell and Kastan, 1994; Weinert et al., 1994). This hypothesis, which has been validated for the cell cycles of animals, fungi and plants, postulates that each checkpoint helps to ensure that replication and cell division are accurately accomplished in sequence

prior to the onset of the next cell cycle stage. The number of checkpoint states exceeds the number required for a four-stage cell cycle, demonstrating that the four stages are artificial and that describing the cell cycle according to checkpoint status may provide a more biologically relevant nomenclature. For example, in tobacco BY-2 cells, it has been demonstrated that the appearance of the PPB depends on the completion of DNA replication, i.e. a replication checkpoint is passed prior to the appearance of the PPB (Yanagawa et al., 2002). Thus, appearance of a PPB actually differentiates the G2 phase of the meristematic cell cycle into at least two substates: marked (post-PPB); and unmarked (pre-PPB band but post-replication). If a cell can be halted or diverted from a checkpoint and transitioned to a different checkpoint, a mechanism for the production of many cell cycles (using a minimal number of components) can be proposed based on the meristematic cycle.

Environmental stimuli both inhibit and promote growth via cell cycle-dependent mechanisms. Increasing temperature results in a shortening of the cell cycle (Tardieu and Granier, 2000) until it reaches the point of inducing stress, at which point cell division is inhibited (Cheikh and Jones, 1994; Commuri and Jones, 1999, 2001). In a majority of plant species tested, elevated atmospheric carbon dioxide leads to an increase in cell divisions and organ size (Pritchard et al., 1999).

Cell cycle manipulation has also contributed to vigor in cereal crops. In summarizing data from earlier publications, Kiesselbach stated that approximately 10% of the increased size of maize hybrids over their inbred parents is due to cell enlargement, and 90% of this increase can be accounted for by a greater number of cells (Kiesselbach,

1922; Kiesselbach, 1949). This observation, that enhanced cell proliferation contributes to increased maize plant vigor, remains unchallenged. Recently it has been demonstrated that transgenic manipulation of cell cycle regulatory components can lead to increased organ initiation (Doerner et al., 1996; Riou-Khamlichi et al., 1999; Cockcroft et al., 2000), stimulation or inhibition of cell proliferation (Hemerly et al., 1995; Hemerly et al., 2000; Gordon-Kamm et al., 2002) and increases in plant and plant organ size (Wyrzykowska et al., 2002).

Variations of the classical mitotic cell cycle are ubiquitous and are involved in events in the development of virtually all plant and metazoan taxa. Many variations on this cell cycle can be conceptualized as reductions of the classical cell cycle, i.e. they are missing one or more stages. Examples of some prevalent variations on the mitotic cell cycle include: the production of multinucleate cells or coenocytes (a cell cycle retaining all but cytoplasmic division and compartmentalization); meiosis II (a cell cycle lacking DNA replication); the rapid cell cycles during embryogenesis (a cell cycle lacking gap phases); endocycles of recurrent replication of the nuclear DNA (lacking nuclear and cytoplasmic division). Additionally, there are cell cycles in which the two daughter cells are not genetically equivalent to the mother, or each other; these cell cycles often contribute to cellular differentiation. In haploid *S. cerevisiae* cells, from which much of the genetic and molecular experiments that contribute to our present understanding of the cell cycle were obtained, normal mitotic division utilizes a cell cycle that violates the classical one. During haploid cell divisions in yeast, the DNA at the mating type locus undergoes a programmed sequence alteration, in which the relative position of genes and

regulatory sequences is altered (Haber, 1992). The consequence of this, as with all cell cycles resulting in genetically distinct daughter cells, is a change in cellular diversity (differentiation). While the cell cycle is often thought of as a modulator of growth, this example illustrates that a cell cycle that produces cells genotypically unequal to the mother cell leads to both growth and differentiation.

Partial endopolyploidy (polysomaty) characterizes most multicellular organisms

In most multicellular eukaryotes, a subset of cells uncouple the processes of DNA replication and cell division such that they re-replicate their DNA. Most frequently, this is accomplished without nuclear division, giving rise to a tissue or organ containing cells with the basal nuclear ploidy and cells with greater-than-basal nuclear ploidy, or endopolyploid cells. The coexistence of cells with differing nuclear ploidy in an organ or tissue is referred to as polysomaty (D'Amato, 1964). Like endopolyploidy, the definition of polysomaty does not implicate a mechanism for DNA re-replication. As with the example of the haploid yeast cell cycle (above), polysomaty accomplishes both growth and differentiation.

A variety of endocycle mechanisms exist. Some stop DNA replication prior to completion, such as the partial re-replication cycles in Drosophila nurse cells (Sauer et al., 1995; Lehner and Lane, 1997; Weiss et al., 1998; Edgar and Orr-Weaver, 2001). Others progress through mitosis, as occurs in the endomitotic cell cycles during mammalian megakaryocyte differentiation (Vitrat et al., 1998) and the abortive mitoses of cells with a compromised mitotic apparatus (Khan and Wahl, 1998; Verdoodt et al.,

1999). In plants, the most common mechanism for the generation of endopolyploid cells is endoreduplication (D'Amato, 1984). Endoreduplication is defined as the complete replication of the nuclear DNA without chromosomal condensation, nuclear division or envelope breakdown. Endoreduplication cycles proceed through the completion of S-phase, have a gap phase, and then repeat the process leading to endonuclear DNA re-replication. If compared to the meristematic cell cycle, endoreduplication would include any variation that ends the cell cycle after completion of DNA replication but before the onset of mitosis. This should give rise to polytene chromosomes, although only a few examples of these have been utilized for the investigation of chromosomal banding in plants (Carvalheira and Guerra, 1998)

Endopolyploidy, regardless of the mechanism, is typically measured by fluorometric determination of the DNA content per nucleus. In early studies, this was accomplished by making sections of fixed tissue stained by the Feulgen reaction and microphotometric measurement of nuclear fluorescence. This method, and variants of it using more DNA-specific and vital dyes, such as DAPI, has the advantage of both quantifying the differences in fluorescence between cells, and recording their relative positions. More recently, with the advent of flow cytometric methods, endopolyploidy can be assayed by extracting nuclei, staining them with a fluorometric dye, typically DAPI or the general nucleic acid stain, propidium iodide, and then passing them in front of an excitation light source and emission detector. In this way, the DNA content of hundreds to hundreds of thousands of nuclei can be measured in a few minutes, allowing for more robust conclusions to be drawn regarding the degree of endopolyploidy in a

sample. However, this approach sacrifices any spatial resolution below the size of the explant used as the source for protoplasts or nuclei. As neither of these methods can critically address the mechanism of endopolyploidization, we often know more about the occurrence and distribution of polysomaty than the mechanisms by which it occurs.

Measurements of endopolyploidy pose problems for the definition of its scale, for quantitation and statistical analyses. The data obtained typically have no single value that accurately describes the degree of endopolyploidy. For instance, in a tissue in which both mitosis and endoreduplication are occurring, cells with a duplicated DNA content are comprised of both G2 cells poised for mitosis and endoreduplicating cells in G1. Thus, the degree of polysomaty does not necessarily reflect the degree of endoreduplication, as the latter is defined as re-replication. However, if a tissue contains cells with duplicated DNA which will not divide, i.e. are terminally doubled, they should be regarded as endopolyploid.

When comparing two or more endoreduplicated samples by most statistical methods, a value or set of values need to be generated from the measurements. The most commonly used value, mean ploidy, can be skewed by a few highly polyploid cells and is not necessarily a linear measure, as increases in DNA content are geometric. Another common measure is the percentage of cells with a greater than basal DNA content, referred to as %E in this dissertation. While %E lumps G2 and G1-endocycle cells together, it is insensitive to a few highly polyploid cells. As is discussed for the specific example of endosperm in Chapter 2, a third measurement, the mean ploidy of

endopolyploid cells, can be used in conjunction with the %E to calculate the mean ploidy and better expresses the effect of endocycles on cells.

Endoreduplication in plants

While conspicuously missing from many textbooks discussing plant growth, polysomaty is a nearly ubiquitous feature of the higher-plant life cycle. Within the angiosperms, endopolyploidy is subject to no major taxonomic limitation and is estimated to occur during the development of at least 90% of all plants; examples of endopolyploid cells derived from all three layers of the meristem are known (D'Amato, 1984). In lower plants, such as the bryophytes, it is a less common occurrence, but polysomaty is an important component of the development of some taxa (Kingham et al., 1995). Polysomaty of the leaves and roots has been identified in arabidopsis, pea, potato, corn, bean, brassica, and others (Dvorak, 1968; Capesius and Stohr, 1974; Bryans and Smith, 1985; Galbraith et al., 1991; Gilissen et al., 1993; Demchenko, 1994; Smulders et al., 1994; Gilissen et al., 1996; Cavallini et al., 1997; Engler et al., 1999; Berta et al., 2000; Lagunes-Espinoza et al., 2000; Kudo and Kimura, 2001, 2002). While the mere existence of a few cells with altered nuclear ploidy may appear to be of little consequence, the near ubiquitous nature of the phenomenon and its degree in certain cases suggest otherwise. For example, in maize endosperm of all the copies of a particular gene, greater than 90% are contained in chromosomes resident in an endopolyploid nucleus (see Chapter 2).

Endopolyploid cells are often associated with ephemeral and metabolically active cells (D'Amato, 1984). These cells are commonly found in support and storage tissues,

or exist as specialized storage cells. Examples include the endosperm (Kowles et al., 1990; Brunori et al., 1993), cotyledon (Bryans and Smith, 1985; Smulders et al., 1995; Lemontey et al., 2000), and embryo suspensor of plant seeds(Clutter et al., 1974; Luszczek et al., 2000), the tapetum cells of the anther (Sawicka, 1991; Sherwood, 1995) and idioblasts in leaves (Kausch and Horner, 1984; Melaragno et al., 1993; Perazza et al., 1999). Apart from high metabolic activity, many of these cells also share the feature of having physical barriers to cell division. In these cells, endoreduplication could support increases in cell size and metabolic activity in a way that is not mechanically inhibited by the cytoplasmic contents of the cell. Cases of extreme endopolyploidy are found in the suspensors of many angiosperm embryos. C values upwards of 1024 are not uncommon in suspensors (D'Amato, 1984), and the not insubstantial investment of carbon, nitrogen, and ATP for nucleotide and DNA synthesis in these short lived cells suggest that endoreduplication serves some purpose. These cells endoreduplicate to such a degree and engage in sufficient transcription to produce chromosome puffs, permit G-band staining and in situ localization of the site of rRNA synthesis (D'Amato, 1984; Carvalheira and Guerra, 1998).

Changes in cellular ploidy can be affected by a number of genetic, signaling and pharmaceutical manipulations. Treatment of cells with nucleotide analogs and inhibitors of protein phosphorylation has been shown to induce endopolyploidy in plants (Ronchi et al., 1965; Rosiak et al., 2002). Alteration in the exogenous supply of auxins and gibberellic acid can activate endocycles *in vitro* and in whole plants (Cavallini et al., 1995; Valente et al., 1998; Fujii et al., 1999; Gendreau et al., 1999; Mishiba et al., 2001).

Genetic analyses of arabidopsis mutants defective in the perception of gibberellic acid, ethylene, and cytokinin have also demonstrated hormonal regulation of endopolyploidy (Gendreau et al., 1999). In a number of systems light quality and phytochrome perception have been linked to endopolyploidization. In all cases, endoreduplication is associated with the elongation of plant cells and organs, either in response to genetic ablation of a phytochrome signaling component or etiolation (Cavallini et al., 1995; Gendreau et al., 1997; Gendreau et al., 1998; Cavallini et al., 2001). While this does support the idea that cell elongation and endoreduplication share some functional link, mutation of the blue-light receptor in arabidopsis also leads to cell elongation, but fails to increase the nuclear DNA content, suggesting that elongation and endoreduplication can be uncoupled (Gendreau et al., 1998). However, in *Medicago truncatula*, interference with endoreduplication led to a failure in the expansion of some cells (Cebolla et al., 1999).

Endopolyploidy is also a feature of specialized structures involved in the interaction of plants with microorganisms and animals. As with many of the examples above, plant cells in these structures are often involved in support and nutrient transfer functions and are characterized by high metabolic activity. Endopolyploidy is induced by nematode feeding (Niebel et al., 1996; Engler et al., 1999), the association of roots with arbuscular fungi (Berta et al., 2000), and the process of nodulation in legumes (Foucher and Kondorosi, 2000). In all of these, the transition to endopolyploidy is associated with differentiation into a highly specialized cell type (e.g. syncytial giant cells at nematode feeding sites).

Consequences and correlates of endopolyploidy

Organ growth is accomplished by a combination of cellular proliferation and expansion. Animal cells often increase in volume only modestly following cell division. Animals differ in size by five orders of magnitude between taxa, yet cells increase in volume only modestly. The result is that differences in body size are primarily the result of changes in cell number (Flemming et al., 2000). Plant cells, on the other hand, routinely expand many fold during organ growth (e.g. leaf parenchyma cells), and periods of plant organ growth are often divided into proliferative and expansive stages.

The ubiquitous nature of endoreduplication and other mechanisms that increase nuclear DNA content, suggest these phenomena should have some function in organismal activity. Authors have speculated that endoreduplication is critical for cellular differentiation, cell size, cell fate determinacy, and increasing metabolic activity. Each of these processes is certainly correlated with the occurrence of endoreduplication, and all of them may be caused by endopolyploidy in some systems. Critical experiments testing these hypotheses have not been performed for most endopolyploid tissues, and so the meaning of the correlation has had to stand as suggestive. In general, endoreduplication is associated with ephemeral, highly determined, metabolically active cells (D'Amato, 1977; D'Amato, 1984).

The correlation between cell size and endopolyploidy, particularly when done within a tissue type, is perhaps the most widely researched relationship (Nagl, 1976; D'Amato, 1977; D'Amato, 1984; Kowles and Phillips, 1985; Melaragno et al., 1993; Kingham et al., 1995; Gendreau et al., 1997; Cebolla et al., 1999; Engler et al., 1999;

Perazza et al., 1999; Flemming et al., 2000; Foucher and Kondorosi, 2000; Kondorosi et al., 2000; Luszczek et al., 2000; Edgar and Orr-Weaver, 2001; Kudo and Kimura, 2002). As endoreduplication and other forms of DNA re-replication can occur in cells mechanistically recalcitrant to wall formation, e.g. crystal accumulating idioblasts and endosperm storage cells, endoreduplication may help accommodate cytoplasmic accretion and cell expansion. Polysomaty increases during and after leaf expansion, leading investigators to suggest that endoreduplication-mediated cell expansion could play a role in leaf area determination (Melaragno et al., 1993; Traas et al., 1998). As cytoplasmic enlargement is often associated with increases in plastids and mitochondria, some authors have proposed and investigated a connection between endopolyploidy and the maintenance of a balance between nuclear and cytoplasmic genomes (Galbraith et al., 1991). Indeed, when comparing endosperms from the maize inbreds B73 and Mo17, Mo17 endosperms have more cells, of a smaller size, each with fewer amyloplasts (Jones et al., 1996) and a lower degree of endopolyploidy (see Chapter 2). Given the importance of cell expansion to growth in plant systems, these data suggest that endoreduplication could play a role determining the ultimate volume of plant cells and organs.

If endopolyploidy positively impacts growth, one would predict a high correlation between gene expression, metabolic activity, and the number of genome equivalents available for transcription. In the embryos of legumes, microphotometric measurements of polyploid nuclei determined that the RNA/DNA ratio was two fold greater in highly polyploid nuclei, when compared to diploid controls (Clutter et al., 1974). However, this study compared cells within the suspensor to the embryo proper, and therefore did not

account for differences in differentiation state. In *Chara vulgaris*, both RNA and protein synthesis increased with ploidy in the basal cell, suggesting that ploidy, independent of other forms of differentiation, contributed to metabolic activity (Malinowski and Maszewski, 1994). Measurements of rRNA accumulation from leaves of 2C and 4C polyploid maize lines (Guo et al., 1996) provided similar results, arguing in favor of the hypothesis that RNA accumulation can be related to the number of genomes available for transcription. Furthermore, in polyploid series of maize and yeast (e.g. 1C, 2C, 4C), the majority of mRNA transcripts did not deviate in their relative abundance between diploid and tetraploid individuals when normalized to rRNA abundance (Guo et al., 1996; Galitski et al., 1999). Thus, two 2C cells would be roughly equivalent to one 4C cell in their summed transcriptional activity. Additional evidence in favor of this relationship is largely the result of correlative histological studies, demonstrating that metabolically active tissues also contain the highest degree of endopolyploid cells (D'Amato, 1984).

As endoreduplication does not involve the formation of new cell walls or plasma membranes, it serves as an efficient way to maximize gene expression. Neither the energy necessary to engage in cell division nor cell wall and membrane biogenesis needs to be expended, and the cell cycle time can be reduced by eliminating M-phase and all preparations for division. However, this does sacrifice plasticity, in that cells no longer are able to produce haploid gametes, and often divide more slowly; this is reflected in the occurrence of endoreduplication in terminally differentiated, ephemeral and highly determined cells (D'Amato, 1964).

In both plants and animals, body size has been correlated with endoreduplication. A recent study in nematodes demonstrated that the best predictor of body size in 16 taxa was a combined measure of nuclear number and nuclear ploidy (Flemming et al., 2000). In wheat endosperm, dry matter accumulation was effectively modeled by either nuclear number or ploidy, but was best predicted by a combination of the two (Brunori et al., 1993). Pea seed weight and pod size are also best modeled by a combination of cell ploidy and number (Lemontey et al., 2000). In wheat and pea seeds, it is endopolyploidy rather than cell number that best predicts size at maturity. However, in both cases endopolyploidy and cell number are positively correlated. Thus, it is reasonable to propose that endoreduplication can accomplish growth and increases metabolic capacity per cell to support the increased metabolic demands of larger cellular compartments.

The relationship between endopolyploidy and cell differentiation is complex. For example, differentiated cells of the moss, *Frunaria*, typically undergo three rounds of nuclear DNA re-replication (8C), while *Physcomitrella patens* contains no endopolyploid cells in the mature gametophyte (Reski et al., 1994; Kingham et al., 1995). While the majority of plants have cells that endoreduplicate in many of their organs, measurement of thousands of *Helianthus* nuclei from a variety of tissues provided no evidence for polyploid cells (Evans and Vanthof, 1975). These results suggest that increases in ploidy are not strictly required for differentiation of many plant cell types often found in endopolyploid states in the adult plant body. However, changes in cell size and differentiation are often associated with the presence of endopolyploid nuclei, and sometimes ploidy-dependent (Cebolla et al., 1999; Perazza et al., 1999). In the trichomes

of arabidopsis, cell morphogenesis is related to DNA re-replication and nuclear DNA content. Tetraploids, or mutants that over-replicate DNA, produce supernumerary trichome branches while mutants that are defective in replication produce fewer branches (Szymanski and Marks, 1998; Perazza et al., 1999). However, the trichome branching pattern is not an endopolyploidy-dependent phenomena, per se. Mutations that cause ectopic divisions within trichome cells produce trichomes of normal branching pattern composed of multiple cells (Walker et al., 2000; Schnittger et al., 2002). These multinucleate trichomes consist of cells of lower ploidy that sum to a similar number of genomes as the endoreduplicated trichomes of normal leaves, suggesting that the ultimate volume and metabolic output remain linked to the number of genomes available for transcription.

In many cases, endopolyploid cells either cease dividing, or at least slow division below the level of detection. This form of cellular determinacy provides a mechanism for endocycles to limit the size of an organ. If an organ can switch from proliferative cell cycles to endocycles in response to the environment, or developmental cues, it may limit the amount of resources that must be committed to the growth of the organ. If endoreduplication serves to fix the cell number of an organ, it may actually operate as a mechanism to restrict the upper limit of an organ's growth. For example, in leaves endoreduplication is often limited to only a few cycles (Smulders et al., 1994; Cavallini et al., 1995; Cavallini et al., 1997; Szymanski and Marks, 1998; De Veylder et al., 2001). If these endoreduplication cycles preclude later mitotic division, and cell cycle re-entry, cell number can be fixed by the generation of polyploid cells. Cell expansion of these cells

could proceed to a maximum-allowable size and then would cease. To continue growing, neighboring cells would either have to engage in continued expansion of division beyond the boundary of these endopolyploid cells, or overcome the shearing forces within the appressed cell walls. Thus, the diploid cells neighboring these polyploid cells could be limited in their ability to continue dividing and expanding. Growth would only be restricted in the directly abutting dimensions, such that increases in division in the area surrounding an endoreduplicated cell would have an abated effect on organ size (Traas et al., 1998), but divisions elsewhere could continue to expand an organ.

Endopolyploidy does not preclude later mitoses. Plants regenerated from tissue culture of explants of varying ploidy levels occasionally regenerate into tetraploid or greater ploidy individuals (e.g. (Scheid et al., 1996), though polyploid cells regenerate at a reduced efficiency when compared to diploid cells (Gilissen et al., 1996; Ochatt et al., 2000). In animals, cancer cells often become endopolyploid. Considering the requirements of a cancerous tissue, e.g. rapid growth and high metabolic activity, the association with endopolyploidy seems consistent enough with those observed in more "natural" conditions. Tumors consisting of chimeras of mitotically active endopolyploid cells are frequently observed, and in some types of cancer it has been determined that increases in ploidy are negatively correlated with patient survival and response to radiological and chemotherapeutic treatment (Illidge et al., 2000; Ladenstein et al., 2001; Walen, 2002). Thus, endoreduplication can impact many aspects of an organisms growth, from increasing metabolic rates, increases of cell size, increasing the determinacy

of tissues, differentiating cells within a tissue, and facilitating programmed and unprogrammed alterations to the genomic constitution of parts of the organisms body.

Polyteny, the generation of unseparated chromatids through endoreduplication, may have specific consequences of it's own. In studies of maize endosperm, endoreduplication was shown to be associated with increases in the quantity of the HmgI/Y protein (Zhao and Grafi, 2000). This protein is a relatively abundant chromosomal protein associated with the activation of a number of genes in plants (Pedersen et al., 1991; Thanos et al., 1993; Ponte et al., 1994; Zhao and Grafi, 2000; Ascenzi et al., 2001). HmgI/Y binds to the AT-rich enhancer elements in gene regulatory regions (Grasser et al., 1990; Ponte et al., 1994) and stimulates binding of transcription factors (Krohn et al., 2002). The presence of multiple chromatids may also facilitate intrachromatid and intrachromosomal information exchange. If changes in gene regulation result in the activation of a subset of genes, then the metabolic output of an endopolyploid cell may be higher than an equivalent number of genomes distributed into many cells. More conservatively, if we assume a gene template equivalently expressed in cells of any ploidy normally found within a tissue, then 82% of all gene products produced in maize endosperm at 13 days after pollination (DAP), and 91% at 18-DAP, are the result of transcription in polyploid nuclei (Dilkes et al., 2002).

The metabolic activity of an organ is determined by the capacity of each cell and the number of cells in the organ. If the activity of one cell with two genome copies is equivalent to two cells with a single copy, as has been suggested, then *enhanced* endoreduplication should contribute to the activity of a plant organ as much as *enhanced*

cell division. However, if DNA re-replication takes the place of an equivalent number of mitotic cycles, we would expect no change in organ size. Furthermore, if an organism utilizes endoreduplication to fix cell number, higher %E may be associated with smaller organ size. All of these suggestions can be unified if the number of genome equivalents, per organ, determines both the informational capacity of that organ and its sustainable metabolic activity. This proposal unifies the data obtained for worm body size (Flemming et al., 2000), pea seed weight (Lemontey et al., 2000), polyploid and defective kernel mutants in maize (Kowles et al., 1992; Guo et al., 1996), and endosperm weight in wheat (Brunori et al., 1993).

Maize endosperm development

In angiosperms that reproduce via sexual reproduction, seeds are produced through a double fertilization of the haploid female gametophyte (Nawaschin, 1898). One pollen nucleus fuses with the egg cell to form the zygote, and the other pollen nucleus fuses with either two haploid nuclei or a diploid nucleus resulting from their fusion in the central cell of the female gametophyte, forming the endosperm cell. Thus, two genetically distinct structures co-exist in the seed: a diploid embryo (sporophyte) that will carry genetic information into the next generation and a triploid endosperm containing two genome copies from the megagametophyte and one from the microgametophyte.

The endosperm of angiosperms has been demonstrated to be important for embryo development and seedling establishment, and therefore plant fecundity. In seeds with persistent endosperm, such as maize, this tissue engages in critical support functions

storing, and later supplying the embryo and seedling with fixed carbon and amino acids. This storage function has been shown to be important for seedling establishment and embryo development in a number of plants. Even in plants with an ephemeral endosperm, such as arabidopsis, defects in endosperm development are suspected to result in embryo abortion (Scott et al., 1998; Kinoshita et al., 1999; Kiyosue et al., 1999; Adams et al., 2000; Vinkenoog et al., 2000; Baroux et al., 2002).

During the very earliest stages of development, the maize triploid endosperm nucleus proliferates via a syncytial cell cycle, forming a multinucleate coenocyte within the central cell (Olsen, 2001). Each nucleus has an associated region of dense cytoplasm, and the central cell is organized into mitotic subdomains. Replication and nuclear division proceed in a partially synchronous manner, but cell plates do not form between nuclei. Some experiments in barley suggest that phragmoplast formation begins but is aborted or suppressed, leaving traces of microtubule structures associated with daughter nuclei at the site of aborted cell wall formation (Brown et al., 1994; Brown and Lemmon, 2001). During nuclear proliferation, daughter nuclei migrate out of the chalazal pole and distribute throughout the central cell, suggesting that the endosperm coenocyte is polarized. Radial polarity within the coenocyte is also apparent, as nuclei migrate to the periphery of the cell (Olsen, 2001). Cellular polarity does not appear to require active signaling by the sporophyte, through phytohormones or positional cues, as isolated *in vitro* fertilized endosperms show similar organization (Kranz et al., 1998; Laurie et al., 1999).

In vivo and *in vitro* fertilized central cells cellularize between 3-5 DAP (Kranz et al., 1998; Laurie et al., 1999; Olsen, 2001). By this time, approximately eight or nine rounds of nuclear division have taken place, lining the periphery of the central cell with nuclei. Radial microtubule arrays then form around each of the nuclei. Phragmoplasts organize at the junctions between microtubule arrays, and cell wall and plasma membranes are formed anticlinal to the wall of the central cell (Olsen, 2001). These new walls extend until they meet the central cell wall, and then grow into the central cell, forming alveoli that surround each nucleus. The microtubule arrays re-orient during this process, such that they extend to the aperture of the alveolus and connect with the central cell wall anchoring the nucleus to the periphery of the central cell. Division of nuclei within alveoli proceeds via a modified mitotic-type cell cycle that results in cell wall formation periclinal to the central cell. This cell cycle differs from the meristematic cell cycle in that it does not produce a PPB, utilizes an atypical phragmoplast, and there is an unknown mechanism for determining the plane nuclear division (Berger, 1999; Otegui and Staehelin, 2000; Olsen, 2001). Nuclear divisions at the aperture of the alveoli continue to form anticlinal cell walls until the cells have fully invaded the interior of the central cell, when cellularization is complete, and results in ordered cell files. Cellularization is not entirely synchronous, as nuclei in the embryo-proximal region of the endosperm cellularize slightly earlier (Olsen, 2001). Once the endosperm is completely cellularized, mitotic cell cycles continue and most of the cell divisions lack PPBs. Rather than producing periclinal and anticlinal cell walls in an orderly fashion, the

orientation of cell division appears to be random. Thus, after only a few rounds of division, the appearance of orderly cell files is lost from the endosperm.

The cells of the endosperm differentiate into four histologically distinct cell types: aleurone, basal endosperm transfer layer, embryo-proximal region, and starchy endosperm. Genetic analyses of mutants and *in situ* hybridization experiments have confirmed the differentiation of these four cell types (Olsen et al., 1992; Olsen et al., 1999; Olsen, 2001).

The cells at the chalazal pole of the endosperm form the basal endosperm transfer layer (BET). Three to four layers of these cells elongate, and their walls and plasma membranes invaginate, increasing the surface area of the cells, presumably to facilitate transport of nutrients from the maternal sporophyte to the developing endosperm and embryo (Thompson et al., 2001). This region is transcriptionally distinct from the remainder of the endosperm (Hueros et al., 1995; Hueros et al., 1999; Hueros et al., 1999; Gomez et al., 2002). Studies of transport rates, using radio-labeled sugars and amino acids, have demonstrated that this tissue is a primary source of assimilate transport into the developing seed (Shannon, 1972; Shannon and Doughert.Ct, 1972; Felker and Shannon, 1978, 1980). Consistent with a role in metabolite transport, transport-related genes are specifically expressed or upregulated in the BET (Barrieu et al., 1998; Weschke et al., 2000; Weschke et al., 2003).

Cells at the periphery of the remainder of the endosperm differentiate into the aleurone. In late endosperm development, the aleurone is critical for endosperm breakdown and nutrient readsorption by the embryo and developing seedling (Bethke et

al., 1998; Olsen, 2001; Simpson, 2001). During maize endosperm development, aleurone cells divide both periclinally and anticlinally. Periclinal division results in a daughter cell no longer at the surface of the endosperm; this cell subsequently differentiates into a starchy endosperm cell, demonstrating some developmental plasticity within the endosperm. Unlike the remainder of the endosperm, aleurone cell divisions mark the plane of division with a PPB (Brown and Lemmon, 2001; Olsen, 2001). In maize, the appearance of the PPB is the earliest sign of aleurone cell fate specification; however, *in situ* hybridization analyses of gene expression have also identified markers with a similar onset of expression (Doan et al., 1996; Olsen, 2001).

The majority of the maize kernel (>90% by weight) is composed of starchy endosperm cells. These cells accumulate storage proteins in protein bodies, carbohydrate in amyloplasts and various other minerals and metabolites. By weight, the most important of these is starch, which comprises 85-90% of the kernel dry weight at maturity. In the maize seed, it is the starchy endosperm cells that function as the primary storage compartment. Upon germination, these metabolic reserves are mobilized and used by the developing sporophyte. The storage protein is predominantly found in spherical accretions called protein bodies and is mostly comprised of alcohol soluble prolamins called zeins. The composition of the starchy endosperm is not uniform; differentiation displays a radial gradient such that cells at the periphery tend to have fewer and smaller starch grains and protein bodies than those in the interior (Boyer et al., 1976; Lending and Larkins, 1989). *In situ* hybridization with probes corresponding to a number of storage protein genes indicated that in addition to a radial developmental

gradient visible by histological staining, gene expression is spatially and temporally regulated within the starchy endosperm (Woo et al., 2001). The pattern of zein gene expression roughly corresponds to the spatial and temporal distribution of protein body maturity within starchy endosperm.

Less is known about the function of the embryo-proximal region. This region may be critical for the transport of nutrients from the endosperm to the embryo, and for the exchange of hormones and other cues important for development. In maize, a small gene family has been identified as differentially expressed in the embryo proximal region. In situ hybridization and promoter-GUS fusions with these genes have demonstrated that the embryo proximal region is transcriptionally distinct from the remainder of the endosperm (Opsahl-Ferstad et al., 1997; Bonello et al., 2000; Bonello et al., 2002). Differences in cellularization timing and the high cytoplasmic density of these cells early in development also supports the classification of this region as a distinct zone of differentiation within the maize and barley endosperm (Olsen, 2001).

The radial gradient of the endosperm for protein body and starch grain maturation is mirrored in other aspects of cellular differentiation within the starchy endosperm, such as endoreduplication. In maize endosperm, nuclear DNA endoreduplication commences in starchy endosperm cells by 8-9 DAP. The endoreduplication cell cycle is estimated to require 24h for completion (Kowles and Phillips, 1998). As discussed earlier, endosperm cellularization is completed by 5- to 7-DAP, and the rapid onset and progression of endoreduplication appears to contribute to the importance of this process in the development of this tissue. Mitosis all but ceases in the starchy endosperm by 12-DAP

(Kowles and Phillips, 1985). Prior to this, mitosis and endoreduplication overlap such that some new 3C cells are generated, mostly at the periphery (Olsen, 2001), and the 6C cells are comprised of replicated nuclei awaiting division (e.g. G2), cells awaiting DNA replication (e.g. G1), and terminally differentiated cells that are cell cycle arrested (Schweizer et al., 1995). By 13-DAP 82% of the genome equivalents in the endosperm are present in 6C or greater nuclei, and by 18-DAP this has risen to 91% (See Chapter 2). Mitotic cell cycles continue in the periphery of the endosperm in the aleurone and sub-aleurone layers. These divisions presumably increase the number of cells at the periphery to accommodate endosperm expansion.

Microscopic observation of Feulgen-stained nuclei in developing endosperm suggests that endoreduplication is most extensive in the center of the endosperm. A gradient of nuclear sizes and ploidy level is visible at the periphery of the endosperm (Kowles and Phillips, 1985), similar to the gradients in protein body and starch grain sizes (Lending and Larkins, 1989). The concomitant timing of zein gene expression and endoreduplication, and the similarity in spatial distribution of endopolyploid nuclei and mature protein bodies suggest co-regulation of these two traits. At the very least, it would appear that cells in the starchy endosperm increase in size and nuclear DNA content coincidently with storage product biogenesis.

Nuclei with ploidy levels of 96C (representing 5 successive S-phases) are routinely found in endosperms from most maize genotypes, and a substantial proportion of 128C nuclei are not uncommon (Kowles et al., 1997). When single nuclei can be measured, as with Feulgen-stained tissue sections, nuclei of higher ploidy values are

reported at the center of the endosperm (Kowles and Phillips, 1985). However, the mean ploidy of the endosperm is considerably lower than this, owing to the high degree of polysomaty and low frequency of high ploidy nuclei and persistence of all lower ploidy classes throughout development. As a result, mean ploidy values for Midwestern dent varieties are typically around 10-14C at mid-development (Kowles et al., 1990; Kowles et al., 1997).

Endoreduplication in the endosperm continues through 21- to 27-DAP, coincident with the collapse of the starchy endosperm. Endosperm collapse is associated with DNA degradation and cell death (Young et al., 1997; Young and Gallie, 1999, 2000, 2000). As many studies use endopolyploidy as a proxy for endoreduplication, the degeneration of cells at the interior of the endosperm, i.e. those that have engaged in endocycles the longest, makes evaluation of the physiological relevance of endopolyploidy on mature endosperm size difficult.

We do not know the relationship between the degree of endopolyploidy at 18-DAP and the absolute or mean number of endoreduplication cycles experienced by endosperm cells. Neither has the relationship between endopolyploidy at 18-DAP and mature kernel phenotypes, such as starch and protein content, been investigated. By 18-DAP the endosperm has achieved approximately 70% of it's maximal volume and fresh weight and accumulated 50% of the dry matter it will have at maturity (Ritchie et al., 1997). Further increases in cell number are negligible by 17-DAP and cell numbers decrease after 20-DAP (Schweizer et al., 1995). This suggests that the remaining growth of the endosperm is due to ploidy-mediated growth and/or metabolic activity in cell cycle

quiescent cells. As seen in Chapter 2, a majority of the cells in the endosperm exist as cells with greater than 3C ploidy values by 18-DAP. Thus, if endoreduplication affects endosperm size, contributions of endopolyploidy to endosperm size should be evident at this stage.

Storage protein synthesis in maize endosperm

The maize prolamins, zeins, are subdivided into four classes (alpha, beta, gamma and delta) based on SDS-PAGE migration, immunologic similarity and amino acid sequence analyses. Zeins are predominantly expressed in a subset of starchy endosperm cells. The first zeins to be synthesized and to acquire spatially distinct organization are the 14- and 15-kDa beta and 16- and 27-kDa gamma zeins. By 10-DAP, the 27-kDa-gamma zein gene is expressed throughout the starchy endosperm as an abundant mRNA (Woo et al., 2001; Hunter et al., 2002). While the accumulation of storage proteins and endoreduplication are temporally correlated, expression of zein genes and the differentiation of metabolically active starchy endosperm cells does not require endoreduplication. The beta and gamma zeins contain considerable amounts of cysteine (c.a. 7%) and appear to form both intra- and inter-molecular cysteine bridges; these may provide structural support for the protein body (Shewry and Tatham, 1990; Lopes and Larkins, 1993; Shewry and Halford, 2002). At 9-11 DAP in the central endosperm, and in recently differentiated starchy endosperm cells at the periphery of the horny endosperm throughout development, the beta and gamma zeins form a small spheroid that stains densely with uranyl acetate, consistent with the high cysteine content (Lending and Larkins, 1989).

Expression of the genes encoding the alpha zeins commences shortly after the beta and gamma zeins. Alpha zeins can be distinguished into subtypes by immunological and sequence similarity. They are classified by SDS-PAGE migration into 22-kDa and 19-kDa alpha zeins, each with recognizable subtypes (Song et al., 2001). These proteins are co-translationally inserted into the small beta and gamma-containing accretions, increasing the size of the protein body and producing less electron dense "locules" within the beta and gamma zeins (Lending and Larkins, 1989; Lopes et al., 1994; Coleman et al., 1997). As protein bodies mature, beta and gamma zeins are partitioned to the exterior, comprising a shell, while alpha is accumulated in increasing amounts at the center of the endosperm. This process is not uniform throughout the endosperm, and in general it follows a spatial pattern consistent with endosperm differentiation; the youngest cells occur at the periphery and fully mature cells and protein bodies are found towards the interior of the endosperm (Lending and Larkins, 1989).

The onset of storage protein accumulation is associated with dramatic changes in gene expression. The levels of individual zein mRNAs can be extremely high (Marks et al., 1985; Song et al., 2001; Woo et al., 2001; Hunter et al., 2002). For example, 22-kDa α-zein mRNAs are not detectable before 9-DAP by northern blot (Song et al., 2001). Subsequently, single genes can account for up to 4.9 % of the total transcript population by mid-development (Woo et al., 2001). These changes in gene expression are associated with changes in the abundance of chromosomal proteins associated with chromatin de-condensation. During endoreduplication in maize endosperms, there is a reduction in the chromatin-condensing histone H1 protein and increased accumulation of high mobility

group (HMG) protein I/Y (Zhao and Grafi, 2000). HMG proteins bind enhancer-like cis-elements in the promoters of endosperm storage protein genes and assist transcription factor binding (Grasser et al., 1990; Schultz et al., 1996). It has been suggested that this may be one way in which cellular differentiation via an endocycle contributes to endosperm metabolic output and dry matter accumulation (Zhao and Grafi, 2000).

Analysis of zein promoters has identified cis-acting motifs that are conserved in endosperm-specific genes from a number of cereals (Takaiwa et al., 1991; Nakata and Okita, 1996; Russell and Fromm, 1997; Mena et al., 1998; Wu et al., 2000). Promoter fragments have been identified that are necessary and sufficient for high level endosperm-specific gene expression in maize (Takaiwa et al., 1991; Russell and Fromm, 1997). Some of the conserved sequence elements have been demonstrated to bind endosperm-specific transcription factors, and are presumably required for high level expression (Vicente-Carbajosa et al., 1997; Mena et al., 1998; Yunes et al., 1998; Carlini et al., 1999). However, nothing is known about the contribution of these transcription factors, and their binding sites to the spatial regulation of gene expression within the endosperm.

Endoreduplication and endosperm growth and growth inhibition

Much of the increase in kernel volume and mass occurs during the period described as the "effective grain filling period" (EGFP). The onset and duration of this period temporally coincides with that of endocycles within the starchy endosperm (Schweizer et al., 1995; Jones et al., 1996). During the EGFP, starch and storage proteins are rapidly synthesized and accumulated in the starchy endosperm cells. The role of

endoreduplication in the EGFP is undetermined. However, a number of lines of evidence suggest they are related. In wheat, mature kernel weight was predicted by a combination of the number of mitotic divisions and the number of endoreduplication cycles in the endosperm, and it was much better predicted by the latter than the former (Brunori et al., 1993). In maize, both endosperm endocycling and the EGFP are negatively impacted by water stress, heat stress, and exogenous application of ABA. Lastly, both the EGFP (Poneleit and Egli, 1983; Seka and Cross, 1995, 1995; Jones et al., 1996) and some aspects of endoreduplication (Cavallini et al., 1995; Kowles et al., 1997) are subject to maternal control, such that F1's more closely resemble the ear-parent. Given the evidence for endopolyploidy's impacts on plant growth and the correlation between growth, cell expansion, and endoreduplication in maize endosperm, it appears likely that endoreduplication influences the rapid synthesis of starch and storage protein during the EGFP.

Endoreduplication in maize endosperm can be influenced by alterations to kernel physiology. Exogenous application of the auxin analog, 2,4 dichlorophenoxyacetic acid, prior to the period of endoreduplication, increased endopolyploidy at 9- and 11-DAP (Lur and Setter, 1993). However, applications of an auxin inhibitor led to no change in endopolyploidy early in development but decreased it at 13-15-DAP (Lur and Setter, 1993). The stress and ripening hormone, ABA, also impacts the cell cycle in maize endosperm. Exogenous applications of ABA at concentrations of 100 μM reduced the number of cells per endosperm, but had little effect on kernel fresh weight at 11-DAP (Myers et al., 1990; Mambelli and Setter, 1998). Endosperm cell number was most

dramatically affected if ABA was applied early in development, e.g. 5-8-DAP, when mitosis is most active. By contrast, endopolyploidy was less sensitive. In an identical treatment regimen, 1mM ABA was required for a significant decrease in mean ploidy at 11-DAP; the percentage of nuclei in higher ploidy classes was most affected and decreased between 100-300 μM ABA (Mambelli and Setter, 1998).

Water and heat stress influence endoreduplication similar to the effects observed with ABA application. Water stress, early in endosperm development, inhibits cell division but stress during the endoreduplication phase had little effect on mean ploidy. Endopolyploidy, particularly that in nuclei of the highest ploidy classes, as inferred from nuclear size, was dramatically decreased by early water stress, suggesting that after the removal of the stress endoreduplication does not proceed normally (Artlip et al., 1995). Heat stress has been well studied in relation to kernel growth, cell division and endoreduplication. Application of heat stress at 5-DAP for four or six days dramatically reduces kernel dry weight and cell numbers (Commuri and Jones, 2001). As with water stress, endoreduplication is most sensitive to heat stress occurring before the endoreduplication period, such that four days of heat stress from 4-8 or 6-10 DAP halves the mean ploidy at 18-DAP, while later application of the same stress had no effect on mean ploidy at 18-DAP (Engelen-Eigles et al., 2001). In the case of heat stress, correlation coefficients were calculated between fresh weight, nuclear number, and mean ploidy. There was a statistically significant positive correlation between nuclear number and both fresh weight and mean ploidy. However, fresh weight was better predicted by the mean ploidy of the endosperm than nuclear number (Engelen-Eigles et al., 2000),

similar to observations in pea and wheat seeds (Brunori et al., 1993; Lemontey et al., 2000). Interestingly, the dry weight of Mo17 inbred endosperm is substantially more sensitive to heat stress than B73, and Mo17 endosperm cells exhibit less endoreduplication (Commuri and Jones, 2001). The results of the ABA and water stress experiments and the high correlation of fresh weight to mean ploidy, suggest that enhanced endoreduplication positively impacts endosperm growth and exchanging mitotic for endoreduplication cell cycles may lead to stress-resistant endosperm growth.

Maternal control of endoreduplication as quantitative trait

Quantitative genetics and maternal control

Quantitative genetic analyses have at their heart two complementary approaches. On the one hand, are variations in trait means, or suitable non-parametric correlates, which facilitate the identification of populations that vary for the particular trait being studied. However, trait mean values can change due to a number of factors, and defining the degree to which a trait is subject to genetic control is a "mean components" analysis. On the other hand is a variance-components analysis, an investigation of the deviation of individuals within a population from their population mean. It is alterations in variance, and not the mean, that typically provide the most valuable information regarding the populations under observation and the genetic differences between them. Principally, while detection of mean allelic effects suggests the presence of genetic control, it is the variance that increases when genes segregate, providing the best evidence for or against a particular model of genetic effects. Detection of an increase in variance requires the

measurement of generations in which genes do not segregate and generations in which they do. The mode of inheritance expected for a gene would then dictate the crosses and generations necessary to observe segregation variance.

Perhaps in no plant tissue is the importance of proper crossing design more pertinent than in endosperms. First, endosperm is triploid, necessitating a design that accounts for the two identical alleles inherited from the female gametophyte and the single allele from the male gametophyte, as well as the two dominance relationships possible (Foolad and Jones, 1992; Alleman and Doctor, 2000). Second09, maize endosperm development is significantly influenced by the maternal parent. For example, during maize seed development, starch, protein and other nutrients from the maternal parent (sporophyte) are stored in the endosperm of the developing kernel. Kernel size and shape are known to be maternally controlled, such that F1 kernels more closely resemble the ear parent (Birchler, 1993; Alleman and Doctor, 2000). This is also true for the EGFP (Jones et al., 1996) and some aspects of endoreduplication (Cavallini et al., 1995; Kowles et al., 1997). Maternal effects on seed and fruit traits have been demonstrated for many plants, suggesting this is a general feature of angiosperms. (Foolad and Jones, 1992; Birchler, 1993; Cavallini et al., 1995; Cavallini et al., 1997; Kowles et al., 1997; Alonso-Blanco et al., 1999; Alleman and Doctor, 2000; Lagunes-Espinoza et al., 2000; Lemontey et al., 2000). Lastly, the endosperm has a genome balance of two female gametophyte and one male gametophyte genomes (2m:1p). In maize, successful development of the endosperm requires that this balance of maternal and paternal genomes be maintained, as departure from the 2m:1p ratio leads to

endosperm failure and kernel abortion (Cooper, 1951; Lin, 1982; Lin, 1984). This demonstrates that the contributions of the seed and pollen parents to the endosperm are not equivalent. Thus, an additional form of maternal and paternal control are theoretically possible (gametophytic inheritance) in endosperm and should be formally considered, especially for traits with demonstrated parent-specific-effects.

The rule of thumb in quantitative genetics is to test the additive only and additive with dominance models first. Typically, if they prove to be adequate, then it is not necessary to test any additional models. If additional evidence suggests that some other mode of inheritance or complicating factor influences the quantitative trait, then the experimental design should allow a test or provide a control for these factors. For seed traits, and traits influencing early life history in animals and plants, it should be considered necessary to investigate, or control for, the presence of maternal influence. In endosperm, the question becomes which additive or additive and dominance model should be tested first. Models for triploid or diploid inheritance might be appropriate. In addition, factors displaying parent-of-origin specific and maternal sporophytic genetic effects have been identified as modulators of endosperm growth and must be formally considered. Ignoring the differences in predictions for different maternal modes of inheritance also has dangers, as the most common experimental designs (e.g. the diallele), cannot distinguish between parent-of-origin and sporophytic effects.

Quantitative genetics, like all of experimental science, is subject to canalization and distortion by the subjectivity of the researcher. Data are tested against theoretical models of inheritance, and the question is asked if the data and the models are

irreconcilable. As with all evidence, qualities inherent within the experiment determine the precision of estimates drawn from the data and the kinds of differences that can be detected. As such, the experimental design (e.g. the nature of each cross, the number of individuals sampled) as well as the theoretical models tested (e.g. diploid nuclear inheritance with additive and dominant allele effects) can unduly influence interpretation of the data, and hence the conclusions. Investigations cannot always distinguish between sporophytic and gametophytic maternal control. In some studies this is explicitly stated, and conclusions regarding the meaning of the results are carefully drawn (Cavallini et al., 1995; Cavallini et al., 1997). However, in the rather extensive literature on nutrition and cooking quality of rice endosperms, a diallele design was utilized which lumps sporophytic and gametophytic genetic effects, and maternal additive genetic effects were assigned to the sporophyte (Shi et al., 1997; Shi et al., 1999; Shi et al., 1999; Shi et al., 2000). As a further example of the utility in considering multiple modes of inheritance, QTL mapping of endosperm traits using a diploid model led to the identification of significant QTL (Wang and Larkins, 2001; Wang et al., 2001) that was better fit to a triploid inheritance model (Wu et al., 2002). The triploid model detected more loci and had smaller intervals, demonstrating that the precision and sensitivity of genetic estimates are dependent on the mode of inheritance employed.

Evaluation of quantitative traits is frequently complicated by two types of maternal influence. The first and most common is a shared maternal-environment effect. This can be envisioned as similarity, or predictable differential treatment, by the maternal parent in organisms where development is tied to a persistent maternal parent and shared

microenvironmental effects due to co-localization. In maize, this might occur as an influence of the environment on the metabolite source strength of the sporophyte during kernel development. The second type of maternal effect is bonafide genetic effects. Either the genotype of one parent differentially influences a trait, or the genes inherited from only one parent affect the trait. Differential contributions of the maternal and paternal sporophytic generations, as well as the maternally inherited plastid and mitochondrial genomes, can be considered as examples. If not considered directly, all forms of maternal effect can lead to incorrect estimation of the allelic effects and heritability by other modes of inheritance (Falconer and Mackay, 1996; Lynch and Walsh, 1998). As shown in Chapter 2, maternal sporophytic and gametophytic effects can be distinguished by a variance component analysis. Gametophytic expression of alleles will result in segregation and increases in variance, so called break-out variance, when the F1 is used as the ear parent. Cytoplasmic and sporophytic effects will be of no consequence for the variance in the crossing design implemented, but may effect estimates of the mean.

Endoreduplication in maize endosperm has been subjected to six genetic studies, in addition to the one I undertook. In the first, the largest cells from the interior of endosperms from inbred lines, some harboring a mutation of the Opaque2 transcription factor, were analyzed. The authors detected an increase in the average ploidy level of cells in *o2* endosperms when compared to their wild-type counterparts (Kowles and Phillips, 1985). Upon repeating this study with whole endosperms, I was unable to detect a change of this kind. This suggested either the microphotometric method used produced

an artifact in *o2* comparisons or, more likely, that the modest changes in the mean ploidy of a few cells in the interior of the endosperm was below the resolution of detection by whole endosperm analysis (Kowles and Phillips, 1985). This same group followed up the study with an analysis of maize defective kernel mutants. These mutants were analyzed for endosperm cell number as well as ploidy. All the mutants but one were found to contain fewer endosperm cells and decreased levels of endoreduplication. One mutant decreased endosperm cell number without also decreasing ploidy. However, no mutants were found that had decreased levels of endoreduplication and no concomitant effect on cell number (Kowles et al., 1992). In a second study of defective kernel mutations, one mutant was identified with no change in nuclei per endosperm, and an increased mean and median ploidy level (Lur and Setter, 1993). Unfortunately, the number of samples analyzed did not permit a statistical evaluation of significance. Nonetheless, these two studies suggest that endoreduplication and mitotic proliferation can be uncoupled (Kowles et al., 1992; Lur and Setter, 1993).

The last three studies are most relevant to the research I undertook. In one, endosperm endoreduplication levels in Illinois High Protein and Illinois Low protein populations, their reciprocal crosses and F2 generations were measured at low precision using microphotometric methods. The authors concluded their data were consistent with predominantly maternal control over endoreduplication, and the lack of a detectable increase in the variance of the F2 indicated the absence of genetic segregation for the trait (Cavallini et al., 1995). However, the quality of the data and the accuracy of the conclusions are difficult to assess, as it is difficult to imagine that reliable estimates of

mean ploidy could be calculated from the data presented (Cavallini et al., 1995, Fig 1). In a separate study, a large number of maize inbreds and populations, as well as some of their reciprocal crosses, were subjected to flow cytometric analyses of endopolyploidy (Kowles et al., 1997). In some cases, individuals from F1, F2, and F3 generations were measured, and the means and variances compared pair-wise. These comparisons indicated that in most crosses, the mean ploidy of F1 progeny more closely resembled the maternal parent. However, in some crosses the F1 was closer to the paternal mean value, suggesting that the variability in ploidy in the stocks chosen was subject to different modes of inheritance. Furthermore, the variance of endoreduplication in the F1 and F2 generations of the few crosses analyzed was not different, but the variance increased in the F3, as assessed by pair-wise F-tests for variance equivalence. Though no formal genetic analysis was attempted, the authors concluded that the maternal genetic control of endoreduplication is most consistent with maternal sporophytic determination of endopolyploidy levels in the progeny endosperms cells (Kowles et al., 1997). However, the data were not shown, and only the conclusions of statistical tests were shown and discussed. In the last study, the effect of heat stress on endoreduplication in the endosperms of two inbred lines, B73 and Mo17, was investigated. As discussed above, the application of heat stress early in development inhibited later endoreduplication, and the effect was more severe in the lower-endoreduplicating Mo17 genotype (Commuri and Jones, 2001).

The observation of predominantly maternal control of endoreduplication was clear in the Cavallini et al. (1995) and Kowles et al. (1997) studies, but at least three

modes of maternal inheritance were possible, and no formal genetic analysis was attempted. The first possible maternal mode of inheritance was, as the authors suggested, that the maternal sporophytic genotype determines the phenotype of the endosperm (Cavallini et al., 1995; Kowles et al., 1997). A second possibility, suggested by the authors of the IHP vs. ILP study (Cavallini et al., 1995), was that the cytoplasmic genotype of endosperm, which is solely inherited from the ear parent, determines the phenotype of the endosperm. A third possibility, which could not be discounted with the limited number of crosses utilized by the two previous studies, was that alleles affecting endoreduplication in endosperm are disproportionately expressed from the maternally inherited genomes. This possibility could be distinguished from the other two maternal modes of inheritance by means of a 3^2 factorial design, in which three generations, the two parental and one F1, were inter-crossed. If sporophytic or cytoplasmic effects were the sole operating factors, as suggested by the authors of the previous studies, there should be no difference in the variance of endoreduplication traits between endosperms produced by these crosses. If the genotype of the megagametophyte or microspore affected ploidy, an increase in variance should be seen in the F2 and F1 x parental (BC1) generations. Furthermore, maternal gametophytic effects should be manifest by increases in variance when the F1 is used as the ear parent only.

Theoretical models for the inheritance of traits in endosperms, with and without cytoplasmic and sporophytic effects, have been derived (Foolad and Jones, 1992; Shaw and Waser, 1994; Zhu and Weir, 1994). However, I was unable to find a model for gametophytic effects based on a factorial cross design. Thus, for further experimentation

it was necessary to generate models that would also be able to take into consideration the sole expression of the maternal or paternal chromosomal complement, or residual effects of the megagametophyte, on subsequent endosperm development. Results pertaining to the generation and testing of such models is presented in Chapter 2.

In these models, the additive maternal effect on the mean value of endoreduplication cannot necessarily be assigned to gametophytic and sporophytic components. Even if the variance component analysis detected gametophytic effects, the mean component analysis, which lumps both into an indistinguishable additive component, would not be able to distinguish between the contributions of the two given the crossing design of the experiment. Thus, the detection of female gametophytic effects essentially nullifies any conclusions about the nature of maternal additive effects on the mean value of endoreduplication, as they could be due to either sporophytic and gametophytic alleles or gametophytic alleles alone. However, dominance effects on the mean can only be due to maternal sporophytic effects, and this can serve as an indication that both maternal factors are influencing a trait. If female gametophytic effects are not detected, then maternal additive mean components are most likely due to the genotype of the maternal sporophyte and the conclusions of the previous studies would be confirmed.

Mean ploidy, referred to hereafter as E3P, can be expressed algebraically as two potentially separate components, the mean ploidy of the endopolyploid nuclei (E6P) and the percent of nuclei that endoreduplicate (%E). The E6P is calculated similarly to E3P, except the 3C class is excluded from the average. In maize endosperm, after 14-DAP the vast majority of cells with a 6C DNA content are either in G1, or quiescent, and should

be considered endopolyploid rather than part of a mitotically cycling population of cells. Similarly, the %E is calculated by dividing the number of nuclei with a ploidy of 6C or greater by the total number of nuclei. The E6P and %E can be used to calculate the E3P with the following formula: E3P = (%E x E6P) + (3 x (1-%E)). The mechanisms for the initiation and reiteration of the endoreduplication cell cycle need not share the same regulatory components. Thus, the %E and E6P could actually represent wholly different phenomenon regulated by different genetic effects, and their inheritance should be analyzed separately and as a single trait by modeling the E3P. Prior suggestion of differential regulation has been identified as differential sensitivity of the progression of endoreduplication, here best detected in the analysis of E6P, to water stress and ABA levels (Artlip et al., 1995; Mambelli and Setter, 1998). The data produced in a 3^2 factorial design might also allow for the correlation between traits to be calculated, and provided they are not unduly co-linear, to perform an analysis of co-variance. Covariance analyses can be used to test for shared environmental and genetic regulation. Thus, the nature of the maternal influence on endopolyploidy identified in previous studies (Cavallini et al., 1995; Kowles et al., 1997), and the relationship between potentially separable components influencing endopolyploidy was investigated as part of this dissertation.

Molecular mechanisms of cell cycle regulation

As one would expect of a crucial and complicated set of events, regulation of the cell cycle occurs at multiple levels of gene expression (e.g. transcription and protein stability) by a variety of factors. Cell cycle-dependent gene expression (Duronio and

Ofarrell, 1994; Elledge, 1996; Sanchez and Dynlacht, 1996; Dynlacht, 1997; Luo et al., 1998; Chaboute et al., 2000), reversible protein modification (Nasmyth, 1996; Stillman, 1996), and proteolysis (King et al., 1996) are all required to affect the progression through and between cell cycle phases. In animals, and most likely in plants, much of the transcriptional regulation of genes required for DNA replication is coordinated via a family of proteins related to the retinoblastoma (RRB) tumor suppressor gene of humans. Virtually all cell cycle regulatory proteins, and many of the proteins responsible for carrying out the biochemical reactions necessary for accomplishing S-phase and cell division, are modulated by at least some form of post-transcriptional regulation, including stage-specific proteolysis (King et al., 1996; Michael and Newport, 1998) and phosphorylation state changes (Nurse, 2000). The latter of these is the most studied.

Protein phosphorylation events that activate DNA synthesis, prevent DNA synthesis, and promote the progression through S-phase are mediated by the cyclin-dependent kinase (CDK) family (Stillman et al., 1992; Wu et al., 1998; Bell and Dutta, 2002). The RRB and CDK pathways interact directly, as CDKs phosphorylate RRBs to effect the changes in gene expression necessary for S-phase (Ezhevsky et al., 1997; Harbour et al., 1999; Zhang et al., 2000; Ezhevsky et al., 2001). RRBs, in turn, negatively regulate the transcription of CDK components and activators (Dyson, 1994, 1998). Any discussion of endocycles, and how they are regulated or manipulated needs to start with an overview of these controls of the cell cycle.

The current model for cell cycle regulation is based on a highly reticulate network of interacting genes and proteins that comprises or impinges upon an oscillator (Novak

and Tyson, 1993, 1997; Tyson et al., 2002). At the very center of the oscillator is a family of protein kinases, the cyclin dependent kinases (CDK). Active CDK proteins consist minimally of a catalytic subunit, the CDK subunit, and a regulatory component called a cyclin (Morgan, 1995, 1997). Though both subunits are regulated at the transcriptional, translational, and post-translational levels, changes in the abundance of cyclin subunits are more dramatic. Temporal regulation of CDKs is affected by changes in cyclin availability, activation and inhibition of the protein kinase subunit by phosphorylation, and association with multiple interacting proteins and inhibitors. In most organisms, CDKs can partner with multiple cyclin subunits and have different substrate specificity as a consequence (Peeper et al., 1993; Gibbs et al., 1996; Holmes and Solomon, 1996; Horton and Templeton, 1997; Kelly et al., 1998; Endicott et al., 1999).

The retinoblastoma related proteins and growth control

In animals and plants, DNA replication is regulated by the E2F family of transcription factors (Johnson et al., 1993; Qin et al., 1994; Egelkrout et al., 2001; De Veylder et al., 2002; Kosugi and Ohashi, 2002, 2002). E2F proteins form transcriptional activators as heterodimers with members of the Dp protein family (Weinberg, 1996). In plants, E2F target sites have been identified in the promoters of genes important for DNA synthesis and initiation of DNA replication (Chaboute et al., 2000; Castellano et al., 2001; de Jager et al., 2001; Chaboute et al., 2002; Kosugi and Ohashi, 2002). Differentiated and quiescent plant cells can be induced to re-enter the cell cycle and replicate their DNA by the ectopic expression of E2F family members and their partners

(Rossignol et al., 2002). Constitutive co-overexpression of E2F and Dp genes in arabidopsis led to ectopic cell divisions and a smaller cell size, but it disrupted plant development such that plant size decreased (De Veylder et al., 2002). Similarly, in animals E2F/Dp dimers activate genes encoding DNA polymerases, nucleotide biosynthetic enzymes, chromatin structural proteins, and cyclins, leading to the onset of S-phase (Pagano et al., 1992; Pagano et al., 1992). As was true in plants, E2F overexpression in mice had negative consequences for development and body size (Johnson, 2000; Wang et al., 2000). Mammalian E2F DNA-binding activity can be inhibited by phosphorylation via CDKs, which also targets E2F for proteolysis (Krek et al., 1995; Weinberg, 1996). Both plant and animal E2F proteins bind RRBs, and this association results in the repression of E2F activated promoters (Pagano et al., 1992; Du et al., 1996; Ferreira et al., 1998; Huntley et al., 1998). A role for the plant RRBs in cell cycle-dependent gene expression has yet to be demonstrated; however, some promoters with E2F-binding sites have been demonstrated to be repressed in a cell cycle-dependent manner (Egelkrout et al., 2001).

E2F and RRB were identified as interacting partners during investigations of oncogenic DNA viruses in mammalian systems (Chittenden et al., 1991, 1991; Kaelin et al., 1992). Viral promoters were found to be activated by the E2F transcription factors (Kovesdi et al., 1987; Raychaudhuri et al., 1987; Yee et al., 1989; Ben-Israel and Kleinberger, 2002). Viral genomes regulated by E2F-binding sites encode proteins that activate E2F-regulated promoters. Expression of these proteins de-represses the host genes required for viral DNA synthesis, often resulting in the stimulation of host cell

cycles and oncogenic transformation (Moran, 1988; Chellappan et al., 1992; Ben-Israel and Kleinberger, 2002). Adenovirus, cytomegalovirus, human papilloma virus, and SV40 each encode proteins with similar functions that contain an LxCxE motif. These proteins share the ability to interact with members of the RRB family via the LxCxE motif, and mutations to this sequence interfere with viral replication and the expression of viral RNAs (Moran, 1988, 1993; Ben-Israel and Kleinberger, 2002). Furthermore, identification of the binding sites for the LxCxE motif and the E2F transcription factor revealed they are not overlapping. Despite this, these viral LxCxE-containing proteins dissociate RRBs from E2F.

Plant geminiviruses are DNA viruses which encode RRB-binding proteins. Some of these geminiviral-encoded RRB-binding proteins contain LxCxE motifs, like their animal analogs. The best studied of the LxCxE containing proteins are encoded by the complementary strand of the monopartite monocot-infecting masteviruses. Typically, the first complementary strand transcript is alternately spliced to encode two proteins, Rep and RepA (Wright et al., 1997; Palmer and Rybicki, 1998; Gutierrez, 2000, 2000). In wheat dwarf virus, this alternative splicing results in 80% of the mRNA population encoding RepA, while the remaining 20% encodes Rep (Schalk et al., 1989). Both proteins contain the LxCxE motif (Xie et al., 1995), but only RepA interacts with RRBs with high affinity (Horvath et al., 1998; Liu et al., 1999; Missich et al., 2000). The Rep sequence encodes the viral replicase and is absolutely required for viral genome synthesis, while RepA is an accessory factor not strictly required for viral replication in cultured plant cells (Schalk et al., 1989). Viral mutants disrupting the splice junctions

that eliminate the RepA mRNA, or mutations of the LxCxE motif, do not replicate at wildtype levels (Schalk et al., 1989; Xie et al., 1996), suggesting that some function provided by RepA, potentially RRB-inactivation, is important to the viral life cycle. In some wheat cell types, RepA is necessary for high-level transcription of the virion sense strand (Gooding et al., 1999). Like the mammalian oncoviruses, masteviral promoters are cell cycle regulated (Nikovics et al., 2001), and ectopic expression of RRBs interferes with viral gene transcription and DNA replication (Xie et al., 1996). Co-overexpression of RepA overcomes the RRB-mediated block in viral replication (Xie et al., 1996). Thus, it would appear that RepA functions as a transcriptional regulator important for forcing the host cell into a replication-competent state via the opposition of RRB-activity.

Thus far, all sequenced genomes encode at least one RRB protein. The animal and plant genomes sequenced to date encode a variable number of RRBs. Mammalian cells encode three partially redundant RRBs (Hauser et al., 1997; Lacy and Whyte, 1997; Ferreira et al., 1998); drosophila encodes two (Du et al., 1996; Stevaux et al., 2002), *C. elegans* (Lu and Horvitz, 1998) and arabidopsis genomes both encode one; while maize encodes at least three (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Huntley et al., 1998; Shen, 2002). Though regulators of viral life cycles, animal and plant RRBs play critical roles in transcriptional regulation of host cell S-phase (Chittenden et al., 1991; Dunaief et al., 1994; Du et al., 1996; Brehm et al., 1998; Ferreira et al., 1998; Knudsen et al., 1998; Du and Dyson, 1999; Bosco et al., 2001; Charles et al., 2001; de Jager et al., 2001; Zhang and Dean, 2001; Ben-Israel and Kleinberger, 2002; Chaboute et al., 2002). RRBs primarily act as negative regulators of genes expressed in G1 and at the G1-S

transition. As such, RRB overexpression leads to cell cycle arrest, with enlarged cells in animal cell cultures (Qin et al., 1992). However, RRBs themselves appear to lack any enzymatic function that could actively repress gene expression. Binding of E2F by RRB does mask the activation domain of this transcription factor, perhaps resulting in a failure to express genes that require E2F activity (Chan et al., 2001; Xiao et al., 2003). Animal cells deleted for RRB are characterized by relaxed chromatin, providing evidence that RRB proteins have an effect on chromatin architecture (Herrera et al., 1996). A growing body of evidence has now linked RRBs to regulators of chromatin architecture, and it strongly suggests that RRBs act as a coordinating module for the recruitment of multiple co-repressors.

Cell cycle repression by RRB is mediated through its association with multi-protein complexes containing modulators of chromatin architecture. Direct binding has been demonstrated for mammalian DNA helicases (e.g. Brg1 and hBrm) similar to the Sucrose-Non-Fermenting2 gene (Snf2) of yeast, DNA and histone methyltransferases, and histone deacetylases (Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001). Associations have also been found with a group of WD-40 repeat containing proteins, the MSI/RBAP proteins, which are components of histone acetylation modifiers in plants and animals and are part of the RAS signaling pathway in animals (Lu and Horvitz, 1998; Rossi et al., 2001). The association with the Snf2-like helicases, such as the LxCxE containing Brg1 or hBrm, are necessary for RRB function, as helicase knockouts fail to undergo cell cycle arrest when RRBs are ectopically expressed (Dunaief et al., 1994; Strobeck et al., 2000; Strobeck et

al., 2002). Moreover, association with histone deacetylases has also been demonstrated to repress the expression of cell cycle regulated RRB targets in both animals and plants (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Rossi et al., 2003). Additional cell cycle regulated contacts are made with the minichromosome maintenance (MCM) proteins, which are involved in licensing DNA for replication (discussed below), and components of DNA polymerase, potentially targeting RRB and chromatin-level repressors to replicating heterochromatin (Takemura et al., 1997; Sterner et al., 1998; Kennedy et al., 2000; Takemura et al., 2001). Like viral oncoproteins, many of the RRB-interacting proteins encode an LxCxE motif. Unlike the viral proteins, LxCxE-containing histone deacetylases and Snf-2 type helicases can complex with RRB while it is simultaneously bound to E2F sites and repress transcription (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Strobeck et al., 2000; Muchardt and Yaniv, 2001). Indeed, LxCxE and E2F sites are not entirely overlapping in RRB (Chan et al., 2001; Xiao et al., 2003).

RRB is also important for other cellular phenomena; it promotes changes in gene expression independent of the cell cycle and can promote cellular differentiation (Lu and Horvitz, 1998; Sellers et al., 1998; Hsieh et al., 1999; Charles et al., 2001). In *C. elegans*, for instance, RRB is required for the transcriptional repression of tandem repeat gene arrays produced during the production of transgenic animals (Hsieh et al., 1999). This does not result in cell cycle-regulated gene expression, as expression is constitutively off in the wild type strains and on in the mutants. RRBs are also crucial for vulval

development, and RRB and its regulators show a synthetic multivulval phenotype with Ras mutations (Lu and Horvitz, 1998; Hsieh et al., 1999).

For RRB to mediate cell cycle phase-specific expression, its activities, namely the repression of E2F, must be cell cycle regulated. During the cell cycle, repression of RRB activity and the subsequent activation of E2F-regulated promoters is accomplished by phosphorylation of the LxCxE and E2F binding sites in RRB by multiple members of the CDK family (Ezhevsky et al., 1997; Harbour et al., 1999; Zhang et al., 2000; Ezhevsky et al., 2001). Phosphorylation of RRB by CDKs is sequential and begins with the cyclin D-regulated CDKs at the transition from quiescence to G1. Cyclin D-mediated phosphorylation is not sufficient to release the cell from G1, but it may be required for the G0/G1 transition (Ezhevsky et al., 1997; Ezhevsky et al., 2001). In mammalian cells, Cyclin D-targeted phosphorylation of RRBs is sufficient to disrupt interactions with LxCxE containing proteins but insufficient to dissociate RRB from E2F (Harbour et al., 1999). It is not known whether this is also the case in plants, although plant cyclin Ds possess an LxCxE, interact with the retinoblastoma protein, and efficiently use it as a substrate (Huntley et al., 1998; Nakagami et al., 1999; Boniotti and Gutierrez, 2001; Healy et al., 2001; Nakagami et al., 2002). Plant cyclin Ds partner with the P34-type PSTAIRE-containing CDK, rather than a divergent CDK like their animal counterparts, and this complex can efficiently utilize plant RRBs as substrates (Healy et al., 2001).

The second step in RRB inactivation in animal cells is phosphorylation by the cyclin E- and A-containing CDKs. It is these phosphorylation events that fully inactivate RRB as a cell cycle repressor (Harbour et al., 1999; Zhang et al., 2000). As a

consequence, released transcription factors, such as E2F, activate repressed gene promoters by reversing the chromatin modifications effected by RRB. For example, E2F can promoting gene transcription by recruiting histone acetyltransferases (Allard et al., 1999; Ait-Si-Ali et al., 2000; Lang et al., 2001). Cyclin A- and cyclin E-, but not cyclin D- associated CDKs cooperatively induce S-phase in isolated G1 nuclei from animal cells (Hauser et al., 1997; Lacy and Whyte, 1997; Ferreira et al., 1998). Both of these CDKs complex with E2F and pocket proteins (Xu et al., 1994; Ezhevsky et al., 1997; Harbour et al., 1999; Staehling-Hampton et al., 1999; Ezhevsky et al., 2001). However, only cyclin A-containing CDK is capable of hyperphosphorylating the RRBs *in vitro* (Gibbs et al., 1996). In plants, cell cycle regulatory functions have been assigned to cyclins A and D but no clear cyclin E candidates have been reported.

As with increased cell division due to ectopic co-expression of E2F and its partner Dp1 (De Veylder et al., 2002; Rossignol et al., 2002), the expression of genes hypothesized to inactivate RRB leads to cell cycle activation in plants. For example, over-expression of RRB-interacting cyclin-D shortens the duration of the cell cycle and can lead to organ hypertrophy (Riou-Khamlichi et al., 1999; Cockcroft et al., 2000). As these cyclins and E2F are known to interact with RRBs in higher plants, it seems likely that RRBs from higher plants will function as cell cycle repressors like their mammalian counterparts. However, the only study of RRB gene function in plants comes from a single-celled alga, *Chlamydomonas*. In this organism, an RRB was cloned and mutants altered cell size at division, but not the rate of cell division, suggesting that the animal and plant RRBs may have evolved to regulate separate processes (Umen and

Goodenough, 2001). Deletion of RRB led to a smaller cell size before and after division, but not a decreased phase duration, suggesting that growth and not the cell cycle was rate limited by this deletion. As drosophila RRBs have also been implicated in growth , and drosophila cyclin D's are involved in growth control but dispensable for mitotic and endoreduplication cycles (Datar et al., 2000; Meyer et al., 2000; Martin-Castellanos and Edgar, 2002) it is reasonable to propose that the plant RRBs are not directly involved in the control of cell proliferation or endoreduplication. Maize appears to contain multiple pocket proteins, and the conservation of their interactions with transcriptional and cell cycle regulators is the only evidence in favor of cell cycle regulatory function (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Huntley et al., 1998).

Cycling with cyclin dependent kinases

CDK catalytic subunits are members of the Casein Kinase II subfamily and show high sequence similarity to *S. pombe* $p34^{Cdc2}$. Thus far, classification of genes encoding $p34^{Cdc2}$-like proteins from plants by sequence similarity has identified at least five major groups (Magyar et al., 1997; Joubes et al., 2000; Meszaros et al., 2000). Two subgroups are highly related to $p34^{Cdc2}$ of yeasts and animals and contain the conserved PSTAIRE motif, which is present in at least one CDK encoded by the genome of every animal, plant and fungus investigated. In maize, protein(s) immunologically identical to p34 have been demonstrated to be part of a CDK associated with mitotic microtubule structures (Colasanti et al., 1991; Colasanti et al., 1993) and the S-phase CDK (Grafi and Larkins, 1995). Though in mammals the closest relative to $p34^{Cdc2}$ is exclusive to mitosis (van den Heuvel and Harlow, 1993), the closest plant relatives bind G1- and S-phase

specific cyclins (Healy et al., 2001). A divergent CDK type, that does not contain a PSTAIRE-motif is active in a mitotic-specific fashion in plants (Fobert et al., 1996; Magyar et al., 1997; Ayaydin et al., 2000; Meszaros et al., 2000). By contrast, in animal cells divergent CDKs with cell cycle regulatory function act in G1 and complex with the cyclin Ds (Matsushime et al., 1992; Ezhevsky et al., 1997). Northerns for all five classes of CDKs have been reported for synchronized alfalfa cells, and no clear S-phase expression patterns were found for any CDK subunit (Magyar et al., 1997; Meszaros et al., 2000). In arabidopsis, the expression pattern of Cdc2 includes all cells with the capacity to divide or endoreduplicate, further implicating this protein in S-phase control (Hemerly et al., 1993).

Plant CDKs have been demonstrated to regulate the cell cycle. For example, microinjection of active CDK/cyclin complexes from plants can induce mitosis in *Tradescantia* (Hush et al., 1996). Ectopic expression of wild-type, hyper-active, and dominant negative CDKs has also been done. Expression of wild-type or hyper-active arabidopsis Cdc2 in tobacco cell cultures resulted in smaller cells (Hemerly et al., 1995), a result reminiscent of the effects of a hyperactive CDK in yeast cells (Nurse and Thuriaux, 1980). These same constructs had no discernable impact on tobacco plant architecture, however, suggesting that positional information or other developmental cues were able to take compensatory action (Hemerly et al., 1995). Expression of dominant negative mutant CDKs, generated by replacing the residue corresponding to aspartic acid 145 in Cdk2, which is required for ATP binding, or threonine 161, which must be phosphorylated for CDK activation, create a competitive inhibitor of endogenous CDK

that binds cognate cyclins but is inactive. In animal cells, expression of these mutant CDKs arrest the cell cycle and expression in plant cell cultures gave similar results (Hemerly et al., 1995). Expression of these mutant CDKs in whole tobacco plants was also possible. The regenerated plants had a reduced number of cells that compensated by expanding, such that plant size was not affected (Hemerly et al., 1995). In contrast to adult plants, embryos aborted in transgenic progeny, suggesting that the rapid cell cycles of early development are more sensitive to decreases in CDK activity (Hemerly et al., 2000).

Oscillation of CDK activity during the cell cycle is controlled by sophisticated mechanisms involving transcriptional and post-transcriptional regulation (Morgan, 1995). A principal means of regulation is an alteration in cyclin abundance. Cyclin proteins share a region of high similarity, referred to as the cyclin box, which folds into a tight barrel of helices. Upon binding the CDK subunit, cyclins deform the CDK, which exposes the ATP binding site, promotes substrate interactions, and activates the kinase (Morgan, 1997). Oscillations in cyclin abundance are accomplished by transcriptional modulation of cyclin genes (Cross, 1991; Nasmyth and Dirick, 1991), translational regulation of mRNAs (Swenson et al., 1987) and proteolytic degradation (Glotzer et al., 1991; Glotzer, 1995; King et al., 1996; King et al., 1996). Cyclin genes have been isolated from a number of plant species (Hata et al., 1991; Hemerly et al., 1992; Hirt et al., 1992; Renaudin et al., 1994; Dahl et al., 1995; Meskiene et al., 1995; Soni et al., 1995; Sun et al., 1999), although biological activities in some of these species have not been investigated. While activation of CDK components and cell cycle-specific

accumulation of cyclins is common to fungi, animals, and plants, all cell cycle regulatory genes from plants are more similar to their animal counterparts and the nomenclature used for plant genes resembles that of the animal literature (Fobert et al., 1996; Hirt, 1996; Renaudin et al., 1996).

In plants, cyclin transcript and protein abundance are subject to phase-specific control. Like their animal counterparts, plant B and A cyclins are accumulated during M-phase and G2. Additionally, A-type cyclins accumulate additionally during S-phase (Reichheld et al., 1996; Shaul et al., 1996; Meszaros et al., 2000). No consensus for phase-specific expression has emerged for D-type cyclins. Instead, cyclin D transcripts appear to be regulated primarily by nutritional and hormonal factors (Dahl et al., 1995; Soni et al., 1995; Riou-Khamlichi et al., 1999; Meijer and Murray, 2000). This might be seen as partially consistent with the growth-related functions of cyclin D's in drosophila. However, one D-type cyclin has been demonstrated to have an expression pattern restricted to G1 and S phase, similar to that seen in animal systems (Dahl et al., 1995).

In all cases analyzed to date, cyclin overexpression has led to increased cell proliferation and organ hypertrophy. Cyclin B expression in trichomes led to ectopic cell divisions (Schnittger et al., 2002), while expression in roots led to increased rates of root growth and branching (Doerner et al., 1996). Consistent with these results, treatment of roots with auxin induced cyclin B expression in the zone of lateral root initiation (Himanen et al., 2002). Cyclin D overexpression led to cytokinin-independent growth and overproliferation of cells (Riou-Khamlichi et al., 1999). Consistent with this, cyclin Ds expression can be induced by cytokinin application (Soni et al., 1995). An elegant

study of cyclin expression further linked these proteins to plant growth, as cyclin A was shown to predict cell proliferation and leaf growth rate in arabidopsis (Donnelly et al., 1999).

In animal cells, the various CDK complexes preferentially target different substrates. CDK substrate specificity can be modified by the cyclin-type, the kinase subunit, and the presence of multiple interacting proteins (Gibbs et al., 1996; Hauser et al., 1997). *In vitro*, reconstituted CDKs containing cyclin A, B or E show similar substrate specificity (Higashi et al., 1995), but *in vivo* they carry out non-overlapping functions. Of the three, only cyclin A and E form stable complexes with RRB proteins. In animal systems, the cyclin D-associated kinases show vastly different substrate specificity *in vitro,* but much of this may be due to the divergent CDK-type with which they associate (Matsushime et al., 1992; Kitagawa et al., 1996). In animals cyclin A- and E-containing CDKs associate with RRBs and E2Fs, which changes the kinase's substrate specificity (Xu et al., 1994; Hauser et al., 1997; Lacy and Whyte, 1997). While binding of plant cyclins to multiple interacting proteins (e.g. PCNA and RRB) has been demonstrated, their substrate specificity has not been systematically investigated (Ach et al., 1997; de la Paz Sanchez et al., 2002).

The activity of CDKs is regulated by phosphorylation and de-phosphorylation of the protein kinase. Three phosphorylation sites have been documented to be involved in switching CDK activity on and off. Phosphorylation at Thr 161 of human CDK1 (Thr 160 of CDK2) is required for activation the complex (Krek and Nigg, 1992). Members of the Wee1 kinase family, originally identified in *S. pombe*, inactivate CDK by

phosphorylating Thr 14 and Tyr 15 within the ATP-binding domain of the kinase subunit (Russell and Nurse, 1987; Mueller et al., 1995; Mueller et al., 1995; Parker et al., 1995; Leise and Mueller, 2002). A gene encoding a Wee1-like kinase has been cloned from maize and demonstrated to inhibit maize CDK activity (Sun et al., 1999). Over-expression of animal or plant Wee1-like proteins in yeast complements Wee1$^-$ cells and slows the cell cycle in wildtype cells, generating enlarged cells (Igarashi et al., 1991; Sun et al., 1999). In animal and yeast cells, Wee1 is cell cycle regulated by differential localization (Baldin and Ducommun, 1995; Morgan, 1997), and it is rapidly up-regulated in response to environmental stress in plants (Schuppler et al., 1998).

Dephosphorylation of the Wee1 target site of CDKs is accomplished by multiple protein phosphatases. In animals and yeasts, phosphatases of the Cdc25-family are cell cycle regulated activators of CDKs (Kumagai and Dunphy, 1991). In tobacco cell cultures, cytokinin treatment stimulates the dephosphorylation of a Wee1 target site on PSTAIRE-contain CDKs prior to cell division (Zhang et al., 1996). Cdc25 is not the only phosphatase regulating CDKs in animals. Phosphatases that remove the activating phosphate residue from T161 (Karaiskou et al., 1999; De Smedt et al., 2002) have been identified. In plants, no Cdc25-like gene has been identified to date. However, Cdc25 proteins from other systems efficiently dephosphorylate plant CDKs. Transgenic expression of yeast *Cdc25* leads to supernumerary organ initiation from both the root and shoot, and results in earlier flowering in tobacco (Bell et al., 1993; McKibbin et al., 1998). In screens for plant genes that can complement *Cdc25* mutants of yeast, an

unrelated protein phosphatase was uncovered, suggesting that plants regulate the activation of Wee1-inhibited CDKs in a novel way (Ferreira et al., 1993).

The activity of CDKs can also be reversibly inhibited by small inhibitory proteins. These CDK inhibitors (CKIs) control the activity of CDKs by binding both the cyclin and kinase subunits (Fotedar et al., 1996). For example, $p40^{Sic1}$ in *S. cerevisiae* binds to $p34^{Cdc28}$ and reduces its V_{max} and K_m for protein substrates (Mendenhall, 1993; Mendenhall et al., 1995). Multiple CKIs related to the yeast CKIs have been isolated from animal and plants systems (Lui et al., 2000; Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002). In animals, these inhibitors are involved in exit from the cell cycle (de Nooij et al., 1996; Lane et al., 1996), the inhibition of CDKs in response to DNA damage (Deng et al., 1995), and control of the G1/S and G2/M transitions (Harper et al., 1995; Guadagno and Newport, 1996). Plant and animal CKIs share at least two regions of similarity required for binding cyclin and CDK subunits and inhibiting CDK activity (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001). Plant CKI proteins inhibit CDK activity *in vitro* (Wang et al., 1998; Lui et al., 2000; De Veylder et al., 2001) and abrogate cell division when microinjected into mitotic *Tradescantia* stamen hairs (Cleary et al., 2002). Messenger RNA abundance for some CKIs is dramatically increased by stress and ABA (Wang et al., 1998; Wang et al., 2000). Over-expression of CKIs leads to smaller plants and organs with fewer cells and lower degrees of endopolyploidy at maturity (Wang et al., 2000; De Veylder et al., 2001; Jasinski et al., 2002). Plant CKIs, unlike their animal counterparts, directly associate with cyclin Ds (Wang et al., 1998; De Veylder et al., 2001), exhibit expression patterns in synchronized

plant tissue culture cells that mirror cyclin D (Menges and Murray, 2002), and oppose cyclin D3 action in transgenic plants (Jasinski et al., 2002). Additionally, CKIs are expressed in a radial stripe surrounding the initiation of lateral root formation in response to auxin treatment, possibly determining the position of lateral root initiation (Himanen et al., 2002). Thus, while cyclin D links cell proliferation to plant physiology, CKIs connect cell cycle inhibition and hormonal cues.

In animals and yeasts, CKIs are phosphorylated by active CDKs in a cell cycle-dependent manner (Morisaki et al., 1997). Phosphorylated CKIs are targeted for degradation by the proteosome, resulting in self-activation of a latent pool of CDKs (Morisaki et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999). The rapid onset of CDK activity during cell cycle phase changes generates a molecular switch regulated by the total abundance of CDK-CKI complexes and CDK localization (Swanson et al., 2000). The system operates such that constant expression of cyclin, CDK, and CKI generates oscillations in CDK activity (Montagnoli et al., 1999).

An additional protein that is often associated with CDK complexes in yeast, animals, and plants; is the so-called constitutive kinase subunit (CKS1). Over-expression of CKS1 in yeast leads to cell cycle delay, and its deletion nearly abolishes cell division (Hindley et al., 1987). Plant and animal homologues of CKS1 are small proteins varying from 9- to 15-kDa. These proteins bind some CDKs with high affinity. Yeast CKS1 will bind the G2/M- but not the S-phase CDK of plants (Grafi and Larkins, 1995; Bogre et al., 1997) and only one of the two maize and arabidopsis PSTAIRE-containing CDKs (de la Paz Sanchez et al., 2002). By contrast, the arabidopsis CKS1, binds both of them and is

expressed in both mitotic and endoreduplicating cells (Jacqmard et al., 1999; de la Paz Sanchez et al., 2002). In arabidopsis, CKS1 functions as a CDK inhibitor, presumably by directing active CDK to the proteosome (Kaiser et al., 1999), and overexpression of CKS1 leads to smaller organs and decreased apical meristem size (De Veylder et al., 2001).

The actions and interactions of these cell cycle regulators provide the information to initiate and maintain cell cycle progression. In the mitotic cell cycles action is required to initiate DNA replication, ensure complete replication, and prevent re-replication. The regulatory events required for the last of these must be overcome in an endoreduplication cycle.

Molecular mechanisms of endoreduplication

DNA replication is limited to once for each division during the standard mitotic cell cycle by a process termed "replication licensing" (Blow and Laskey, 1988). In a pioneering set of experiments it was demonstrated that DNA synthesis is initiated by two cell cycle-regulated factors present in cell extracts. The first, replication licensing factor (RLF), is required to make chromatin at the site of replication initiation, origins of replication, replication-competent or "licensed" for replication. The second, induces competent origins to initiate replication and was termed the S-phase promoting factor (SPF). In animal and yeast cells, the components of both RLF and SPF have been identified. Despite the simplicity of the conceptual model and the single terms for each function, RLF and SPF are comprised of multiple multiprotein complexes. Chromatographic separation of RLF identified two fractions that were necessary and

sufficient for replication licensing in Xenopus oocytes, RLF-M and RLF-B (Chong et al., 1995).

In higher eukaryotes, replication origins are sites along the chromosomes, often of ill-defined sequence, that initiate DNA replication during S-phase of the cell cycle. Ultimately, these sites recruit DNA polymerase to chromatin and initiate DNA replication. Replication origins are marked by a six subunit origin recognition complex (ORC). This complex has the capacity to bind DNA at high affinity and hydrolyze ATP in a single stranded DNA-stimulated manner (Klemm et al., 1997; Lee et al., 2000). Disruption of ORC ATP hydrolysis activity blocks replication initiation (Klemm and Bell, 2001). As ORC is the first complex bound to the DNA at replication origins, observations that DNA becomes "saturated" with ORC at a spacing of approximately 16kB, the lower limit for replicon sizes, suggest that the ORC complex determines the sites of replication (Bell and Dutta, 2002). In addition to providing a platform for the association of other proteins with pre-replication functions , the ORC interacts with RRBs and E2F in drosophila and the interaction with RRB disrupts origin function (Bosco et al., 2001). However, there is no biochemical evidence that ORC proteins contribute to the replication process per se, as fully assembled pre-replicative complexes no longer require the ORC to support replication (Hua and Newport, 1998; Rowles et al., 1999). However, the ORC is required for localization and chromatin association of proteins that make DNA competent for genomic replication.

In the process of assembling a pre-replication complex at ORC sites, the next step is the binding of two proteins Cdt1p and Cdc6p. These proteins associate with the ORC

on chromatin and facilitate the loading of an additional complex to complete the pre-replication complex (Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997; Hua and Newport, 1998; Rowles et al., 1999; Tada et al., 1999; Tada et al., 2001). Like the ORC proteins, both Cdt1 and Cdc6 have ATP binding, and presumably hydrolyzing activity (Bell and Dutta, 2002). The Cdc6 gene provides a link between the RRB and CDK pathways and replication licensing. The Cdc6 homologs of plants and animals are E2F-regulated, and likely to be RRB-repressed (Castellano et al., 2001; de Jager et al., 2001). A connection between CDKs and replication licensing is also provided by Cdc6. Cdc6 is an efficient substrate for CDK phosphorylation, and this phosphorylation promotes its proteolysis and inhibits replication complex assembly (Jallepalli et al., 1997; Lopez-Girona et al., 1998; Findeisen et al., 1999; Jiang et al., 1999). Phosphorylation and subsequent degradation of Cdc6 is required to limit re-replication, as the overexpression of wildtype Cdc6 in plants or the expression of phosphorylation-defective mutants of Cdc6 in yeast induces endocycles (Jallepalli et al., 1997; Castellano et al., 2001). Recently, it has been demonstrated that RLF-B is Cdt1 (Tada et al., 1999; Tada et al., 2001). However, immunodepletion of Cdc6 blocks the initiation of replication demonstrating that Cdc6 is also required in Xenopus oocytes, but may already be bound to ORC complexes in the RLF assay used (Bell and Dutta, 2002). Consistent with this, Cdt1 has been reported to associate with chromatin in an Cdc6-dependent manner (Tada et al., 1999) and both were required to produce licensing factor from highly purified proteins (Gillespie et al., 2001).

RLF-M, on the other hand, is composed of yet another protein complex with ATP hydrolyzing capacity, the MCM proteins. MCM proteins bind to chromatin in an ORC dependent *and* independent manner, requiring Cdc6 and Cdt1 for association with the ORC (Donovan et al., 1997; Tanaka et al., 1997; Cook et al., 2002). Binding of MCM proteins is associated with a decreased affinity of ORC and Cdc6 for chromatin, and removal of either of these from DNA after MCM binding does not block subsequent replication (Bell and Dutta, 2002). Some biochemical evidence suggests that the MCM protein complex can act as a DNA helicase and facilitate local DNA unwinding, which may be required for the initiation of DNA replication (Ishimi, 1997; You et al., 1999; You et al., 2002; Masuda et al., 2003). One of the MCM proteins, MCM7 has been demonstrated to bind RRB, and may be responsible for the association of RRB with replication foci (Sterner et al., 1998).

The last element to be assembled at the presumptive site of replication is the Cdc45p protein. Originally identified in *S. cerevisiae* as a cold-sensitive mutation, Cdc45p binds to the MCM complex following activation of the origin by SPF (Aparicio et al., 1997; Aparicio et al., 1999; Bell and Dutta, 2002). Binding of Cdc45p appears to activate the DNA unwinding activity of the MCM complex, and the subsequent association of the single stranded DNA binding protein, RPA, and the replicative DNA polymerases, alpha and epsilon (Stillman et al., 1992; Bell and Dutta, 2002). Like the MCM proteins, Cdc45p remains associated with DNA polymerase during replication. Thus, the factors required to recruit replication machinery to the origins, licensing

factors, disassociate from the site of initiation when replication begins allowing each origin to fire once.

The SPF is composed of at least three protein complexes. It was shown that a mixture of the Cdk2/Cyclin A and Cdk2/Cyclin E CDKs was sufficient to act as the SPF in cell free extracts with mammalian nuclei (Krude et al., 1997). In each case, purified recombinant enzymes were able to substitute for the partially purified SPF nuclear fraction, and immunodepletion of these enzymes from the same nuclear extract eliminated it's activity. Maintenance of CDK activity throughout S-phase is required for replication continuation. In addition to potentially stimulating and inhibiting DNA polymerase activity (Takemura et al., 1997; Voitenleitner et al., 1999; Schub et al., 2001; Takemura et al., 2001), a decrease in CDK activity can lead to the cessation of DNA synthesis (Flores-Rozas et al., 1994; Shivji et al., 1994; Strausfeld et al., 1994). An additional kinase, Dbf4, is also involved and genetic experiments have demonstrated that it is required for DNA replication and shows allele specific interactions with MCM proteins in yeast (Lei et al., 1997).

In addition to acting as the inducing signal and promoting the complete replication of nuclear DNA, CDKs are critical to blocking DNA re-replication (Aparicio et al., 1997; Tanaka et al., 1997; Aparicio et al., 1999; Jiang et al., 1999; Snaith and Forsburg, 1999; Furstenthal et al., 2001). Cdc6 does not accumulate if CDKs are active, and phosphorylation of MCM proteins by CDKs blocks their nuclear localization and association with origins of replication (Donovan and Diffley, 1996; Aparicio et al., 1997; Donovan et al., 1997; Tanaka et al., 1997; Aparicio et al., 1999; Cook et al., 2002).

These observations may explain why, in their original experiments, Blow and Laskey were able to promote chromatin licensing by disrupting the nuclear membrane (Blow and Laskey, 1988; Blow et al., 1989). If there is not a drop in CDK activity between the onset of DNA replication and induction of the S-phase CDK, the DNA cannot replicate. Maintenance of CDK activity throughout the remainder of the cell cycle prevents re-replication. In the mitotic cell cycle, cyclin A-dependent kinase activity is maintained throughout G2 in most cells, at which time cyclin B accumulates; when cyclin B-dependent kinase is activated, mitosis ensues. At the end of mitosis, prior to spindle separation, cyclins A and B are rapidly proteolyzed in a ubiquitin-dependent manner by the proteosome via a protein complex referred to as the "anaphase promoting complex" (Glotzer et al., 1991; King et al., 1996; Michael and Newport, 1998). In drosophila and plants, deletion or suppression of an activator of this complex inhibits the formation of endopolyploid cells (Sigrist and Lehner, 1997; Cebolla et al., 1999). The proteolysis of CDKs results in a lull of CDK-mediated phosphorylation, allowing MCM proteins to re-localize to chromatin and Cdc6 and Cdt1 to accumulate to levels that will support re-licensing of the DNA. Thus, while the addition of CDK activity will speed the onset of mitosis and S-phase, the removal of CDK activity is required for cells to re-enter G1.

Endoreduplication poses a special case of licensing, in that the signal inhibiting licensing must be disrupted, but mitosis cannot be induced. As DNA is completely replicated it is reasonable to propose that DNA is licensed and replication regulated by ordinary mechanisms. In cultured mammalian cells, RRB inhibition by genetic ablation or expression of viral LxCxE-containing proteins, together with the constitutive

expression of CKI, is sufficient to induce recurrent S-phases without intervening mitoses, i.e. endoreduplication (Niculescu et al., 1998). Inactivation of RRBs by phosphorylation might also play a role in endoreduplication. In mammals, progression through the G1/S transition involves inactivation of RRB by phosphorylation, not genetic ablation (Garcia and Cales, 1996; MacAuley et al., 1998). If the same were to be true of endocycling cells, then the Gap-phase preceding re-replication might be enforced by the presence of transiently hypophosphorylated RRB. Endoreduplication in plants can be activated by expression of E2Fs, suggesting that constitutive activity of downstream RRB-repressed transcription factors may be sufficient to induce it (De Veylder et al., 2002). In endoreduplicating drosophila cells, oscillations in cyclin E expression (Sauer et al., 1995), or cyclin degradation (Sigrist and Lehner, 1997) are required to induce endocycles. Indeed, in drosophila cell cycles CKIs, cyclin E and RRBs show allele-specific interaction effects. (de Nooij et al., 1996). Accumulation of the $p57^{Kip2}$ CKI in mouse trophoblast cells is associated with endoreduplication (Hattori et al., 2000). In this case CKI mRNA is synthesized constitutively but periodic proteolysis drives cycles of inhibitor concentration and therefore CDK activity (Hattori et al., 2000).

Given the regulation of replication licensing in yeast and animals, a three-step process for endoreduplication could be proposed. First, the cell cycle program required for repression of S-phase gene expression, e.g. RRB suppression of E2F activity, should be disabled. As E2F is a known regulator of RLF components in plants and animals (Castellano et al., 2001; de Jager et al., 2001; Egelkrout et al., 2001; Ramos et al., 2001), this would activate the synthesis of regulatory components that initiate S-phase and the

structural genes required for DNA synthesis. Second, processes regulating the cell cycle must be diverted such that mitosis is not initiated. As cyclin B is a known component of the MPF, subjecting it to transcriptional repression, constitutive proteolysis or post-translational inhibition could suffice. In fact, deletion of $p56^{Cdc13}$, the B-type cyclin in *S. pombe*, causes cells to undergo repeated rounds of S-phase, without progressing to M-phase. As a result, the ploidy can be increased to greater than 32C (Hayles et al., 1994). Likewise, overexpression of Rum1, a CKI in *S. pombe*, leads to increased DNA content and nuclear enlargement (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995). Mathematical models of cell cycle control, based on fission yeast genetics and biochemistry, predict that an oscillation of CDK activity in the absence of MPF is sufficient to induce endoreduplication in yeast, where the RRB pathway does not exist (Novak and Tyson, 1997). Third, a mechanism for destabilizing the licensing mechanism should be periodically disrupted. This would require the proteolysis mechanism and nuclear exclusion of MCM proteins to be overridden, allowing the accumulation of RLF and support of DNA re-replication. One mechanism to achieve this would be the oscillation of CDK activity. Simply put, any oscillation of CDK activity of sufficient period to allow re-licensing should result in DNA re-replication, provided MPF is not subsequently activated and S-phase gene expression is active.

Regulation of endoreduplication in maize endosperm

The cell cycle mechanisms that trigger endoreduplication in maize endosperm are unknown, but several studies illustrate that there are alterations to the normal cell cycle during endosperm development. First, components of the M-phase CDK are altered

during endoreduplication in maize endosperm. Cyclin B transcripts are alternatively spliced to yield an aberrant and putatively inactive protein at the beginning of the endoreduplicative period (Sun et al., 1997). Additionally, during the endoreduplication phase of development, accumulation of cyclin B mRNA is decreased below the level of detection (Sun et al., 1999). Second, a CKI-activity accumulates during the period of endoreduplication (Grafi and Larkins, 1995). The expression of a maize Wee1-like protein, the CDK-inhibitory kinase, is also increased concomitant with the highest rates of endoreduplication (Sun et al., 1999). These events are associated with a dramatic decrease in the activity of an affinity adsorbed M-phase CDK activity (Grafi and Larkins, 1995). Third, there is a marked increase in a CDK activity that is likely to function as an S-phase promoting factor (Grafi and Larkins, 1995; Grafi et al., 1996). This CDK activity can be adsorbed onto RRB- and E2F-agarose, and it phosphorylates histone H1, ZmRb1, and E2F-1, characteristic of cyclin A- and D-complexes (Grafi and Larkins, 1995). This is associated with the phosphorylation, and presumably inactivation, of RRBs expressed in endosperm (Grafi et al., 1996). CKI expression and RRB inactivation may be sufficient to induce endoreduplication in plants, in which case these changes in cell cycle regulatory components should be sufficient to explain the endoreduplication cycles of endosperm. However, these predictions need to be tested.

As maize encodes multiple RRB proteins that are expressed throughout the plant, testing the role of RRB proteins on endoreduplication in endosperm presented a technical challenge. To address the problem of creating a three-gene knockdown phenotype in endosperm, such as by RNA-mediated gene suppression, I chose instead to analyze the

effects of the RRB-interacting protein, RepA, from wheat dwarf virus. It was previously shown that this protein can oppose RRB action in the suppression of viral DNA replication (Xie et al., 1996). If RRBs from higher plants are important negative regulators of the cell cycle, then ectopic expression of RepA should activate the normal cell cycle. Furthermore, if RRB inactivation is a rate-limiting step for endoreduplication in endosperm, then expression of RepA in this tissue during the period of endoreduplication should result in higher degrees of endopolyploidy. Alternately, if the RRB present in endosperm is constitutively inactivated by phosphorylation, RepA should have no effect on endoreduplication. Additionally, if RRB inactivation is an important step in the initiation of endocycles, RepA expression may induce ectopic endoreduplication in some cell types.

As the production of transgenic maize is not trivial, I developed an efficient bioassay for cell cycle regulation in the fast-cycling BY-2 (Nagata et al., 1992) cell line of tobacco. This was used to test the cell cycle regulatory activity of both the ZmRb1 gene and the wheat dwarf virus RepA protein. As predicted by protein-protein interactions, sequence similarity, and analogy to mammalian models, RepA stimulated, and ZmRb1 inhibited, the cell cycle in BY-2 cells. ZmRb1 and RepA were demonstrated to act in opposition to each other, and RepA expression overcame the cell density-dependent G1-block in these cells. For the analysis of transgenic maize materials, antisera to recombinant RepA was generated and characterized. The effect of RepA on mitotic maize cells was investigated by flow cytometric analyses of embryogenic maize calli expressing one of two constitutive RepA constructs and accumulating the RepA

protein. In these cells, the accumulation of RepA was associated with increased endopolyploidy, suggesting that RepA expression was sufficient to induce endoreduplication in these cells. Following regeneration of transgenic maize expressing RepA in an endosperm-specific manner, transgenic endosperm cells were also tested for alterations in endopolyploidy and RepA accumulation. The presence of RepA protein in endosperm during the period of endoreduplication, even at high levels, was not sufficient to affect increased endopolyploidy by 18-DAP. Thus, it seems unlikely that RRB activity represents a rate-limiting step for endocycle progression in these cells. In addition, RepA accumulation did affect kernel growth and the accumulation of storage products, both of which were documented.

CHAPTER 2

GENETIC ANALYSES OF ENDOREDUPLICATION IN ZEA MAYS ENDOSPERM: EVIDENCE OF SPOROPHYTIC AND ZYGOTIC MATERNAL CONTROL

Introduction

During maize seed development, starch, protein, and other nutrients from the maternal parent (sporophyte) are stored in the endosperm of the developing kernel. The endosperm is formed by the fusion of two nuclei from the female gametophyte and one nucleus from the male gametophyte, such that the balance of genomes is 2m:1p. Endosperm development is characterized by three distinct cell cycles: syncytial karyokinesis, mitosis, and endoreduplication. The latter, which occurs by reiterative cycles of nuclear DNA replication, begins 8– to 9 -days after pollination (DAP) and is associated with the terminal differentiation of endosperm cells (Berger, 1999; Olsen et al., 1999). Subsequently, endosperm cells cease mitotic divisions, and the number of cells undergoing endoreduplication dramatically increases, such that the mitotic index falls to nearly zero by 14-DAP (Kowles and Phillips, 1985). Endoreduplication continues until 21– to 27-DAP, when the central cells of the starchy endosperm begin to undergo cell death (Young et al., 1997; Young and Gallie, 2000).

Endoreduplication has been implicated in a number of important physiological processes in endosperm and other tissues. Much of the increase in kernel volume and

mass occurs during the period of effective grain filling (EGF). During the EGF, which is temporally correlated with endoreduplication, starch and storage proteins are rapidly synthesized and accumulated in the endosperm (Schweizer et al., 1995; Jones et al., 1996). In wheat, kernel size was predicted by a combination of the number of mitotic divisions and the number of endoreduplication cycles in the endosperm (Brunori et al., 1993). Thus, endoreduplication might assist in the rapid synthesis of starch and storage protein during the EGF. Endoreduplication is thought to increase the metabolic activity of cells by increasing the number of DNA templates from which RNA transcripts are generated. Recently, it was shown that during endoreduplication in maize endosperm there is a reduction in chromatin condensing proteins and increased accumulation of an HMG protein associated with an open chromatin conformation (Zhao and Grafi, 2000). HMG proteins bind enhancer-like elements in the promoters of endosperm storage protein genes and assist with the binding of transcription factors (Grasser et al., 1990; Schultz et al., 1996).

Endosperm development is significantly influenced by the maternal parent. Kernel size and shape are known to be matroclinally controlled, such that F1 kernels more closely resemble the ear parent (Birchler, 1993; Alleman and Doctor, 2000). This is also true for the EGF (Jones et al., 1996) and some aspects of endoreduplication (Cavallini et al., 1995; Kowles et al., 1997). Endosperm development also requires a balanced number of maternal and paternal genomes, as departure from the 2m:1p ratio leads to kernel abortion (Cooper, 1951; Lin, 1984). Kernel development is further tied to the physiology of the maternal plant. Cessation of kernel growth can be induced by heat

stress, water stress, the exogenous application of stress-associated growth regulators, or a disruption in the delivery of photosynthate (Myers et al., 1990, 1992; Engelen-Eigles et al., 2000). While the endoreduplication cell cycle can be inhibited, it is an order of magnitude less sensitive than the mitotic cell cycle to physiological perturbation (Myers et al., 1990; Mambelli and Setter, 1998).

In this study, we investigated the genetic regulation of endoreduplication in maize endosperm using inbred lines that differ in the quantity of endoreduplication. Measurements of nuclear DNA content were scored as quantitative traits, and their inheritance was investigated. Analyses of the variability of these traits predicted genetic regulation through the maternal lineage as the predominant mode of inheritance. By monitoring endoreduplication through multiple generations, we confirmed it is a heritable trait. Consequently, reciprocal backcrosses were initiated to introgress alleles causing a high level of endoreduplication into a low- endoreduplicating inbred background to assess the importance of this trait for kernel development.

Materials and Methods

Plant material

Maize plants were grown at the University of Arizona West Campus Agricultural Facility in the spring and fall seasons of 1998, 1999, and 2000 and in the greenhouse during the winter of 2000. Pollinations and harvesting were performed in the morning. Ears were broken from the stalk at the shank and placed in crushed ice for transport to the laboratory. Stocks of the inbred lines A188, B37, B73, Mo17, Oh545, Va99, W64A,

B37o2, B73o2, Oh545o2, Va99o2, and W64Ao2, open- pollinated varieties of 8-Row Flint and Yellow Flint, and the sweet corn "Florida StaySweet" are maintained in our laboratory. South American accessions Bolivia 1044 and Venezula 568, inbred lines Nc282 Nc306, Nc318, and Nc320, were provided by M. Goodman (North Carolina State University). The popcorn inbred lines A1-6, Hp58, Kp58k, Sg18, Sg32, and Sg1533 were provided by R. B. Ashmann (Purdue University). For the analyses of variance and mean components, a 3^2 design was employed where nine generations were created by crossing two parents and their F1 in each pair wise combination. For these experiments B73 was crossed with the dent corn Mo17 or the popcorns A1-6, Kp58k, and Sg18. For each set of crosses, the parental types, reciprocal F1, and four BC1 reciprocal generations were produced, a total of nine generations. Crosses between B73 and Kp58k were affected by dent sterility factors. These factors exist in some popcorns and lead to allele-specific pollen abortion and poor seed set when the popcorn serves as the maternal parent (Glover and Mertz, 1987; Ziegler, 2001). Thus, all F1 plants used to generate F2 and BC1 data in B73 x popcorn crosses were the progeny of popcorn pollen on B73 ears. In all cases except Kp58k x B73 F1, kernel abortion was not complete and enough F1 and BC1 kernels were obtained for analysis when the popcorn inbred was used as the female parent. Sg18 does not contain alleles affecting dent sterility (Kindiger and Beckett, 1992).

Flow cytometric analyses

Developing kernels were carefully removed from the middle third of ears with a razor blade or scalpel. A razor blade was used to make an incision through the pericarp,

and the pericarp, nucellus, and developing embryo were removed. The dissected endosperm was placed in a 60 x 15- cm round polystyrene petri plate on a ceramic floor tile resting on a bed of ice. The tissue was overlaid with 0.5 ml of ice-cold lysis buffer (200 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 0.1% Triton X-100, filtered through a 22 μm filter) and chopped using a new single-edged razor blade (no. 55411-050, VWR, South. Plainfield, NJ). The homogenate was taken up into a 2- ml plastic pipette through four layers of cheesecloth and transferred to a 3- cc syringe housing (Becton-Dickinson, Franklin Lakes, NJ) fitted with 100 μm nylon mesh (Sefar International, Kansas City, MO) at the tip. The petri plate was rinsed with an additional 0.5 ml ice-cold lysis buffer, and this wash was also passed through the nylon mesh. The filtrate was collected in a 55 x 12- mm polystyrene tube (no. 55.484, Sarstedt, Newton, NC). Lysis buffer was added to adjust the volume of the homogenate to 1 ml, and 4,6-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO) was added to a final concentration of 4 mg/ml. Samples were held on ice no longer than 20 min prior to analysis. All samples were analyzed with a Partec CAIII flow analyzer (Partec, Münster, Germany) fitted with the standard filter set for DAPI analysis. Sheath fluid consisted of 22 μM filtered deionized water. The flow cytometer was controlled by Partec's CA3 software package at a resolution of 512 channels. Offline data analysis and figure preparation were performed using WinMDI software (Ver 2.8) kindly donated by J. Trotter (Trotter, 2000).

During the initial screening of germplasm, three to five endosperms were chopped together in a single petri dish and data from a minimum of 10,000 nuclei were collected for each sample. For all analyses subjected to statistical evaluation, endosperms were

chopped individually, and at least five kernels were assayed from each ear. Samples were analyzed immediately after chopping, and a minimum of 15,000 nuclei were counted. Numerous buffer systems (TRIS, MOPS, phosphate, and HEPES), DAPI concentrations (2-20 mg/ml), additives (citrate, polyvinyl pyrrolidone, dithiothreitol, Ficoll, and glycerol), incubation times (0-300 min post-chopping), tissue storage procedures (0-6 days at 4°), and buffer component concentrations were tested. Flow cytometric analysis and microscopic analysis of DAPI-stained samples clearly indicated that the procedure described above was optimal for obtaining intact nuclei from fresh, developing maize endosperm.

Embryo rescue

Ears were harvested and sterilized by immersion in a solution of commercial bleach and water (20% v/v) with a drop of Tween-20 for 30 min. They were then immersed in sterile water two times for 15 min and placed in a sterile beaker. Kernels were removed from the ear using a sterile scalpel and placed in a sterile petri dish. The pericarp was removed with a razor blade, the endosperm placed in a petri plate for flow cytometric processing, and the embryo placed with the scutellum facing up in a 12 x 150-mm culture tube containing 3 ml of MS-RD rescue medium (4.33 g Murashige and Skoog salts supplemented with 100 mg/liter myo-inositol, 0.4 mg/liter nicotinic acid, 0.2 mg/liter thiamine-HCl , 20% sucrose, adjusted to pH 5.7- 5.8 with 1M KOH, solidified with 0.8% agar) (Murashige and Skoog, 1962). Flow cytometric analysis of endosperm nuclei was performed as described above. Embryos were placed in the dark at 25°, checked daily, and moved to a growth chamber (28°, 16L:8D) following seedling

emergence. Seedlings were allowed to green and then transplanted to soil for growth to maturity in the greenhouse.

Statistical analyses

We routinely analyzed data of five kernels from three ears at 19-DAP for each generation. In the A1-6 x B73 and Kp58K x B73 experiments one generation failed. In all other cases, at least two ears and no less than eight kernels were analyzed per generation. The statistical output from manually gated histograms produced in the WinMDI software program were imported into an Excel spreadsheet (Microsoft Inc., Redmond, WA) where three values describing the nuclear DNA content were calculated for each endosperm. The mean ploidy of all nuclei, E3P, was calculated by multiplying the number of nuclei in each ploidy class by the number of genome copies (C) value and dividing the sum of these products by the total number of nuclei. The mean ploidy of nuclei with a C value greater than three, referred to as E6P, was calculated as for E3P, except the 3 C class was excluded. The recruitment of nuclei to an endoreduplication cell cycle was estimated by calculating the percentage of nuclei with greater than 3 C DNA content, and is referred to as %E.

The variances of E3P, E6P, and %E values from each cross were tested for variance components. Coefficients for genetic variance components were taken from Foolad and Jones (1992) with the exception of additional additive variance components considering zygotic parent-of-origin specific genetic components. All components tested are summarized in Table 2.1. Lowercase 'a' stands for additive and 'd' for dominance inheritance patterns. The subscripts refer to the mode of inheritance predicted by the

coefficients with t, d, m, zm, and p referring to triploid, diploid, sporophytic maternal, zygotic maternal, and zygotic paternal patterns of inheritance, respectively. The subscripts one and two differentiate the two dominance coefficients predicted for the allelic interactions Aaa and Aaa in the triploid model. Mathematically indistinguishable models for variance components are obtained when models containing additive zygotic maternal and zygotic paternal components (Va_{zm} and Va_p) are compared with models containing additive zygotic maternal and additive diploid components (Va_{zm} and Va_d). The difference is simply that when Va_{zm} and Va_d are estimated, variance preferentially partitioned into Va_d with respect to the Va_{zm} and Va_p predictions.

A maximum likelihood (ML) method (Hayman, 1960), as modified in Lynch and Walsh (1998), was used to estimate variance components from the endoreduplication data. The variance of endoreduplication measurements from each ear were utilized as the dependent variable. These variances were weighted with the inverse of the unbiased estimate of their variance (Lynch and Walsh, 1998). Regression was then carried out using the coefficients of genetic and environmental variance components presented in Table 2.1. The variances for each ear predicted by the regression results were tabulated, and the inverse of the unbiased estimates of these expected variances were calculated and used to weight the observed variances for a second iteration of regression. Regressions were repeated, recalculating the weighting term at each iteration. The weighted chi-square value was then calculated by summing the deviation of the observed and expected variances divided by the weighting factor. ML iterations were ceased when the weighted chi-square values changed less than 1×10^{-5} for three consecutive iterations. After

Table 2.1. Coefficients for genetic components of the variance

		Triploid zygotic			Diploid zygotic		Maternal sporophytic		Maternal zygotic	Paternal zygotic
Coefficient name	*Ve*	Va_t	Vd_{t1}	Vd_{t2}	Va_d	Vd_d	Va_m	Vd_m	Va_{zm}	Va_p
B73	1	0	0	0	0	0	0	0	0	0
Parent2	1	0	0	0	0	0	0	0	0	0
B73 x Parent2	1	0	0	1	0	0	0	0	0	0
Parent2 x B73	1	0	0	0	0	0	0	0	0	0
F2	1	5/9	3/16	3/16	1/2	1/4	0	0	1	1
F1 x B73	1	4/9	1/4	0	1/4	1/4	0	0	1	0
F1 x Parent2	1	4/9	1/4	0	1/4	1/4	0	0	1	0
B73 x F1	1	1/9	0	1/4	1/4	1/4	0	0	0	1
Parent2 x F1	1	1/9	0	1/4	1/4	1/4	0	0	0	1

a additive effect, d dominance effect, e error and environment, subscripts t, d, m, zm, and p refer to triploid, diploid, maternal sporophytic, zygotic maternal, and zygotic paternal modes of inheritance, respectively

convergence, goodness of fit was evaluated by means of a weighted chi-square. Weighted chi-squares involving categories with five or fewer members are suspect as measures of the fit for a model. As a solution, measurements were pooled for each genotype and the nine or eight generation variances were used as the observed variances to perform the weighted chi-square as before. The p-value was calculated at one less than the number of generations minus the number of estimated parameters. As a further test of the models, the generation variances were used to estimate genetic parameters. Both methods returned qualitatively similar results. Estimates from ear variances are reported, as they are generally more conservative. The narrow sense heritabilities for each component and the total were calculated according to Falconer and Mackay (1996). Analysis of components of the covariance between E3P, E6P, and %E for genetic and environmental components was performed similarly to that for variance analysis. Co-variances were generated by multiplying the standard deviations of two traits for each individual and averaging the total for each ear. Coefficients and ML methods were identical to that for variance components.

Multiple genetic models describing the additive and dominance components of mean values were also tested. Coefficients for sporophytic, standard triploid, and diploid inheritance were taken from the testa, endosperm, and embryo models, respectively, from Foolad and Jones (1992). A model was generated for zygotic parent-of-origin expression, which is mathematically equivalent to a completely additive sporophytic model. All coefficients discussed in the text are presented in Table 2.2. Lower case letters and subscripts are used identically to those described for variance components.

Table 2.2. Coefficients for genetic components of the mean

		Triploid zygotic			Diploid zygotic		Maternal sporophytic		Maternal zygotic	Paternal zygotic
	Mean	a_t	d_{t1}	d_{t2}	a_d	d_d	a_m	d_m	a_{zm}	a_p
B73	1	-1	0	0	-1	0	-1	0	-1	-1
Parent2	1	1	0	0	1	0	1	0	1	1
B73 x Parent2	1	-1/3	0	1	0	0	-1	0	-1	1
Parent2 x B73	1	1/3	1	0	0	0	1	0	1	-1
F2	1	0	1/4	1/4	0	1/2	0	1	0	0
F1 x B73	1	-1/3	1/2	0	-1/2	1/2	0	1	0	-1
F1 x Parent2	1	1/3	0	1/2	1/2	1/2	0	1	0	1
B73 x F1	1	-2/3	1/2	0	-1/2	1/2	-1	0	-1	0
Parent2 x F1	1	2/3	0	1/2	1/2	1/2	1	0	1	0

a additive effect, d dominance effect, e error and environment, subscripts t, d, m, zm, and p refer to triploid, diploid, maternal sporophytic, zygotic maternal, and zygotic paternal modes of inheritance respectively

The mathematical conundrum between maternal models including paternal or diploid additive components described for variance components is also true for mean components in this crossing design. To perform tests for components of the mean and investigate models of inheritance, weighted least-squares regression analysis was used to estimate the genetic contribution to mean E3P, E6P, and %E using the SPSS statistical package (v. 10.01 SPSS Inc., Chicago, IL). Mean values were calculated for each ear and considered the dependent variable. The genetic components described in Table 2.2 were used as the independent variables. Each mean was weighted with the inverse of its variance. Regression was used to estimate the values of the population mean, each component, and the component significance. To assess the significance of the model, the p-value was calculated according to a chi-square distribution for the residuals of the regression where d.f. = $(n - p) - 1$, n being the number of ears and p being the number of parameters estimated. A variety of models were tested in each case. First, the mean component models consisting of genetic factors present in the variance component analysis were tested. These models were supplemented with the maternal additive and dominance coefficients for the maternal sporophytic model, as they cannot be detected in the variance component analysis. If inclusion of maternal coefficients not detected in the variance component analysis did not improve the fit of the data, they were not reported. To confirm and extend these analyses, a model consisting of all the coefficients was simultaneously evaluated by weighted least-squares regression. Coefficients were systematically added and removed until an optimal model was produced. Finally, each of the individual modes of inheritance was tested singly. The result of a mean component

analysis using the additive and dominance triploid model, which is the assumed genetic model for an endosperm trait, is reported. For example, if the variance component analysis detected zygotic paternal effects (Va_p), the mean value predicted by this model for the last BC1 generation is given by the equation, $y = 1 \times a_p + 1 \times m$ if no maternal sporophytic effects are present, and by, $y = 1 \times a_p + 1 \times a_m + 0 \times d_m + 1 \times m$ if sporophytic additive and dominance effects are expected, where y is the observed variable, m is the mean of the population, and all other variables are from Table 2.2. In this way the values for each genetic effect can be estimated by generating a series of equations from Table 2.2 specific to any combination of genetic predictions, by estimating a solution by regression, and by evaluating the solutions for fit to the data.

For the analysis of variance and covariance components in the recurrent backcross progeny, family means of the offspring were compared to individual maternal parent values. The narrow sense heritability was then estimated by the slope (b) of the regression line (Falconer and Mackay, 1996). As segregation occurred only on the maternal side, only the maternal contributions to the genetic variance were calculated from these offspring-parent regressions. Modes of inheritance, other than the simple diploid model, were taken into consideration such that $h^2 = 2/3 \times b$ for triploid inheritance and $h^2 = b$ in maternal zygotic parent-of-origin-specific inheritance. Progeny family means were weighted to take into consideration the number of individuals and the family variance (Falconer and Mackay, 1996).

Statement of collaboration

Flow cytometric data collection was done in collaboration with Cintia Coelho and Ricardo Dante at the University of Arizona.

Results

Identification of maize inbreds differing in the level of endoreduplication in developing endosperm

To assess the phenotypic variation in the degree of endoreduplication among various types of maize, we performed flow cytometric measurements of endosperm at several stages of development. In general, we found little variation in the maximum measured mean C value (E3P) among most midwestern dent (A188, B37, Mo17, Va99, W64A, Oh545), South American landraces (Bolivia 1044 and Venezuela 568), flint (8-Row Flint and Yellow Flint), and sweet corn genotypes. In contrast, a number of popcorn inbreds (A1-6, Sg18, Sg1533, Sg32, HP58) exhibited dramatically higher levels of endoreduplication.

Figure 2.1 shows flow cytometric histograms illustrating the highest E3P detected for selected genotypes, and it illustrates the variation in the degree of endoreduplication among maize popcorn and dent genotypes. Sg1533 and A1-6 have nearly identical E3P values, but they are clearly different with regard to the proportion of endoreduplicated nuclei (%E). In Sg1533, fewer nuclei undergo endoreduplication, but those that do engage in more cycles of DNA replication. In A1-6 endosperm, nearly all nuclei participate in endoreduplication. Similar differences in the relationship between E3P and %E can be observed by comparing the histograms of nuclei from Sg18, Kp58k, and B73.

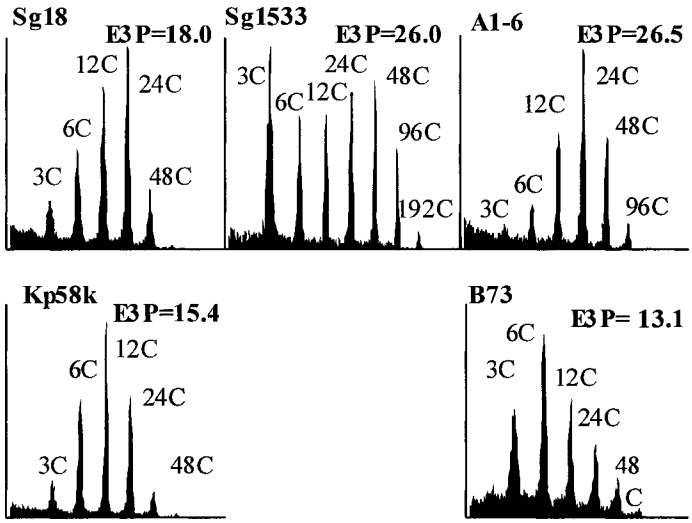


Figure 2.1. Flow cytometric measurement of mean nuclear ploidy (E3P) in selected inbred lines. Multiple endosperms from a single ear were combined for analysis. Histograms representing the endosperm with the highest measured ploidy for each inbred are illustrated. The X-axes are the log of the fluorescence intensity and the Y-axes correspond to the relative frequency of a given intensity. The C value indicates the number of genome copies, and the E3P value describes the mean ploidy of all nuclei. The highest peak mean ploidy was attained at different stages of development for different inbreds as follows: B73, 23-DAP; Kp58k, 25-DAP; Sg18, 26-DAP; Sg1533, 29-DAP; A1-6, 27-DAP.

The phenotypic variability we detected between these inbreds suggested that endoreduplication occurs earlier, is more rapid, involves a greater proportion of cells, or proceeds for a longer period of time in popcorns than in dent inbreds.

Developmental analysis of Sg18 x B73 crosses

To determine the timing of endoreduplication in genotypes with different levels of endoreduplication, we examined endosperms from B73 and Sg18, and (B73 x Sg18) x B73 at 13-, 15,- and 18-DAP (Fig. 2.2). By 13-DAP, the E3P value for B73 endosperm was 9 C, and it increased to greater than 10 C by 18-DAP. In Sg18, the E3P value at 13-DAP was about 13 C, and it did not increase up to 18-DAP. Similarly, the E3P value in the BC1 cross was 12 C at 13-DAP, and there was no further increase. The difference between the E3P values of the inbreds was significant by Student's t-test ($p<0.01$) at each developmental stage. We attempted to measure endoreduplication in endosperm at later stages of development. However, samples from endosperms older than 19-DAP frequently clogged the tip at the base of the flow cell, making a large-scale analysis at developmental stages later than 19-DAP unfeasible. The observation of significant differences in endoreduplication between genotypes at 18-DAP suggested that a study of the genetic regulation of this trait was feasible.

Genetic analysis of endosperm endoreduplication in B73 x Mo17 crosses

To assay genetic factors influencing endoreduplication, crosses were made according to a 3^2 design, in which all nine pair-wise crosses between B73, Mo17, and the F1 were generated. The nine means and variances calculated from this crossing design

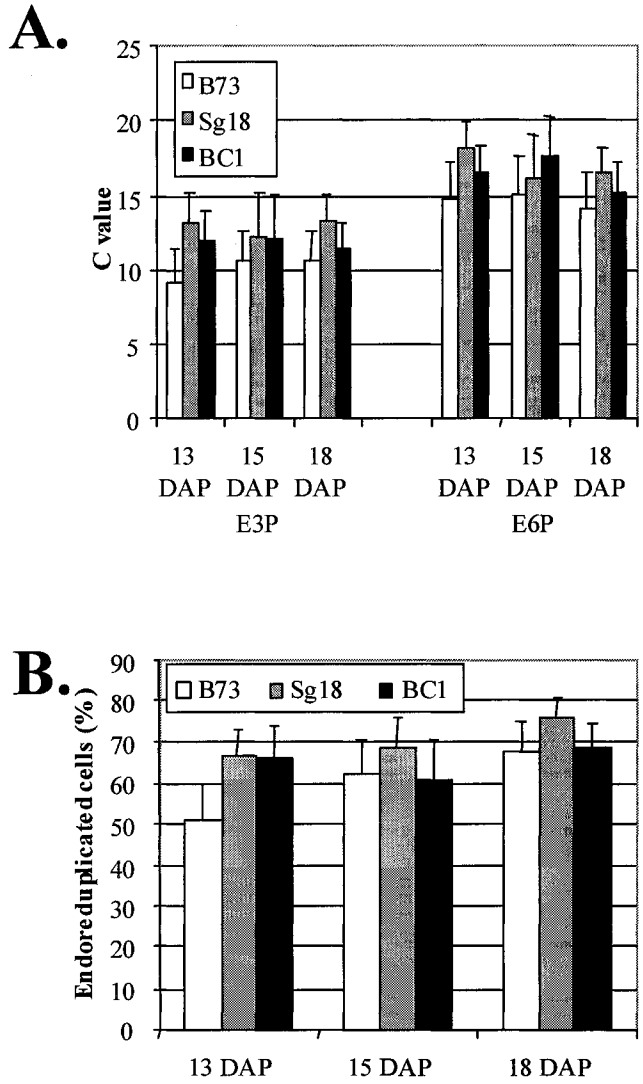
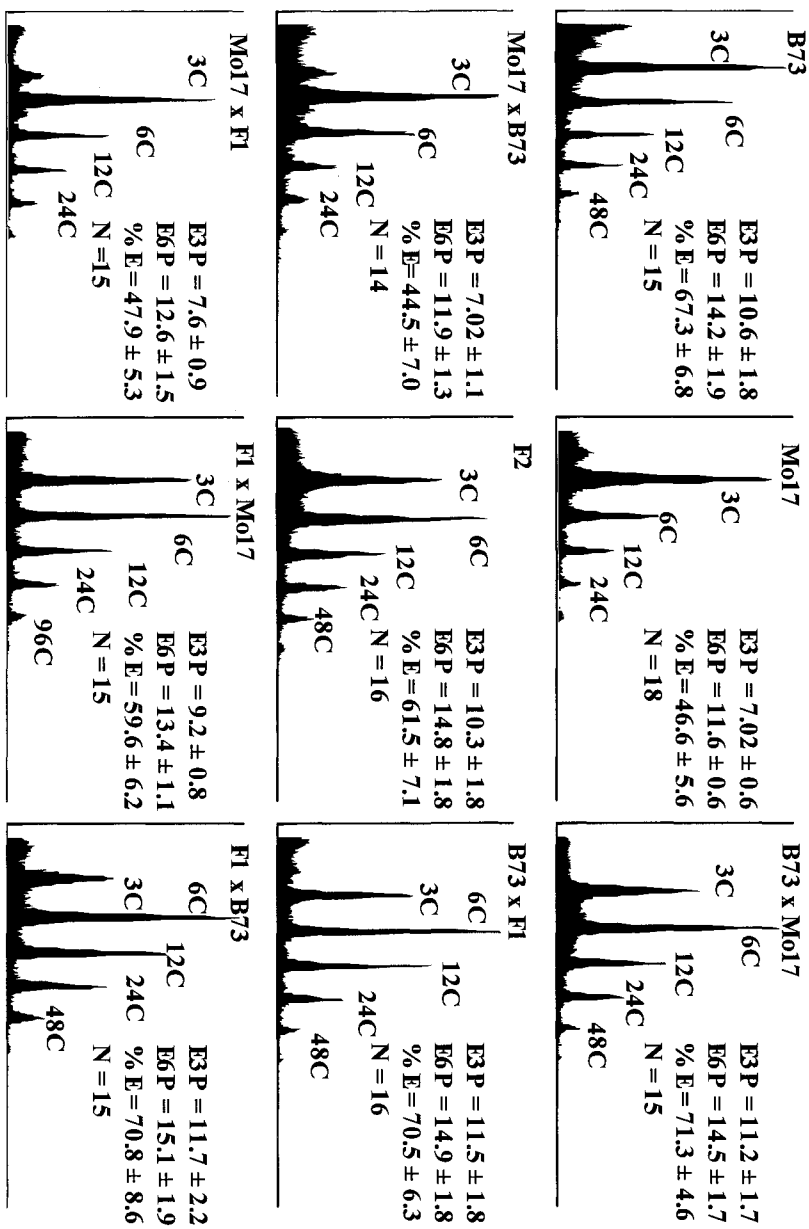


Figure 2.2. Measurement of ploidy level in B73, Sg18, and their BC1 endosperms at 13- to 18-DAP. (A.) The E3P and E6P values indicate the mean ploidy and mean ploidy of endoreduplicated cells in the endosperms, while the error-bars show the standard deviations of these measurements. (B) The %E indicates the frequency of cells with greater

allowed for the detection of genetic components affecting endoreduplication expressed from the genome of the endosperm itself (zygotic expression) and the progenitor (sporophytic expression). Thus, this design facilitates the testing of models in which genetic factors affecting differences in endoreduplication exhibit zygotic parent-of-origin specific inheritance, commonly referred to as parental imprinting. Each of the nine generations of B73 x Mo17 were grown in the fall of the 1999 field season, and single endosperms were subjected to flow cytometric analysis at 19-DAP.

To more completely describe the state of endoreduplication in the endosperm, three measurements of nuclear ploidy were calculated from the flow cytometric data, E3P, E6P, and %E. The mean ploidy (E3P) was calculated by weighting each nucleus by its respective ploidy. However, this value does not consider the relative distribution of nuclei in each ploidy class. The E3P value can be expressed algebraically as two potentially separate components, the mean ploidy of the endopolyploid nuclei (E6P) and the percentage of nuclei that endoreduplicate (%E). These two components can be used to calculate the E3P with the following formula: E3P = (%E x E6P) + (3 x (1-%E)). The E6P was calculated similarly to E3P, except the 3 C class was excluded. The %E was calculated by dividing the number of nuclei with a ploidy of 6 C or greater by the total number of nuclei. Figure 2.3 shows the E3P, E6P, and %E for each of the nine B73 x Mo17 generations, with the corresponding standard deviation and a representative flow cytometric histogram. Student's t-tests comparing the values for E3P, E6P, and %E between the parental lines were statistically significant ($p<0.01$, data not shown).

Figure 2.3. Endoreduplication phenotypes of nine B73 x Mo17 generations. Single endosperms were subjected to flow cytometric analysis. Representative histograms were selected for illustration based on their approximation of the mean ploidy value of the generation. The X-axes are the log of the fluorescence intensity and the Y-axes correspond to the relative frequency of a given intensity. Means and standard errors were calculated from no fewer than 14 kernels. The C value indicates the


B73
3C
6C
12C
24C
48C
E3P = 10.6 ± 1.8
E6P = 14.2 ± 1.9
% E = 67.3 ± 6.8
N = 15
Mo17
3C
6C
12C
24C
E3P = 7.02 ± 0.6
E6P = 11.6 ± 0.6
% E = 46.6 ± 5.6
N = 18
B73 x Mo17
3C
6C
12C
24C
48C
E3P = 11.2 ± 1.7
E6P = 14.5 ± 1.7
% E = 71.3 ± 4.6
N = 15
Mo17 x B73
3C
6C
12C
24C
E3P = 7.02 ± 1.1
E6P = 11.9 ± 1.3
% E= 44.5 ± 7.0
N = 14
F2
3C
6C
12C
24C
48C
E3P = 10.3 ± 1.8
E6P = 14.8 ± 1.8
% E = 61.5 ± 7.1
N = 16
B73 x F1
6C
3C
12C
24C
48C
E3P = 11.5 ± 1.8
E6P = 14.9 ± 1.8
% E = 70.5 ± 6.3
N = 16
Mo17 x F1
3C
6C
12C
24C
E3P = 7.6 ± 0.9
E6P = 12.6 ± 1.5
% E = 47.9 ± 5.3
N =15
F1 x Mo17
3C
6C
12C
24C
96C
E3P = 9.2 ± 0.8
E6P = 13.4 ± 1.1
% E = 59.6 ± 6.2
N = 15
F1 x B73
6C
3C
12C
24C
48C
E3P = 11.7 ± 2.2
E6P = 15.1 ± 1.9
% E = 70.8 ± 8.6
N = 15

Moreover, Student's t-tests were unable to distinguish F1's from their respective ear parent ($p>0.05$).

To investigate the mode of inheritance for the three endoreduplication traits, an analysis of variance components was performed. Maximum likelihood (ML) was employed to estimate the contribution of each of the factors in Table 2.1 to the nine generation variances in B73 x Mo17. ML estimates and their corresponding standard errors and p values and weighted chi-square values for each model are presented in Table 2.3. The variance in %E and E6P values were best predicted by a maternal parent of origin specific component, Va_{zm}. Variance component analyses of these data yielded estimates with a rather poor fit (Table 2.3). The p-values calculated were insufficiently small to reject these models ($p > 0.05$), and the addition of further genetic coefficients did not improve the fit. The large chi-square values suggest that these results should be interpreted with caution. In all three analyses, the Mo17 parent was associated with lower means and variances (Fig. 2.3), and this aspect of the data inflated the chi-square values for all models. A logarithmic scale transformation did not improve the fit of the model to the data (not shown). Variances from B73 and Mo17 generations were not equivalent for both E3P and E6P data (F-test, $p<0.05$), suggesting that the two inbred lines differed in their sensitivity to the environment. Significant differences were also detected between Mo17 x B73 and either parental line, with the F1 variance being significantly greater than Mo17 and less than B73 (F-test, $p<0.05$). However, our experimental design does not allow for an in-depth analysis of genotype by environment interactions. Nonetheless, it suggests that the assumption of variance equivalence in two

Table 2.3. Variance component analysis of the B73 x Mo17 cross

Trait	Va_{zm}	Ve	p value	χ^2
E3P	No significant models detected			
E6P	0.17+/-0.63	1.61**+/-0.34	0.06	14.9
%E	8.3+/-9.7	29.9**+/-4.7	0.07	14.6

* significantly greater than zero at $p<0.05$, ** significant at $p<0.01$

inbred lines is not valid for this cross. The inclusion of a coefficient for the contributions of unequal variance by the parentals did improve the fit of the data (not shown). When this was done, chi-square values dropped and much of the error variance partitioned into the unequal variance term, but neither the coefficients of the genetic model nor the significance of Va_{zm} changed. Given the statistical power of this variance component analysis and the rather modest estimates of Va_{zm} provided, a much larger sample size would be required to identify significant components of the amplitudes described in Table 2.3. The failure to reject these models and the inability of a scale transformation to improve the fit suggest that the segregation variance in these crosses is simply insignificant.

To further test for the presence of genetic components in the variation of E3P, E6P, and %E in B73 x Mo17, an analysis of mean components was performed. Estimates of mean components were made, and the fit of each model was tested by weighted chi-square (Foolad and Jones, 1992; Lynch and Walsh, 1998). A summary of the results describing the inheritance of mean values for endoreduplication traits in B73 x Mo17 is presented in Table 2.4. For all three traits, the data can be accounted for with a single maternal additive coefficient. Simultaneous regression of all coefficients resulted in significance only for the maternal additive coefficient (not shown). Neither the addition of a paternal additive component nor the inclusion of a dominant maternal coefficient significantly improved the fit of the data for any of the three traits. For E3P and E6P, the significance and size of the maternal additive mean components are inconsistent with maternal zygotic effects on the scale detected in the variance component analysis. These

Table 2.4. Models describing the inheritance of endosperm ploidy in the B73 x Mo17 generations

		Triploid zygotic			Maternal			
Trait/model tested[1]	*Mean*	a_t	d_{t1}	d_{t2}	a_m	d_m	p value	χ^2
E3P/varcomp				no models suggested				
E3P/sporophytic	9.33				-2.1**		0.98	2.0
E3P/triploid zygotic	9.3	-2.3*	-1.0	1.3			0.93	1.9
E6P/varcomp	13.5				-1.6**		0.98	2.1
E6P/triploid zygotic	13.4	-1.7*	-0.3	0.9			0.95	1.6
%E/varcomp	59.5				-11.7**		0.97	2.4
%E/triploid zygotic	58.9	-11.9*	-6.3	8.5			0.88	2.4

* significantly different from zero at $p<0.05$, ** significant at $p<0.01$

[1] varcomp refers to the model defined in the variance component analysis and described in the text all other models are explicitly specified in table 2.2

results are more consonant with additive sporophytic control of endosperm endoreduplication levels in B73 x Mo17.

The *a priori* genetic models for endosperm traits, the triploid models, were also tested for fit to the data. No models provided a better fit than that of the maternal sporophytic additive model. However, E3P, E6P, and %E can all be explained by the standard zygotic model (2m:1p). If a trait is under zygotic parent-specific control, the two dominance coefficients (d_{t1} and d_{t2}) are expected to have opposite signs, compensating for the predicted additive effect (Table 2.2). Furthermore, if it were predominantly under maternal control, one would expect the a_t and d_{t1} coefficients to have the same sign (Table 2.2). This is the case for all three traits. Reworking the zygotic model for diploid zygotic inheritance, a phenomenon known to occur for some genes in the endosperm (Alleman and Doctor, 2000) and possible if embryo genes control this phenotype in the endosperm, did not improve the fit of the data (data not shown).

Genetic analysis of endoreduplication in endosperm of B73 x popcorn crosses

To further address the genetic regulation of endoreduplication in maize endosperm, popcorn inbreds Sg18, A1-6, and Kp58k were crossed with B73, and a 3^2 design was used, as for the B73 x Mo17 crosses above. In the case of Kp58k and A1-6, one of the crosses failed (see Materials and Methods). The nine (or eight) generations for each of the three B73 x popcorn crosses were grown in the fall of 1999 field season, and endosperms from individual kernels were subjected to flow cytometric analyses at 19-DAP. As in the previous analysis, the models presented in Tables 2.1 and 2.2 were used

to test for E3P, E6P, and %E variance and mean components by ML and weighted least-squares regression, respectively.

A set of representative histograms for the B73 x Sg18 cross is shown in Figure 2.4, which presents the mean E3P, E6P, and %E for each of the nine generations with the corresponding standard deviations. Student's t-tests between B73 and Sg18 inbred lines were significant for E3P and E6P ($p<0.01$), but not for %E ($p>0.05$). Again, as with B73 x Mo17, F1's could not be distinguished from their maternal progenitor ($p>0.05$).

The mode of inheritance for each endoreduplication trait was first tested by variance component analysis. The results from the ML estimation of variance components for all three traits in the B73 x Sg18 generations are summarized in Table 2.5. Variance component analyses of E6P data identified maternal zygotic, Va_{zm}, and paternal zygotic, Va_p, components as the major genetic contributions to the variance. Variance component estimation from E3P and %E data, however, identified a single maternal zygotic component. Unlike the case for B73 x Mo17, Va_{zm} estimates from E3P and E6P data were statistically significant. The maternal coefficient is not significantly different from zero in the best model to describe the %E variances. As with all three traits in B73 x Mo17, the values of Va_{zm} calculated for the %E data and Va_p for E6P are rather modest, and an increased sample size is required to detect significant components of this size. Together, these data strongly suggest that maternal zygotic effects are operating in E3P and E6P data sets in B73 x Sg18.

To confirm and extend these analyses of genetic components, an analysis of trait means was performed. The different modes of inheritance presented in Table 2.2 were

Figure 2.4. Endoreduplication phenotypes of nine B73 x Sg18 generations. Measurements and histogram selection were carried out as

B73
3C
6C
12C
24C
48C
E3P = 10.6 ± 1.8
E6P = 14.2 ± 1.9
% E = 67.3 ± 6.8
N = 15
Sg18
3C
6C
12C
24C
48C
96C
E3P = 14.0 ± 0.9
E6P = 19.0 ± 0.9
% E = 68.8 ± 3.4
N = 8
B73 x Sg18
3C
6C
12C
24C
48C
96C
E3P = 9.6 ± 1.8
E6P = 16.0 ± 1.9
% E = 50.3 ± 7.4
N = 13
Sg18 x B73
3C
6C
12C
24C
48C
E3P = 12.2 ± 1.1
E6P = 14.9 ± 1.6
% E = 77.6 ± 8.2
N = 10
F2
3C
6C
12C
24C
48C
96C
E3P = 14.8 ± 3.0
E6P = 19.0 ± 2.6
% E = 72.8 ± 7.9
N = 12
B73 x F1
3C
6C
12C
24C
48C
E3P = 12.0 ± 1.9
E6P = 15.6 ± 1.7
% E = 70.9 ± 6.8
N = 13
Sg18 x F1
3C
6C
12C
24C
48C
96C
E3P = 12.2 ± 2.1
E6P = 17.2 ± 1.7
% E = 64.6 ± 6.8
N = 10
F1 x Sg18
3C
6C
12C
24C
48C
96C
E3P = 14.9 ± 2.8
E6P = 19.2 ± 2.3
% E = 73.0 ± 7.3
N = 11
F1 x B73
3C
6C
12C
24C
48C
E3P = 13.8 ± 2.4
E6P = 18.4 ± 2.3
% E = 69.4 ± 7.6
N = 14

Table 2.5. Variance component analysis of B73 x popcorn crosses

		Coefficients				
Popcorn	Trait	Va_p	Va_{zm}	Ve	p value	χ^2
Sg18	E3P		5.5**+/-2.0	2.6**+/-0.37	0.26	10.0
	E6P	1.04+/-1.5	3.7*+/-2.7	1.9**+/-0.51	0.85	4.0
	%E		12.9+/-13.7	45.0**+/-6.6	0.77	4.9
A1-6	E3P		4.4**+/-1.6	2.5**+/-0.50	0.80	3.8
	E6P	2.6+/-1.7	4.4*+/-1.8	2.9**+/-0.70	0.69	3.9
	%E		5.9+/-10.3	56.2**+/-5.5	.20	10.9

* significantly greater than zero at $p<0.05$, ** significant at $p<0.01$

tested as described above. Estimated mean components for the three endoreduplication traits of B73 x Sg18 crosses are summarized in Table 2.6. Analyses of E3P, E6P, and %E data using the coefficients predicted by the variance component analysis were significant. In all three cases, optimal models were produced when the maternal dominance coefficient from the maternal sporophytic model was included. All three mean components were significant for E6P, but only the maternal additive coefficient was significant for E3P. A model for %E mean values consisting of maternal additive and dominance coefficients could not be rejected despite the insignificance of either genetic coefficient. Additive and dominance maternal and additive paternal components were positive in sign for all three traits. Simultaneous regression of all coefficients on the data for any of the three traits led to the same model predicted by the variance component analysis. The standard triploid zygotic model could not be rejected for any of the three traits. However, coefficient signs were consistent with a trait under strong maternal zygotic influence for the E3P and E6P data. Thus, Sg18 alleles increase endoreduplication levels via zygotic parent-of-origin and maternal sporophytic effects.

The variances of E3P, E6P, and %E from B73 x A1-6 were also tested for the presence of genetic components. The E3P, E6P, and %E for these crosses are summarized in Figure 2.5 along with representative flow cytometric histograms. Endoreduplication measures of the parental, F1, and BC1 generations were indistinguishable from their ear parent by t-test ($p>0.5$). Variance components for each of the three traits were estimated by ML using the coefficients presented in Table 2.1 and are summarized in Table 2.5. Models fitting the variance data for all three traits were

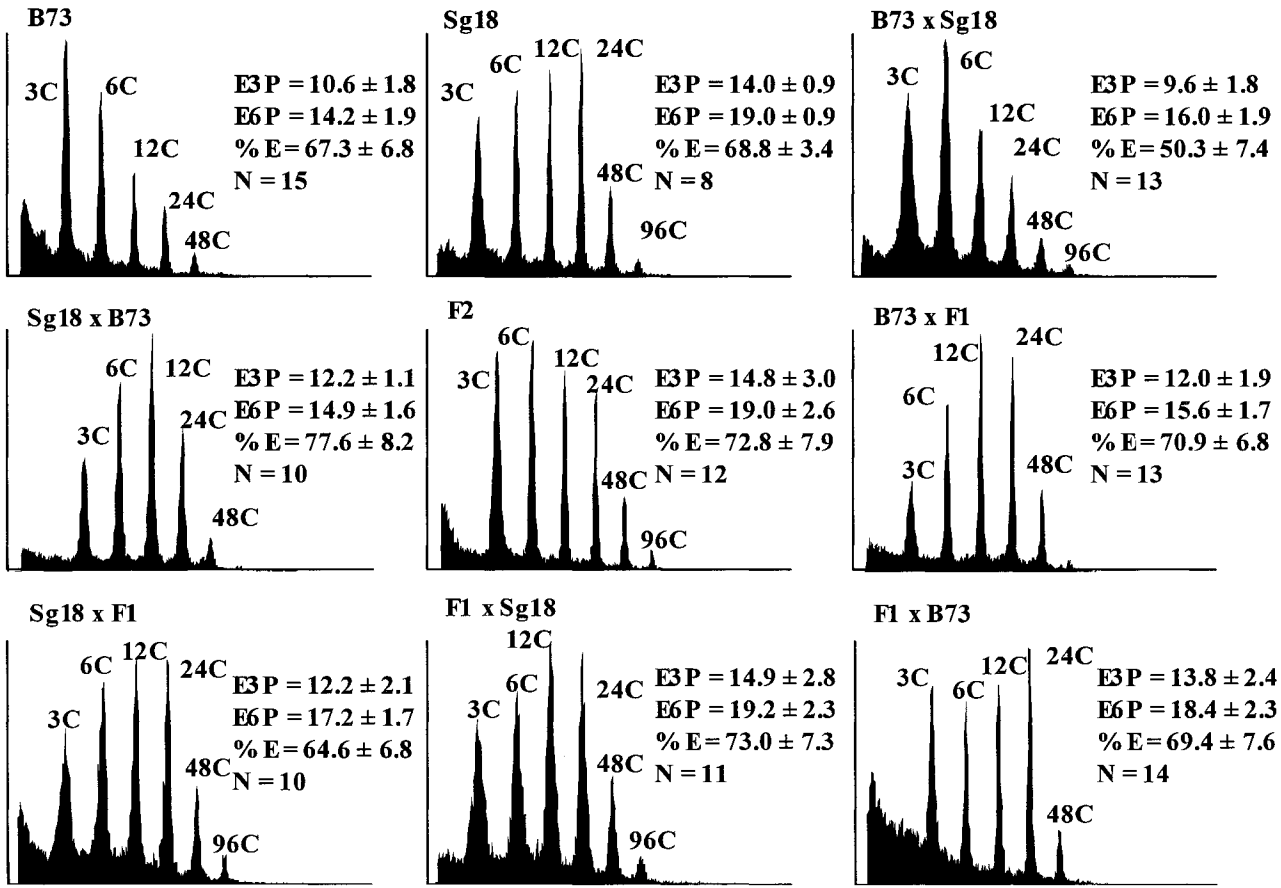
Table 2.6. Models describing the inheritance of mean endosperm ploidy traits in the B73 x Sg18 generations

		Triploid zygotic			Maternal		Paternal		
Trait/model tested[1]	*Mean*	a_t	d_{t1}	d_{t2}	a_m	d_m	a_p	p value	χ^2
E3P/varcomp	11.9				1.2**	2.5		0.91	2.1
E3P/triploid zygotic	12.5	1.5	3.2	-0.5				0.75	3.4
E6P/varcomp	16.3				1.0*	2.6*	1.3*	0.94	1.9
E6P/triploid zygotic	16.6	2.0	4.4	-1.2				0.55	5.0
%E/varcomp	66.3				2.8	5.4		0.50	6.3
%E/triploid zygotic	69.2	1.3	1.5	2.6				0.93	1.9

* significantly different from zero at $p<0.05$, ** significant at $p<0.01$

[1] varcomp refers to the model defined in the variance component analysis and described in the text all other models are explicitly specified in table 2.2

Figure 2.5. Endoreduplication phenotypes of eight B73 x A1-6 generations. Measurements and histogram selection were carried out as described in Figure 2.3. Means and standard errors were calculated from no


B73
3C
6C
12C
24C
48C
E3P = 10.6 ± 1.8
E6P = 14.2 ± 1.9
% E = 67.3 ± 6.8
N = 15
Sg18
3C
6C
12C
24C
48C
96C
E3P = 14.0 ± 0.9
E6P = 19.0 ± 0.9
% E = 68.8 ± 3.4
N = 8
B73 x Sg18
3C
6C
12C
24C
48C
96C
E3P = 9.6 ± 1.8
E6P = 16.0 ± 1.9
% E = 50.3 ± 7.4
N = 13
Sg18 x B73
3C
6C
12C
24C
48C
E3P = 12.2 ± 1.1
E6P = 14.9 ± 1.6
% E = 77.6 ± 8.2
N = 10
F2
3C
6C
12C
24C
48C
96C
E3P = 14.8 ± 3.0
E6P = 19.0 ± 2.6
% E = 72.8 ± 7.9
N = 12
B73 x F1
3C
6C
12C
24C
48C
E3P = 12.0 ± 1.9
E6P = 15.6 ± 1.7
% E = 70.9 ± 6.8
N = 13
Sg18 x F1
3C
6C
12C
24C
48C
96C
E3P = 12.2 ± 2.1
E6P = 17.2 ± 1.7
% E = 64.6 ± 6.8
N = 10
F1 x Sg18
3C
6C
12C
24C
48C
96C
E3P = 14.9 ± 2.8
E6P = 19.2 ± 2.3
% E = 73.0 ± 7.3
N = 11
F1 x B73
3C
6C
12C
24C
48C
E3P = 13.8 ± 2.4
E6P = 18.4 ± 2.3
% E = 69.4 ± 7.6
N = 14

identified. Data from E3P measurements were the best fit to a model consisting of a maternal zygotic component, Va_{zm}, while E6P variances were best described by a combination of zygotic paternal and maternal effects, Va_p and Va_{zm}, respectively. Coefficients for maternal effects were significant for E3P and E6P models and were the only significant genetic coefficients in the analysis of B73 x A16. Failure to detect significance of Va_p may be attributable either to a lack of involvement in segregation variance or to the statistical power of this test, given the sample size. No genetic models contained significant coefficients in the analysis of the %E data. However, a model containing a maternal additive component could not be rejected and was the best fit to the data. These results strongly suggest that genes expressed from the maternal genome of the developing endosperm control E3P and E6P. These data weakly suggest paternal effects also play a role in the control of E6P.

Genetic components for the means of the three endoreduplication traits from B73 x A1-6 crosses were evaluated as described above, and the regression analyses are summarized in Table 2.7. Tests of the mean component models for E3P, E6P, and %E suggested by variance component analyses were significant for both E3P and E6P. Only the maternal additive coefficient was significant, and the inclusion of maternal dominance coefficients decreased the fit of the data. The paternal additive component of E6P was not significant, and unlike in the B73 x Sg18 experiment, was negative. As with the B73 x Mo17 crosses, the triploid zygotic additive and dominance model adequately described E3P or E6P (Table 2.4), but the sign of the coefficients is consistent with maternal control. Results of the analysis of %E means in this experiment were most

Table 2.7. Models describing the inheritance of mean endosperm ploidy traits in the B73 x A1-6 generations

		Triploid zygotic			Maternal		Paternal		
Trait/model tested[1]	*Mean*	a_t	d_{t1}	d_{t2}	a_m	d_m	a_p	p value	χ^2
E3P/varcomp	13.2				1.9**			p>0.99	0.54
E3P/triploid zygotic	12.5	1.5	3.2	-0.5				0.99	0.42
E6P/varcomp	17.3				2.7**		-0.2	p>0.99	0.97
E6P/triploid zygotic	16.6	2.0	4.4	-1.2				0.98	0.81
%E/varcomp	70.1				0.5			p>0.99	0.29
%E/triploid zygotic	69.2	1.3	1.5	2.6				p>0.99	0.15

* significantly different from zero at $p<0.05$, ** significant at $p<0.01$

[1] varcomp refers to the model defined in the variance component analysis and described in the text all other models are explicitly specified in Table 2.2

consistent with no genetic influence, as the low chi-square value has more to do with low variation between generations for this trait (Table 2.7 and, Fig. 2.5). Thus, the analyses of E3P and E6P data from B73 x A16 strongly implicate zygotic maternal parent-of-origin specific inheritance.

Endoreduplication traits in the third popcorn cross, B73 x Kp58k, were subjected to the same types of statistical analyses described above. The mean values of E3P, E6P, and %E and the flow cytometric histograms for each of the generations are summarized in Figure 2.6. Student's t-tests of E3P, E6P, and %E at 19-DAP between parental inbreds were not significant ($p>0.05$) for this cross, despite the apparent differences at later stages of development (Fig. 2.1). Neither variance components nor mean components for the three endoreduplication measures could be identified from the data collected for these crosses (data not shown). We concluded that higher endoreduplication levels at 19-DAP is not a general feature of popcorn germplasm.

Pleiotropy in the genetic control of endoreduplication traits

To assess the degree to which common genetic factors influence the three measurements of endoreduplication, an analysis of the components of covariance between them was done. A high phenotypic correlation between each of these traits was detected in all inbreds (data not shown). However, not all generations showed positive phenotypic correlation. To minimize reporting spurious associations, covariance component analyses were attempted only for crosses in which significant variance components were identified.

Figure 2.6. Flow cytometric phenotypes of eight B73 x Kp58k generations. Measurements and histogram selection were carried out as described in Figure 2.3, except that means and standard errors are from no

B73
3C
6C
12C
24C
48C
E3P = 10.6 ± 1.8
E6P = 14.2 ± 1.9
% E = 67.3 ± 6.8
N = 15
Kp58k
3C
6C
12C
24C
48C
E3P = 10.7 ± 2.0
E6P = 14.2 ± 1.6
% E = 68.0 ± 7.6
N = 15
B73 x Kp58k
3C
6C
12C
24C
48C
E3P = 6.9 ± 1.3
E6P = 11.4 ± 1.4
% E = 45.3 ± 8.3
N = 10
F2
3C
6C
12C
24C
48C
E3P = 9.7 ± 1.1
E6P = 14.1 ± 1.3
% E = 60.3 ± 5.5
N = 19
B73 x F1
3C
6C
12C
24C
48C
E3P = 10.4 ± 1.6
E6P = 13.8 ± 1.9
% E = 67.6 ± 4.7
N = 15
Kp58k x F1
3C
6C
12C
24C
48C
E3P = 8.8 ± 1.2
E6P = 12.8 ± 1.2
% E = 59.9 ± 6.4
N = 16
F1 x Kp58k
3C
6C
12C
24C
48C
E3P = 8.3 ± 1.2
E6P = 12.7 ± 1.6
% E = 54.7 ± 5.6
N = 19
F1 x B73
3C
6C
12C
24C
48C
E3P = 10.3 ± 1.1
E6P = 15.1 ± 2.0
% E = 61.0 ± 8.0
N = 20

Genetic components of the covariance were estimated by ML with the same coefficients used for variance component estimation (Table 2.1), and these are presented in Table 2.8. Of all the trait combinations in both crosses, only the analysis of E3P and E6P covariance in B73 x A1-6 produced a significant model. Shared zygotic maternal regulation was predicted in this analysis. The covariance between E3P and %E data displayed positive phenotypic correlation and coefficients of the covariance could be estimated, but they failed to fit the data ($p<0.01$; data not shown). Estimation of the components of the covariance for E6P and %E was unsuccessful due to a failure of the genetic models to fit the data, as above.

Selection for high endoreduplication individuals and experimental validation of heritability

One value of finding genetic variation in endoreduplication is that it allows the identification of kernel traits influenced by this process. Thus, we sought to introgress genes for high endoreduplication from Sg18 into B73 by a recurrent backcross procedure. For these crosses, pollen from B73 was used to fertilize B73 x Sg18 F1 ears and generate BC1 kernels. Ears were harvested at 19-DAP and, surface sterilized and the embryo and endosperm were dissected from individual kernels. Embryos were placed in germination medium while the corresponding endosperm was used for flow cytometric analysis. We processed 86 BC1 kernels by this method. Subsequently, ears from 11 BC1 plants were fertilized with B73 pollen to generate BC2 kernels. Table 2.9 is a summary of the endoreduplication data collected from the BC1 and BC2 endosperms.

Table 2.8. Covariance component analysis of B73 x popcorn crosses

		Coefficients				
Popcorn	Trait	$CoVa_{zm}$	$CoVa_p$	CoVe	p value	χ^2
Sg18	E3P/E6P	no significant models detected				
	E3P/%E	no significant models detected				
	E6P/%E	no significant models detected				
A1-6	E3P/E6P	3.7**+/-1.4		2.2**+/-0.5	0.52	6.13
	E3P/%E	no significant models detected				
	E6P%E	no significant models detected				

* significantly different than zero at $p<0.05$, ** significant at $p<0.01$

Table 2.9. B73 x Sg18 BC1 and mean BC2 values for endosperm endoreduplication traits

Plant	Family size	BC1 trait			Mean BC2 trait +/-S.D.		
		E3P	E6P	%E	E3P	E6P	%E
15	45	23.6	29.2	76.2	17.8 +/-3.7	21.2 +/-3.0	80.4 +/-8.8
18	19	21.1	25.0	82.1	18.2 +/-3.2	22.1 +/-3.0	79.4 +/-7.1
24	18	10.4	16.2	55.8	11.2 +/-1.1	15.9 +/-1.3	63.9 +/-5.0
26	20	16.3	20.8	74.5	17.7 +/-2.7	21.6 +/-3.1	79.3 +/-4.6
59	20	16.7	22.4	70.6	19.2 +/-2.9	22.3 +/-2.7	83.8 +/-6.0
67	18	16.3	22.4	68.5	14.4 +/-1.8	18.0 +/-1.4	75.7 +/-6.0
73	9	11.8	16.5	65.3	12.5 +/-2.0	17.2 +/-1.6	66.1 +/-7.2
77	25	16.3	21.5	72.0	15.4 +/-2.3	19.0 +/-2.5	77.3 +/-3.7
79	15	16.0	21.1	71.8	15.4 +/-1.8	19.1 +/-1.8	76.9 +/-4.7
82	20	17.4	25.4	58.8	16.5 +/-2.7	20.3 +/-3.1	77.5 +/-4.6
83	23	9.0	16.6	44.3	15.2 +/-2.0	20.7 +/-1.5	68.4 +/-7.4

Regression analysis was used to determine if the endoreduplication characteristics of BC1 endosperms were accurate predictors of BC2 endoreduplication traits. The slope of the regression lines from these analyses are proportional to the narrow-sense heritability values (Falconer and Mackay, 1996). However the relationship between the slope and the heritability is dependent on the mode of inheritance and crossing design (see Materials and Methods). The correlation, slope, and corresponding p values and standard errors calculated from these data are presented in Table 2.10, along with the heritability expected in such backcross progeny based on the variance components presented in Table 2.5. Heritabilities calculated for maternal zygotic inheritance from the backcross progeny data are well within the standard errors of those calculated for the 3^2 experiment. A high phenotypic correlation was observed for all three variables between the BC1 and BC2 generations. However, attempts to calculate the genetic correlation were unsuccessful and resulted in estimates greater than one (data not shown). This is expected for samples of this size (Lynch and Walsh, 1998), and may be exacerbated by the shared environment within ears.

Discussion

Two previous studies implicated maternal effects in the control of endoreduplication in maize endosperm. A difference in the mean ploidy (E3P) of nuclei in the central endosperm of Illinois High Protein and Illinois Low Protein maize was observed following Feulgen staining (Cavallini et al., 1995). When the E3P values were compared in reciprocal crosses, the F1 progeny were indistinguishable from the maternal parent. However, we found it difficult to determine how mean ploidies were reliably

Table 2.10. Heritability of endosperm endoreduplication traits in B73 x Sg18

	Offspring-parent			3^2 design
Trait	Slope	Std. E	p value	h^2a_{zm}
E3P	0.409	0.13	0.014	0.579
E6P	0.300	0.14	0.064	0.557
%E	0.412	0.14	0.018	0.222

calculated on the basis of the data presented in the histograms (Cavallini *et al.*, 1995; Fig 1). In another study, flow cytometry was used to evaluate endoreduplication in a variety of maize inbreds and their reciprocal crosses, F2, and F3 generations (Kowles et al., 1997). In this case, differences were observed in flow cytometric histogram peak asymmetry from endosperms of different inbred backgrounds. These peak asymmetries appeared more like the maternal than the paternal parent in 86% of the crosses analyzed, suggesting maternal control of this phenotype. When the variance of endosperm E3P values was compared between generations, the parental, F1, and F2 generations had similar variance, while the variance of the F3 generation was four-fold higher. The authors discussed the results of crosses in which maternal or paternal control of endosperm mean ploidy were observed, but the data were not presented (Kowles et al., 1997). The statistical method employed was an F-test for variance equivalence between the variances of generations, rather than the test of a genetic model. In both the Cavallini *et al.* (1995) and Kowles *et al.* (1997) studies, the crossing design of the experiments did not permit a thorough analysis of the inheritance of endoreduplication traits.

In this study, we set out to measure the genetic contribution to variation in endoreduplication between maize genotypes. Our approach differed from previously described studies of endoreduplication in several ways. First, we labeled nuclei with a more DNA-specific fluorochrome, and we did not fix tissue samples prior to nuclear isolation and analysis. This might explain why we did not observe genotype-dependent peak shapes in flow cytometric histograms (Kowles et al., 1997). Rather, our flow cytometric data showed a normal distribution of fluorescence intensity around peak

means in all genotypes. Second, we did not attempt to measure endoreduplication in selected regions of the kernel. Perhaps this is why we did not observe dramatic differences in E3P values between normal and *o2* endosperms in the B37, B73, Oh545, Va99, or W64A inbreds (data not shown), as was observed by microphotometry of the central endosperm cells (Kowles and Phillips, 1985). Third, three traits were calculated from each flow cytometric histogram to more accurately describe endoreduplication. Despite the differences between our data and those previously reported, measurements of E3P in dent inbreds by flow cytometry were similar (Kowles et al., 1990; Schweizer et al., 1995; Kowles et al., 1997).

The three endoreduplication traits (E3P, E6P, and %E) calculated from the flow cytometric data address different aspects of endoreduplication. Previous studies typically reported the mean ploidy (E3P) of the endosperm, which is a combined measure of the frequency of endoreduplicated cells in a population of cells and the average number of DNA re-replication cycles in these cells. We propose that the mechanisms for initiation and reiteration of the endoreduplication cell cycle need not share the same regulatory components. Indeed, when a covariance component analysis was performed on E6P and %E data, no significant relationship was detected (Table 2.8). The mean ploidy of nuclei greater than 3 C, the E6P value, is perhaps the most accurate single estimate of the number of rounds of DNA re-replication. Although 6 C nuclei are typically considered in G2, we included 6 C nuclei in our calculations of %E and E6P. Previous observations of a negligible mitotic index by 14-DAP (Kowles and Phillips, 1985) suggest that the vast majority of 19-DAP 6 C nuclei are not poised to divide.

We observed differences in endoreduplication between inbred lines as early as 13-DAP, and routine flow cytometric analysis of nuclei from single endosperms was possible between 13- and 19-DAP (Figs. 2.2-2.6). In some cases, we were able to assay endoreduplication at later stages of development, and the results suggested this process continues beyond 19-DAP (cf. Fig. 2.1 and Figs. 2.2-2.6). The interpretation of data regarding ploidy level after 20-DAP is complicated by the fact that cells in the central starchy endosperm begin to undergo cell death at this time (Young et al., 1997; Young and Gallie, 2000). We do not know the relationship between endoreduplication traits at 19-DAP and the final levels of endoreduplication. There is also no clear relationship between endoreduplication at 19-DAP and mature kernel phenotypes, such as starch and protein content. However, we selected 19-DAP as the best developmental stage for large-scale analyses of endoreduplication, because, among the possible stages for measurement, it should be closest to the final level of endoreduplication.

Modes of inheritance

In this study, we attempted to partition maternal genetic contributions into sporophytic and gametophytic components via quantitative genetics. A number of published plant genetic studies have utilized maternal genetic components without considering zygotic parent-of-origin-specific effects. Up to this point, most studies of zygotic parent-of-origin-specific inheritance relied on embryo lethal phenotypes or molecular markers (e.g., differential mRNA accumulation or DNA methylation). Quantitative genetic experiments, such as the one described in this work, allow for the detection of zygotic parent-of-origin inheritance, if such a mechanism is operating on the

measured trait. This could benefit the study of gametophyte-regulated events and parental imprinting during seed development. The study of non-lethal phenotypes affected by uni-parental gene expression may be more informative about the traits influencing seed development, and under selection, in the natural and cultivated history of maize (see below).

The analyses presented here strongly suggest maternal genetic control of endoreduplication in maize endosperm as a general phenomenon. Robust results were obtained for maternal influence on endoreduplication levels in the B73 x Mo17, B73 x Sg18 and B73 x A1-6 crosses (Tables 2.3-2.7). Variance component analyses did not detect any significant genetic components for B73 x Mo17, a result consistent with sporophytic or cytoplasmic inheritance of endoreduplication traits. Comparison of variances from parental and F1 generations by F-tests demonstrated significant differences. Theoretically, the variances of these four generations should be equivalent and reflect the deviation in measurements caused by error and the environment. The simplest explanation for these results is a differential sensitivity to the environment for these two genotypes. While an intriguing suggestion, the design of our experiments did not allow for a more thorough examination of genotype by environment interactions for this cross. Nonetheless, the failure to detect segregational or "break-out" variance in the F2 and BC1 generations is unlikely to be due to a masking effect by the genotype by environment interaction effect. Mean component analyses in this cross of the three endoreduplication traits detected additive maternal effects (Table 2.4) and t-tests between generations were consistent with maternal control. These results suggest that the genes

affecting variation in E3P, E6P, and %E traits in B73 x Mo17 are expressed from the ear parent. As B73 and Mo17 are midwestern dent-type inbreds, these results are consistent with previous conclusions about maternal sporophytic control made by Kowles and colleagues (1997) from crosses between multiple midwestern dent varieties. However, our crossing design is not optimal for the detection of cytoplasmic influence and cytoplasmic effects may be important maternal components influencing endoreduplication trait mean values. In contrast to these results, significant genetic components of the variance were detected in both B73 x A1-6 and B73 x Sg18 crosses. In both crosses, maternal zygotic components were significant in models describing E3P and E6P variances (Table 2.5). Mean component analyses detected significant maternal additive effects, consistent with zygotic maternal effects (Tables 2.6 and 2.7). In the popcorn analyses, additional maternal sporophytic effects cannot be ruled out, and sporophytic maternal effects may influence the magnitude of the maternal additive mean components. In the B73 x Sg18 mean component analyses, the addition of a maternal dominance coefficient, which is specific to maternal sporophytic inheritance, improved the fit of the mean component model to the data, but was significant only for E6P data.

Despite the narrow germplasm base of popcorns (Senior et al., 1998), no one model of inheritance was able to explain the variation in endoreduplication between popcorns and B73 (Tables 2.5-2.7). Indeed, there was no detectable genetic variability between Kp58k and B73 at 19-DAP for any of the traits analyzed. Additionally, the inclusion of a paternal zygotic component improved the fit for E6P data for the B73 x Sg18 and B7s x A1-6 experiments. The paternal additive coefficient was not significant

in the variance component analyses but was in the mean component analysis of B73 x Sg18 (Tables 2.5 and 2.6). As noted previously, it is impossible to distinguish between a maternal and paternal zygotic model, and a maternal zygotic and diploid additive model in this crossing design. An experiment in which individual loci, and not the mass action of multiple independent loci, are taken into account (e.g., QTL mapping) is one method to distinguish between maternal effects with paternal parent-of-origin or additive diploid effects in these crosses.

The presence of additive zygotic genetic effects influencing endoreduplication traits was partially confirmed by offspring-parent regression in B73 x Sg18. Significant regressions were returned for both E3P and %E, but not E6P (Table 2.10). Design of the offspring-parent experiment excluded paternally expressed genes, which were detected for E6P. The relationship of the slope of the regression line (b) to the heritability (h^2) in this experiment is difficult to judge. If zygotic maternal expression is the only mode of inheritance, then the slope and the h^2 value are the same. However, if multiple modes are operating simultaneously, the relationship between the two numbers must be arrived at experimentally. Moreover, if any genes influence both sporophytic and zygotic maternal effects, our estimate of h^2 will be upwardly biased.

Conclusions regarding the %E values for the crosses analyzed in this study are the most problematic. Mo17, which has a relatively larger kernel and less endoreduplication than B73, was the only inbred in which Student's t-tests on %E values were significantly different from those of B73. One possible explanation for this is that a delay in the onset of endoreduplication in B73 x Mo17, with no change in endocycle rate, was measured at

19-DAP. The %E of inbreds A1-6 and Sg18, which have smaller kernels and greater levels of endoreduplication than B73, were not different from B73 at 19-DAP by t-test, nor were any significant genetic components detected. If cell ploidy and size are correlated, this suggests that cell number is dramatically reduced in these popcorns as compared to Mo17, B73, and Kp58k. Unlike the other two traits, the *Ve* components of the %E variance component analyses were by far the largest, suggesting that the majority of the variation in %E cannot be accounted for in this experimental design. In contrast, the estimate of h^2 obtained in the offspring-parent regression analysis of B73 x Sg18 was the highest of the three traits (Table 2.10).

The results of this study neither demonstrate nor detract from the hypothesis that the phase-change from a mitotic to an endoreduplication cell cycle and the iteration of endoreduplication cycles up to 19-DAP share regulatory genetic mechanisms. In all crosses, the %E and E6P measurements of parental inbreds showed positive correlation (data not shown). The co-linearity of %E and E6P in inbred lines suggests environmental variation might similarly influence both traits. However, of the four trait/cross combinations with significant genetic variance components, only the covariance of one combination was described by a significant model (Table 2.8). Additionally, covariance analysis was not possible by offspring-parent regression. Non-genetic maternal co-regulation of these traits, causing sibling co-variance, is a reasonable explanation for the failures of both of these tests, given that multiple kernels were sampled from fewer ears. Thus, these covariance analyses are inconclusive and suggest that while E3P, E6P, and

%E are correlated characters, the nature of their relationships and the presence or absence of genetic and environmental co-regulation remain uncertain.

Relationship between endoreduplication and kernel growth

The detection of zygotic maternal control of endoreduplication could be the result of either gametophytically expressed genes exerting an effect on endosperm development, or imprinting of genes expressed in the developing endosperm. None of the results obtained from this study are able to distinguish between these two mechanisms; however, previous data suggest that phenomena linked to genetic imprinting are operating in maize endosperm and are related to endoreduplication. Interploidy crosses in which a paternal excess is delivered to the endosperm produce a normal phenotype until the endoreduplication phase of development. Defects in endosperm cell differentiation and storage product accumulation then become evident (Charlton et al., 1995). The popcorn inbreds Sg18 and A1-6, but not the lesser endoreduplicated Kp58k, are highly fecund ear parents for maize x *Tripsacum dactyloides* crosses (Kindiger and Beckett, 1992). If the presence and dosage of imprinted factors explains endosperm-based fertility barriers in Angiosperms (Birchler, 1993), then increased fecundity and endoreduplication can be explained by an alteration in the effective dose of parent-of-origin effect genes. Most models for the establishment and maintenance of genetic imprinting postulate that it is a mechanism to alter the effective dose of genes affecting fitness (Hurst and McVean, 1998). As imprinting is a stable evolutionary strategy, traits displaying this property need not currently contribute to fitness or be under selection. Thus, endoreduplication could have been selected before

or after the domestication of maize. Indeed, teosinte endosperm undergoes endoreduplication (Dilkes unpublished results).

Endosperm has a well- established role in providing nutrition to the developing embryo and seedling. If early endoreduplication in this tissue serves to fix endosperm cell number, this process could influence resource management. Consonant with this possibility, the alleles responsible for an increase in endoreduplication (by 19 DAP) were acquired from the parent with the smaller kernel (Tables 2.4, 2.6, and 2.7). Likewise, if the number of chromatids possessing a copy of a gene, and not the number of cells possessing the gene, determines expression level, iterations of endoreduplication might be expected to increase metabolic output. Indeed, endoreduplication in maize endosperm is associated with an increase in chromosomal HMG proteins, which are thought to influence activation of storage protein gene transcription (Zhao and Grafi, 2000). In this light, it is interesting that paternal effects were estimable for E6P but not for the other traits.

CHAPTER 3

REPA EXPRESSION IS SUFFICIENT FOR PLANT CELL CYCLE ACTIVATION AND RECURRENT S-PHASES, BUT ALTERS GENE EXPRESSION AND NOT ENDOREDUPLICATION IN MAIZE ENDOSPERM

Introduction

Endoreduplication, reiterated rounds of DNA synthesis without mitosis, is a widespread phenomenon in Angiosperms, where it affects an estimated 90% of extant taxa (D'Amato, 1984). This novel cell cycle is frequently associated with rapidly growing, highly determined tissues, such as the endosperm, cotyledons, and suspensor of developing seeds (D'Amato, 1984). In maize endosperm, cells cease mitosis and begin cycles of endoreduplication around nine days after pollination (DAP), concomitant with a transition in gene expression, especially those genes encoding storage proteins and starch biosynthetic enzymes. These changes in gene expression are dramatic, and the levels of specific mRNA transcripts can be extremely high (Marks et al., 1985; Song et al., 2001; Woo et al., 2001; Hunter et al., 2002). For example, 22-kDa α-zein mRNAs (Song et al., 2001) are not detectable early in endosperm development (before 9-DAP), but single genes can account for 2.5-5% of total transcripts by 18-DAP (Marks et al., 1985; Woo et al., 2001; Hunter et al., 2002). It is possible this high level of gene expression is directly related to the process of endoreduplication.

The switch from a mitotic to an endoreduplication cell cycle in maize endosperm is temporally associated with the loss of mitotic cyclins, activation of S-phase CDK, an increase in cyclin-dependent kinase inhibitor activity, and phosphorylation of the maize retinoblastoma-related proteins (RRBs) (Grafi and Larkins, 1995; Grafi et al., 1996). However, the mechanism by which this modulation of CDK activity creates the endoreduplication cell cycle is poorly understood (Larkins et al., 2001), and the potential role of RRBs in the endoreduplication of maize endosperm has not been thoroughly investigated.

Studies in other eukaryotes have shown that RRBs' effects on gene expression are complex, but typically the protein is a repressor (Markey et al., 2002). This activity is mediated by RRB's association with multi-protein complexes containing modulators of chromatin architecture, including the Snf2-type DNA helicases (e.g. Brg1 and hBrm), DNA and histone methyltransferases, and histone deacetylases (Brehm et al., 1998; Ferreira et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998; Nielsen et al., 2001). These associations are necessary for RRB function, as helicase knockouts fail to undergo cell cycle arrest when RRBs are ectopically expressed (Dunaief et al., 1994; Strobeck et al., 2000; Strobeck et al., 2002). In addition, RRBs affect non-cell cycle-regulated gene expression and promote cellular differentiation independent of their cell cycle effects (Lu and Horvitz, 1998; Sellers et al., 1998; Hsieh et al., 1999; Charles et al., 2001).

RRB repression of cell cycle-regulated gene expression is alleviated through the action of a number of proteins. In the standard cell cycle, RRB is inactivated via phosphorylation by cyclin-dependant kinases (Harbour et al., 1999; Zhang et al., 2000).

As a consequence, transcription factors such as E2F activate repressed promoters by reversing the chromatin modifications effected by RRB and recruiting RNA polymerase. In plants, over-expression of RRB-interacting cyclin-D shortens the duration of the cell cycle and can lead to cytokinin-independent growth of cultured cells and organ hypertrophy (Doerner et al., 1996; Riou-Khamlichi et al., 1999; Cockcroft et al., 2000). Likewise, ectopic co-expression of E2F and its partner, Dp1, leads to increased cell division, presumably by bypassing the need for RRB inactivation (De Veylder et al., 2002; Rossignol et al., 2002).

In animal cells, RRBs regulate more cellular phenomena than just the mitotic cell cycle (Lu and Horvitz, 1998; Sellers et al., 1998; Hsieh et al., 1999; Charles et al., 2001). In *C. elegans*, for instance, RRB is required for the repression of tandem repeat gene arrays and is crucial for vulval development (Lu and Horvitz, 1998; Hsieh et al., 1999). In cultured mammalian cells, RRB inhibition by genetic ablation or expression of a viral LxCxE-containing protein, together with the constitutive expression of cyclin-dependent kinase inhibitors, are sufficient to induce recurrent S-phases without intervening mitoses, i.e. endoreduplication (Niculescu et al., 1998). RRBs are also important for the regulation of non-cell cycle genes.

By analogy to animal systems, plant RRBs may function as regulators of endoreduplication. If phosphorylation of RRB in maize endosperm is important for S-phase progression (Grafi et al., 1996), then RRB should be inactivated during endoreduplication. Furthermore, if RRBs modulate gene expression in a phosphorylation-independent manner, as seen for some differentiation-associated

functions of RRBs in animals (Harbour et al., 1999; Zhang et al., 2000; Hansen et al., 2001; Pradhan and Kim, 2002), their regulation could alter endosperm development and maturation.

In this study we sought to influence endoreduplication in maize endosperm by altering the RRB pathway with the wheat dwarf virus (WDV) RepA protein. WDV is a monopartite geminivirus (Mastrevirus) (Hanley-Bowdoin et al., 2000). The RNA generated from its complementary strand is alternatively spliced to encode two proteins, Rep and RepA (Wright et al., 1997; Palmer and Rybicki, 1998; Gutierrez, 2000). RepA binds the long inverted repeat (LIR) promoter of WDV and is hypothesized to play a role in regulating viral gene transcription (Collin et al., 1996). Unlike Rep, RepA is dispensable for viral replication (Collin et al., 1996); however, RepA expression is sufficient to overcome a block in viral replication imposed by ectopic RRB expression (Xie et al., 1996) and is required for high levels of viral replication (Schalk et al., 1989).

During viral infection of mammalian cells, activation of S-phase genes and DNA synthesis are regulated by the expression of proteins containing the RRB-interacting motif, LxCxE (Moran, 1988; Chellappan et al., 1992; Ben-Israel and Kleinberger, 2002). Both the Rep and RepA proteins contain an LxCxE, but only RepA binds plant RRB proteins with high affinity (Horvath et al., 1998; Liu et al., 1999; Missich et al., 2000). Analogous to the expression of RRB-interacting viral proteins in animals (Moran, 1988; Chellappan et al., 1992; Ben-Israel and Kleinberger, 2002), RepA expression enhances transformation rate and growth in transgenic maize callus (Gordon-Kamm et al., 2002).

We hypothesized that if RRB is acting to enforce G1 in endoreduplicating maize endosperm, then ectopic expression of RepA during the period of endoreduplication would stimulate S-phase, leading to higher ploidy levels. However, we found differential effects of RepA expression on endoreduplication in cultured somatic and differentiated starchy endosperm cells. Transient expression of RepA in tobacco BY-2 cells lead to greater cell division within 24h, and this stimulation of the cell cycle could be opposed by ZmRb1 expression. This demonstrated cell cycle effects for both of these proteins. Flow cytometric analysis of transgenic maize calli stably expressing RepA showed that the protein is sufficient to induce endoreduplication. However, endosperm cells expressing RepA revealed no evidence of increased endopolyploidy by 18-DAP. Starchy endosperm cells expressing RepA manifested alterations in protein synthesis by 18-DAP that affected starch and storage protein accumulation, as well as proteins regulating maturation. At maturity, the phenotype of these kernels was abnormal, exhibiting an opaque, wrinkled appearance. Based on affinity chromatography experiments, proteins associated with RepA were found to reside in the nucleus and at least one of them belongs to the high mobility group (Hmg) protein family.

Results

RepA promotes, and ZmRB1 inhibits, cell division in tobacco BY-2 cells

A transfection-based assay for cell cycle stimulation and repression was generated using mitotically active tobacco BY-2 cell cultures. Particle bombardment was used to co-deliver a 35S:GFP construct and putative cell cycle regulatory genes. After 24h, GFP-

expressing foci were counted and scored as consisting of divided or single cells. When mitotically active cultures (4d post-subculture) were bombarded with 35S:GFP alone, 42% of the fluorescent foci consisted of divided pairs (Table 3.1). Simultaneous delivery of 35S:RepA increased the proportion of divided pairs to 60%, and this increase was significant by Student's t-Test ($p<0.01$), demonstrating that 35S:RepA can stimulate the cell cycle in actively dividing cells.

If BY-2 cells are not subcultured continuously, they enter a stationary phase after approximately 7d in culture. This is characterized by, no further growth, a low mitotic index, and a halt of the cell cycle in G0/G1 (Nagata et al., 1992; Planchais et al., 1997). Bombardments of 7 and 14d-old cell cultures with 35S:RepA resulted in significantly fewer divided pairs than 4d-old cultures (t-test, $p<0.01$). However co-bombardment of these cultures with 35S:RepA and 35S:GFP resulted in a significant increase of divided cells ($p<0.01$) and a greater-fold increase in the number of divided cells than that seen in 4d subcultures (Table 3.1). This suggests that 35S:RepA is able to overcome the G0/G1 block imposed on aging cell cultures.

Table 3.1. RepA-stimulates cell division in BY-2 cells.

Culture age	Treatment	%Divided (+/-SD)	n	Sig.
4d	Control	42.0 (5.8)	307	
	35S:RepA	59.8(12.1)	326	**
7d	Control	25.3 (5.8)	529	
	35S:RepA	50.4 (6.8)	569	**
14d	Control	19.9 (0.5)	450	
	35S:RepA	35.2 (5.3)	553	**

Significant difference (Sig.) determined by t-Test with '**'. denoting p values < 0.01. All cells, control and 35S:RepA treatments, were bombarded with 35S:GFP.

RepA is hypothesized to act on the plant cell cycle via the disruption of the RRB cell cycle inhibitor family. However, plant RRB proteins are purported to have cell cycle effects based solely on sequence similarity to animal cell cycle regulatory proteins. To test both these hypothesis co-bombardments were carried out with 35S:ZmRb1$^{341\text{-}866}$, encoding the A and B pocket and C-terminus of ZmRb1, and a mutant thereof which lacks the ability to bind LxCxE-containing proteins, ZmRb1^{C706G}. Bombardments of 4d old cultures with 35S:GFP or 35S:GFP and 35S:RepA returned similar results to those obtained in Table 3.1 (compare Tables 3.1 and 3.2). Co-delivery of 35S:GFP and 35S:ZmRb1, but not the point mutant 35S:ZmRb1^{C706G}, significantly inhibited the number of divided cells ($p<0.01$). Consistent with RepA and ZmRb1 acting on the same pathway, co-delivery of 35S:RepA and 35:ZmRb1$^{341\text{-}866}$ had no effect on the rate of cell division, relative to controls. As with the treatment of cells with ZmRb1^{C706G} alone, co-delivery of 35S:RepA and 35S:ZmRb1^{C706G} was indistinguishable from 35S:RepA treatments, further demonstrating the inability of this mutant to inhibit cell cycle progression. Thus RepA overcomes G1/G0 blocks on the cell cycle and opposes ZmRb1$^{341\text{-}866}$ cell cycle inhibition consistent with the hypothesis that RepA stimulates and RRB inhibits entry into S-phase.

Expression of RepA in maize callus induces endoreduplication

Transgenic maize calli were generated with two different constructs for the ectopic expression of RepA, one using the maize ubiquitin promoter and first intron (Ubi), and the other using the *Agrobacterium tumefaciens* nopaline synthase (Nos) promoter (Gordon-Kamm et al., 2002). All bombardments included a plasmid containing

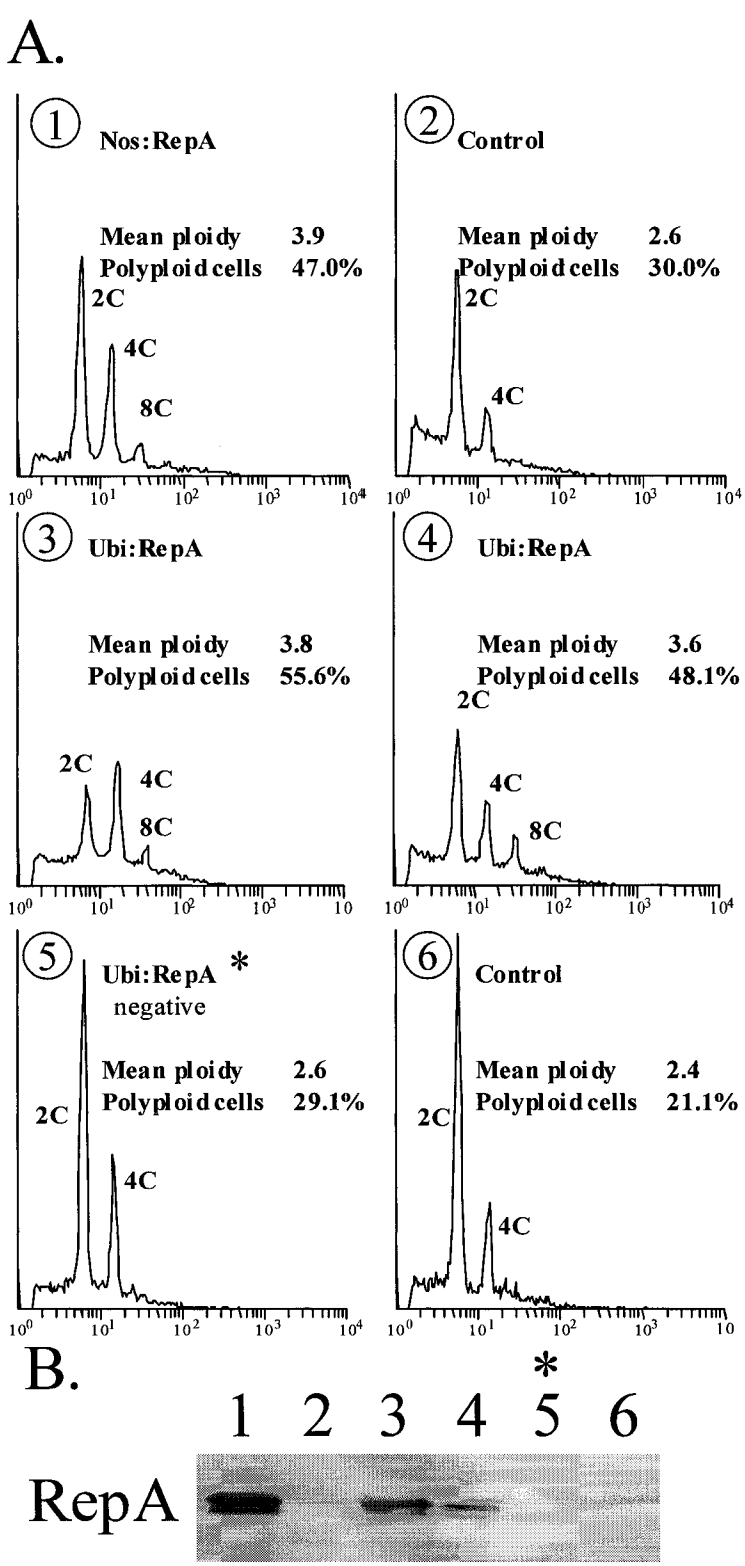
Table 3.2. ZmRb1 opposes RepA in BY-2 cells.

Treatment	% Divided (+/- SD)	n	Sig.
Control	37.6 (2.8)	1009	
35S:ZmRb1$^{341\text{-}866}$	15.9 (3.7)	252	**
35S:ZmRb1^{C706G}	37.7 (4.8)	372	n.s.
35S:RepA	54.3 (9.9)	408	**
35S:ZmRb1$^{341\text{-}866}$, 35S:RepA	37.5 (0.2)	304	n.s.
35S:ZmRb1^{C706G}, 35S:RepA	59.6 (5.6)	339	**

Significant difference (Sig.) determined by t-Test with '**' and n.s. denoting p values of < 0.01, and > 0.05, respectively. All cells, control and all treatments, were bombarded with 35S:GFP.

a 35S:BAR cassette for selection of transformants on BASTA or Bialaphos-containing medium. After four weeks of subculturing, herbicide resistant calli recovered from experiments with and without RepA expression cassettes were subjected to flow cytometric analysis for nuclear DNA content and immunoblot analysis for RepA protein accumulation. Examples of flow cytometric histograms and immunoblots are illustrated in Figure 3.1. Multiple calli representing independent embryos and transformation events for expression cassettes and controls were assayed. Nuclei from herbicide resistant controls had either 2C or 4C DNA content and displayed a flow cytometric phenotype typical of embryogenic calli (Fig. 3.1, and data not shown). Herbicide resistant calli from Ubi:RepA or Nos:RepA treatments had one of two phenotypes. A few calli displayed a phenotype reminiscent of controls, with a similar ploidy distribution (Fig. 3.1, c.f. control and Ubi:RepA*). The majority of Ubi:RepA and Nos:RepA- treated calli exhibited a mean ploidy level higher than the control, including the presence of 8C nuclei. Immunoblot analyses of proteins extracted from calli revealed that the absence of RepA protein from Ubi:RepA and Nos:RepA treated calli correlated with the control cytometric phenotype (e.g. UbiRepA*). Herbicide-resistant calli from Ubi:RepA and Nos:RepA treatments with 8C nuclei accumulated RepA protein, whereas herbicide resistant calli lacking the 8C peak, despite bombardment with the RepA construct, contained no or undetectable levels of RepA. Thus, it appears that in mitotic maize calli, the RepA protein was sufficient to induce endoreduplication.

Figure 3.1. Flow cytometric histograms of maize calli ectopically expressing the RepA gene. A. Histograms showing relative fluorescence intensity of DAPI-stained nuclei from maize calli following transformation with Nos:RepA, Ubi:RepA or control transgenes; the C-value indicates the number of genome copies. B. Immunoblot analysis of protein extracts from the same calli as in panel A showing RepA protein accumulation. The asterix in A and B mark a RepA-negative sample from the Ubi:RepA treatment.


A.
1
Nos:RepA
Mean ploidy 3.9
Polyploid cells 47.0%
2C
4C
8C
2
Control
Mean ploidy 2.6
Polyploid cells 30.0%
2C
4C
3
Ubi:RepA
Mean ploidy 3.8
Polyploid cells 55.6%
2C
4C
8C
4
Ubi:RepA
Mean ploidy 3.6
Polyploid cells 48.1%
2C
4C
8C
5
Ubi:RepA *
negative
Mean ploidy 2.6
Polyploid cells 29.1%
2C
4C
6
Control
Mean ploidy 2.4
Polyploid cells 21.1%
2C
4C
B.
*
1 2 3 4 5 6
RepA

Expression of RepA in maize endosperm does not alter the level of endoreduplication by 18 DAP

The observation of endoreduplication in maize calli expressing RepA suggested that this protein might increase endoreduplication in endosperm tissue. To test this hypothesis, transgenic maize plants were created in which RepA expression was directed by the endosperm-specific rice GluB1 promoter and maize 27-kDa γ-zein terminator (Coleman et al., 1997; Russell and Fromm, 1997). This promoter directs endosperm-specific gene expression beginning around 10-DAP, coincident with the on-set of endoreduplication (Russell and Fromm, 1997). Developing embryos were bombarded with GluB1:RepA, along with a BAR cassette, and selected on bialaphos-containing media. Seven herbicide resistant plants (T0 generation) were regenerated from independent transformation events and crossed with pollen from the B73 inbred to generate T1 individuals. DNA was isolated from the leaves of T0 and T1 plants, and the presence of the GluB1:RepA transgene was analyzed by PCR and DNA blot hybridization. PCR with primers complementary to the GluB1:RepA transgene produced a DNA fragment of the predicted size (800 bp) in four of the seven herbicide resistant lines, which were designated 11, 14, 26, and 28. No bands of this size were seen in reactions with DNA from control (BAR only) or untransformed plants (data not shown). Integration of the RepA gene into the maize genome was confirmed by DNA blot hybridization (Fig. 3.2), which demonstrated a different number of coding sequences in the four transformants. When nuclei from leaf tissue of these plants were analyzed by

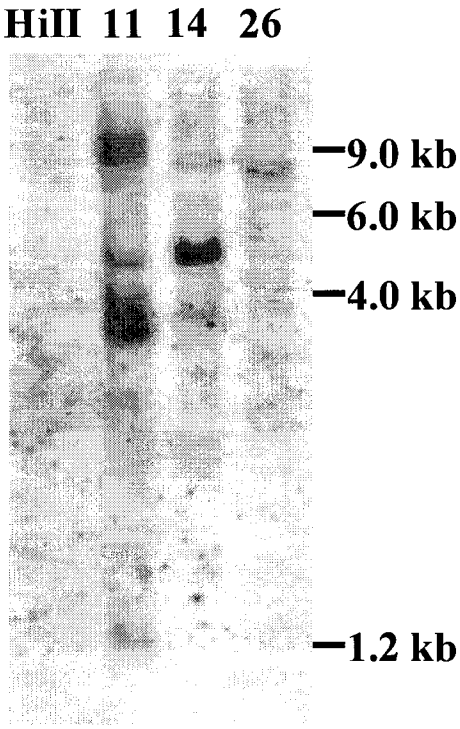


Figure 3.2. DNA blot analysis of Glub1 :RepA transgenic plants. Genomic DNA was digested with *NcoI,* separated by 0.8% agarose gel electrophoresis, and hybridized with a 32P -labeled RepA probe. Lanes contain DNA from wild-type Hi II and GluB1 :RepA transformants 11, 14, and 26. The molecular weight of size markers is indicated on the right.

flow cytometry, transformant 28 was found to be a tetraploid, and it was excluded from further analyses.

T2 kernels obtained after fertilization of B73 ears with pollen from hemizygous T1 plants were harvested at 18-DAP and analyzed by flow cytometry. Remaining extracts, unused in the flow-cytometric analysis, were processed for immunoblotting with anti-RepA antiserum. Endosperm-derived protein extracts from ears segregating for events 14 and 26 tested positive for RepA (Fig. 3.3B) at a ratio of approximately 1:1 Wt:RepA$^+$ (chi-squared $p>0.05$), as expected for a single integration locus. Endosperms from transgenic line 11 did not accumulate sufficient RepA to allow detection by this method (data not shown), but the transgene was expressed and segregated approximately 1:1 (chi-squared $p>0.05$; see below). The C-value of individual endosperms was calculated from the flow cytometric histograms, and the mean ploidy of wild-type and RepA-accumulating endosperms were calculated. A summary of these data is presented in Table 3.3. Mean ploidy of wild-type and RepA-expressing kernels were indistinguishable by Student's t-test (Table 3.3, $p>0.05$; Student 1907), and were similar to those obtained for the B73 inbred (Dilkes et al., 2002) and Hi-II hybrid (data not shown) at similar developmental stages. Thus, expression of RepA in maize endosperm did not alter endopolyploidy by 18-DAP.

Accumulation of RepA leads to a reduction in storage protein accumulation

Mature kernels from transformants 14 and 26 were small and wrinkled, with a chalky, opaque endosperm (Fig. 3.4A and B). However, endosperm size and shape at 18-DAP were not visibly affected by the expression of the GluB1:RepA transgene (Fig.

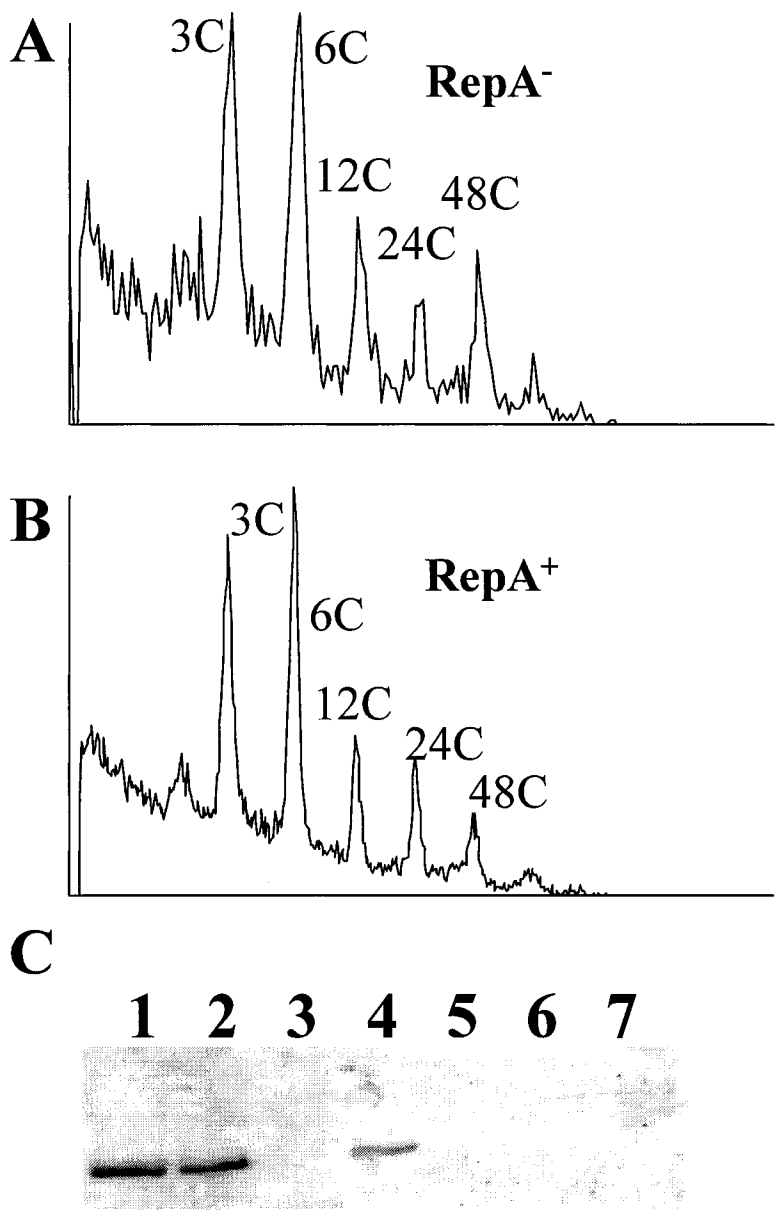
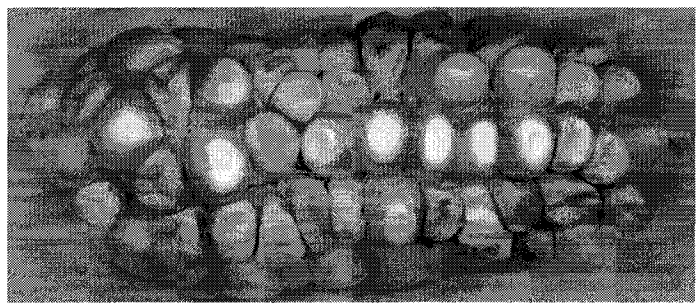


Figure 3.3. Sample flow cytometric histograms and immunoblot analysis of GluB1:RepA transgenic and wild-type sibling endosperms from event 26. Isolated nuclei from single 18-DAP endosperms were treated with DAPI and subjected to flow cytometric analysis (A and B); the remaining lysate was used for SDS-PAGE and immunoblotting with RepA antiserum. Panel C shows a representative immunoblot illustrating transgenic kernels expressing and not expressing RepA.

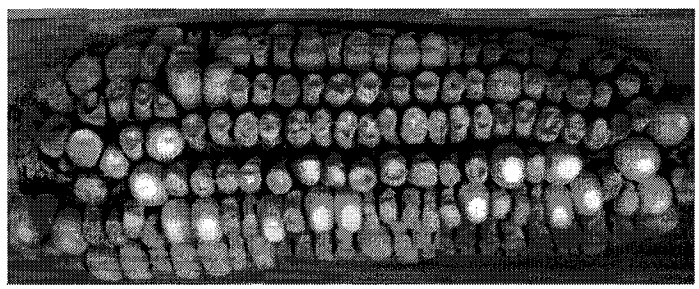
Table 3.3. Effect of RepA accumulation on endosperm endopolyploidy

Event	RepA	Endosperm ploidy +/- SD
14-5	-	10.6 +/- 3.5
	+	9.7 +/- 2.0
26-3	-	9.2 +/- 2.5
	+	11.3 +/- 3.5

A.



B.



C.

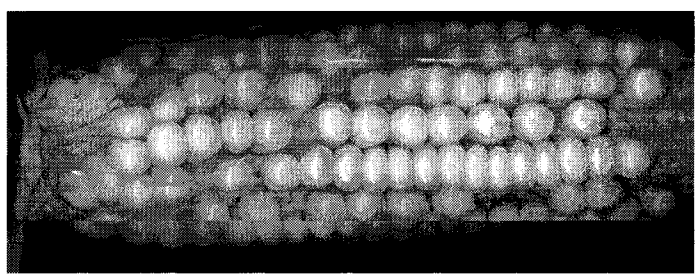


Figure 3.4. Phenotypes of GluB1:RepA transgenic kernels. Photographs of T2 ears hemizygous for GluB1:RepA event 26 (A) and event 14(B) at maturity and event 26 at 18 DAP (C).

3.4C). The lowest expressing transgenic line, line 11, did not manifest this phenotype, but it did produce some ears segregating for opaque kernels (not shown). This phenotype was inherited over multiple generations and only occurred in kernels developed from crosses with GluB1:RepA-positive transgenic plants, and not wild-type siblings (data not shown).

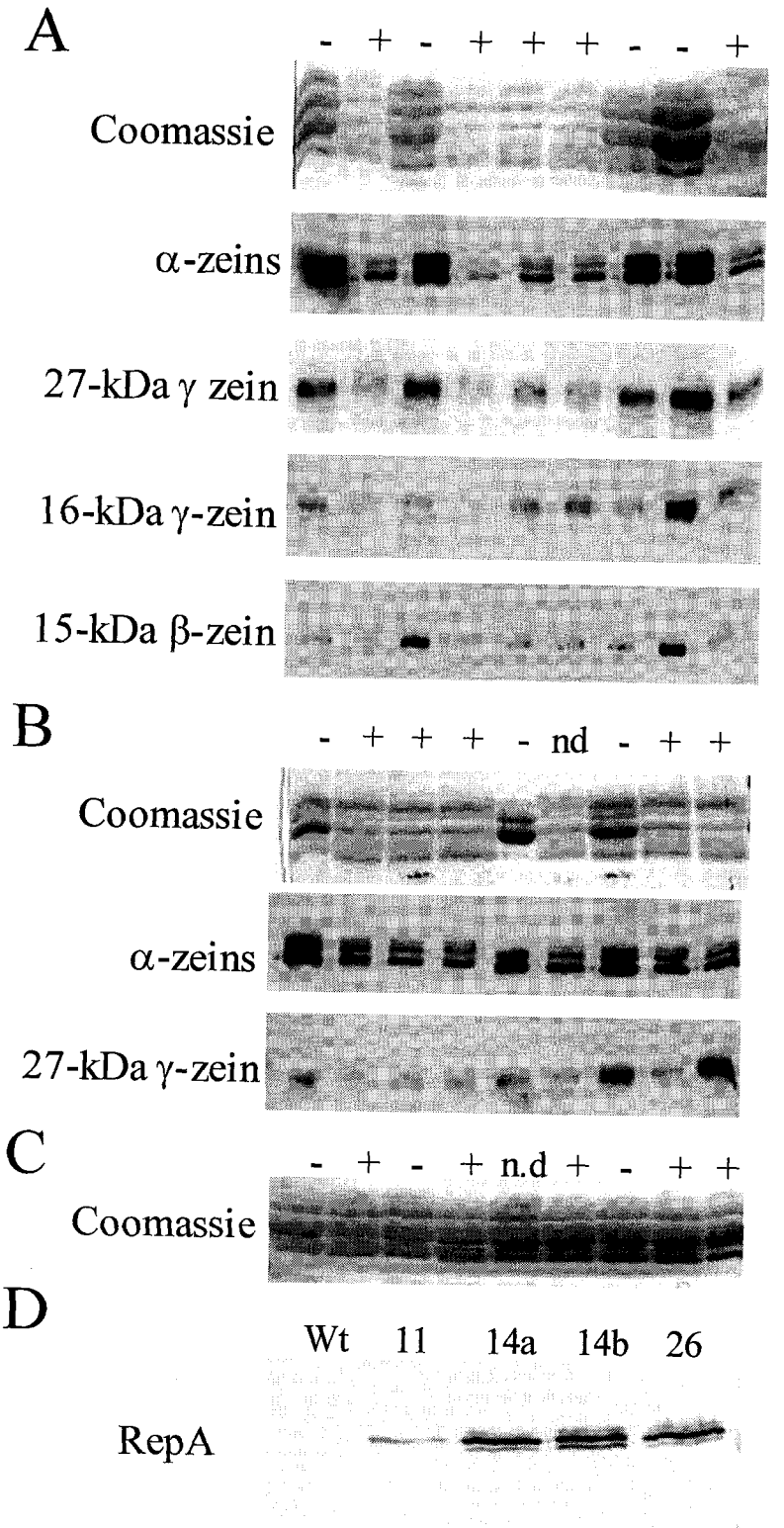
Transgenic and wild-type kernels were harvested from mature hemizygous ears of lines 14 and 26 and weighed; a summary of these data is presented in Table 3.4. Seed weight was reduced to 33% and 36% of wild-type in transformants 14 and 26, respectively. As the kernel is approximately 90% starch by weight, these reductions must be primarily due to less starch synthesis. Observations made during the flow cytometric analysis were consistent with this hypothesis. Extracts from 18-DAP endosperms of event 14 and 26 kernels, later determined to be RepA positive, contained less solid material, i.e. were less milky than wild-type siblings.

Maize mutants with an opaque endosperm are often associated with a reduction in zein storage protein synthesis. To investigate whether or not this was true of the GluB1:RepA-expressing kernels, endosperm proteins from segregating ears were separated into zein and non-zein fractions (Wallace et al., 1990). Following SDS-PAGE, zeins were detected either by Coomassie Blue staining or immunoblot analyses with zein antibodies. The non-zein proteins were analyzed by immunoblotting analysis with RepA antisera to identify transgenic endosperms. Photographs of gels and blots from these analyses are presented in Figure 3.5. Relative to wild-type siblings, there was a general reduction in zein contents in the high GluB1:RepA-expressing transformants. Gel

Table 3.4. Weight of mature GluB1:RepA kernels

Event	Generation	RepA	mean(STD)	N	Sig
26	BC2	+	115.3 (22.9)	33	**
		-	314.8 (38.5)	31	
14	BC2	+	57.2 (10.0)	15	**
		-	173.7 (18.0)	15	
26	BC3	+	86.8 (29.1)	46	**
		-	239.3 (24.7)	45	

Figure 3.5. Analysis of protein accumulation in 18-DAP GluB1:RepA and wild-type sibling endosperms. Coomassie Blue staining and immunodetection of zein proteins in transgenic and non-transgenic sibling endosperms from events 14 (A), 26 (B) and 11 (C). The "+" and "-" in A, B, and C indicate zein extracts from GluB1:RepA and wild-type endosperms, respectively. In one endosperm from events 11 and 14, non-zein extraction failed and RepA accumulation was not determined (marked as n.d.). A representative immunoblot displaying the relative accumulation of RepA protein in the non-zein fractions of wild-type, and hemizygous endosperms of event 11 , two lineages of event 14, and event 26 is shown in panel D.


A
- + - + + + - - +
Coomassie
α-zeins
27-kDa γ zein
16-kDa γ-zein
15-kDa β-zein
B
- + + + - nd - + +
Coomassie
α-zeins
27-kDa γ-zein
C
- + - + n.d + - + +
Coomassie
D
Wt 11 14a 14b 26
RepA

scanning revealed a greater reduction in α-zeins, (migrating approximately 22- to 17-kDa, Fig. 3.5a) than 27- and 16-kDa γ- and 15-kDa β-zeins, suggesting a differential effect on storage protein accumulation (Fig. 3.5 A and B, and data not shown). Immunoblotting with zein-specific antisera showed endosperms from the highest expressing transformant, 14, had a reduced accumulation of 22- and 19-kDa α-zeins, 27-kDa and 16-kDa γ-zeins, and a slight reduction in β-zein accumulation (Fig. 3.5A). Line 26 endosperm extracts also had a reduced accumulation of 22-kDa α- and 27-kDa γ-zein proteins (Fig. 3.5B). There was no consistent difference in accumulation of either the 16-kDa γ- or the 15-kDa β-zein in event 26 kernels (data not shown). Endosperm extracts of event 11, the lowest RepA-expressing line, did not display dramatic changes in zein protein accumulation as assessed by Coomassie Blue staining or immunoblotting (Fig. 3.5C and data not shown).

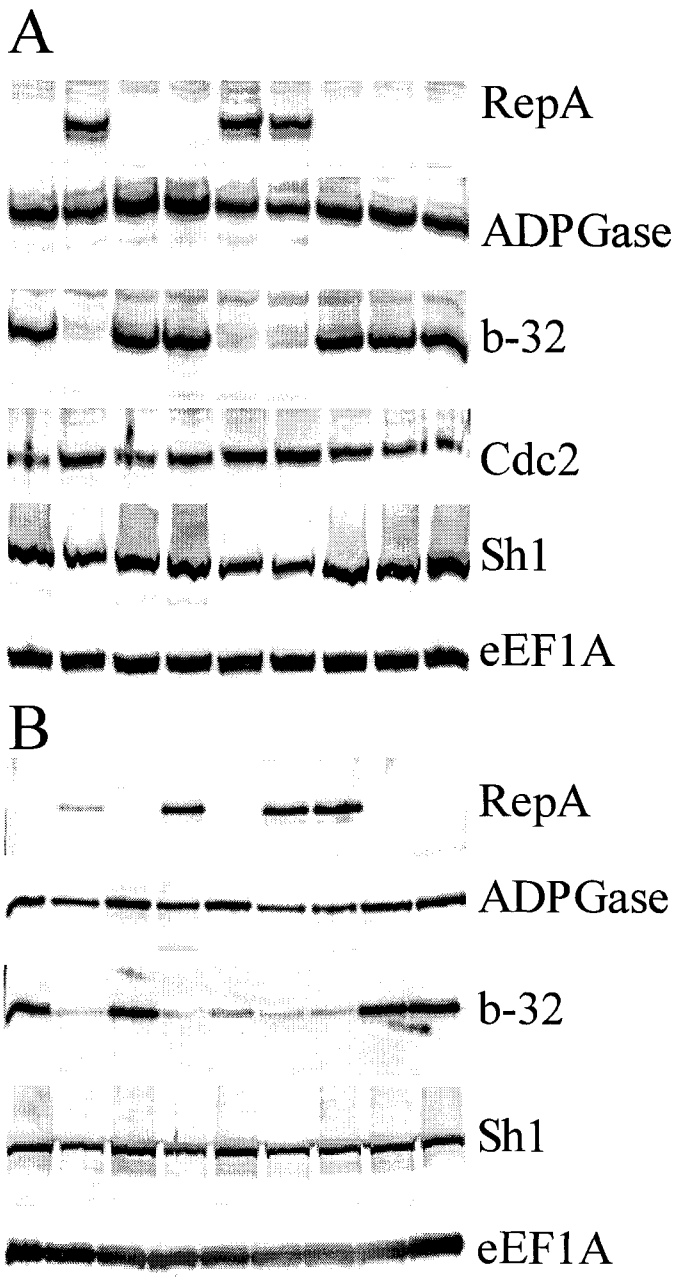
Immunoblot analysis of the non-zein protein fraction from whole kernels was more sensitive than when the flow cytometry extracts were used (c.f. Fig. 3.2B and Fig. 3.5D). RepA protein was detected in approximately half of the T2 endosperms from transformants 11, 14 and 26 (Fig. 3.5D; chi-square, $p>0.05$). As illustrated in Figure 3.5D, the accumulation of RepA was highest in transformant 14, substantially lower in transformant 11, with transformant 26 endosperms accumulating an intermediate amount. Thus, the level of RepA accumulation was related to the reduction in zein proteins as well as the severity of the seed phenotype.

RepA transgenic endosperms are altered in the accumulation of o2-responsive and starch biosynthesis-associated proteins

The decrease in storage protein synthesis could be related to defects in particular transcriptional programs, and it does not explain the decrease in starch accumulation. Therefore, we analyzed the synthesis of a variety of other endosperm proteins. SDS-PAGE separations of non-zein proteins from 18-DAP endosperms of transformants 14 and 26 were analyzed by immunoblotting (Fig. 3.6A and B) with antisera against ADP-glucose pyrophosphorylase (ADPGase), sucrose synthase (the *Shrunken1* gene product) and b-32, an Opaque2 (O2)-regulated gene product encoding a ribosome inactivating protein or RIP (Bass et al., 1992). Accumulation of all three proteins was reduced, and the reduction was more noticeable in transformant 14 than 26 (Fig. 3.6). This suggests that accumulation of these proteins was inversely related to RepA expression. In addition, accumulation of the cell cycle regulatory kinase, Cdc2, may be slightly greater in transformant 14 (Fig. 3.6A). RepA-accumulating endosperms (Fig. 3.6A). Endosperms accumulating RepA did not contain significantly less total non-zein proteins per-endosperm, as assessed by Bradford assay (t-test p value > 0.05, data not shown). Furthermore, RepA expression did not detectably alter the accumulation of eEF1A (Fig. 3.6A and B), an abundant protein associated with translational and cytoskeleton functions (Motorin et al., 1988; Yang et al., 1990; Edmonds, 1993; Durso and Cyr, 1994; Shiina et al., 1994; Condeelis, 1995; Liu et al., 1996; Sun et al., 1997; Clore and Larkins, 1998).

Seed development, particularly close to maturity, is affected by the plant growth regulator ABA. The G-Box Binding Factor 1 (GBF1) protein is known to regulate ABA-

Figure 3.6. RepA expression in endosperm affects the level of a variety of proteins. Immunoblots were prepared with equal amounts of non-zein proteins from 18-DAP endosperms of ears hemizygous for transgenic events 14 (A) and 26 (B). Antibodies to ADP-glucose pyrophosphorylase, b-32, Cdc2, sucrose synthase (Sh1), and eEF1A are described in Materials and Methods.


A
RepA
ADPGase
b-32
Cdc2
Sh1
eEF1A
B
RepA
ADPGase
b-32
Sh1
eEF1A

responsive gene expression in cereals and be up-regulated by both hypoxia and exogenous ABA application (de Vetten and Ferl, 1995; Nakagawa et al., 1996; Hollung et al., 1997; Kosugi and Ohashi, 1997). Immunoblot analyses were performed to determine if RepA expression alters GBF1 accumulation in 18-DAP GluB1:RepA transgenic and wild-type kernels (Fig 3.7). As was true of most zeins and b-32, GBF1 accumulation was negatively affected in endosperms of transformants 14 and 26, but not transformant 11.

RepA associates with nuclear proteins from maize endosperm

The observation of dose-dependent RepA effects on gene expression and the detection of RepA in nuclear and cytoplasmic compartments (data not shown) led us to investigate the nature of RepA-interacting proteins. Maize kernels were harvested at 18-DAP and separated into cytoplasmic and nuclear fractions by differential extraction and centrifugation. Proteins from these fractions were pre-adsorbed on Ni^{2+}-agarose in the presence of 20 mM imidazole buffered saline (IBS). The pre-adsorbed extracts were incubated with native or denatured recombinant His-RepA immobilized on Ni^{2+} agarose, washed with five column volumes of 40 mM imidazole in IBS to remove non-specific protein interactions. The bound proteins were eluted by the addition of 1M NaCl. Protein in aliquots from the eluant, wash, and pre-column sample were resolved by SDS-PAGE and detected with Coomassie Blue staining or immunoblot analysis with antisera to the maize Retinoblastoma-1 protein (ZmRb1) and High mobility group I/Y (Hmg I/Y; Fig. 3.8 and data not shown). Coomassie Blue staining of SDS-PAGE gels loaded with RepA-bound cytoplasmic proteins did not resolve any bands (data not shown). However,

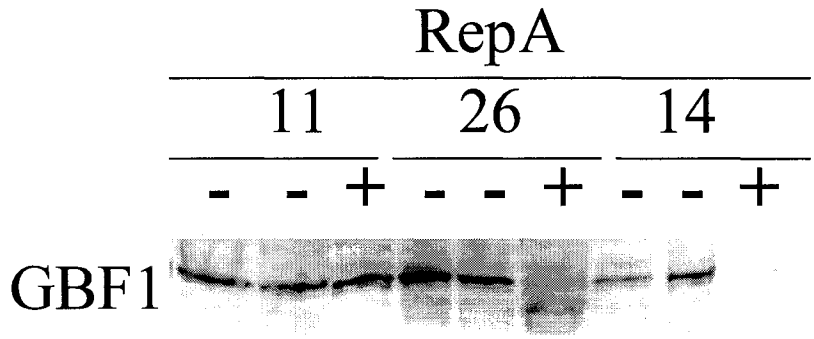
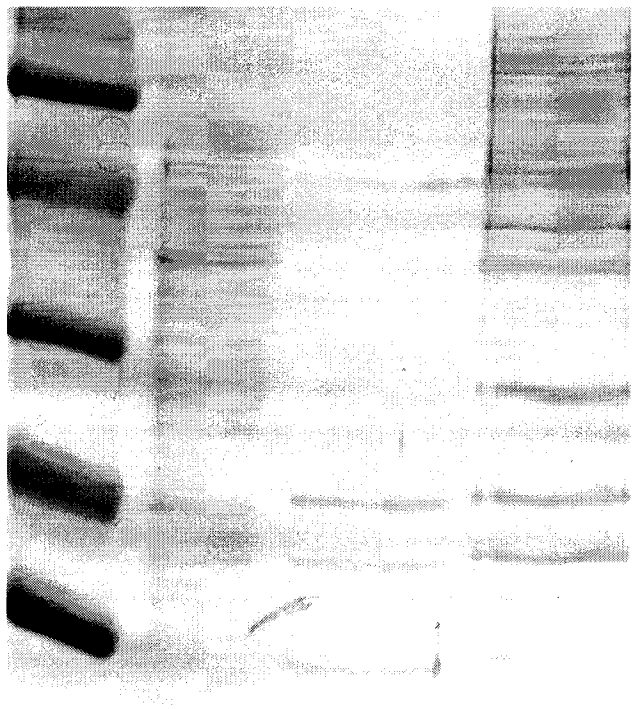


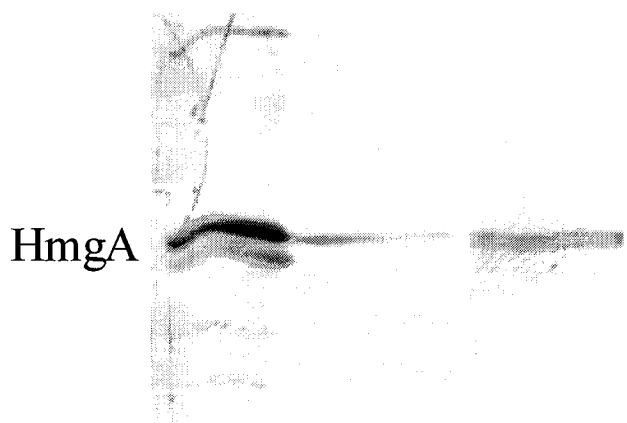
Figure 3.7. RepA expression affects G-box binding factor-1 accumulation in maize endosperm. Non-zein protein extracts were made from 18-DAP endosperms of hemizygous ears segregating for GluB1:RepA events 11, 14, and 26. Following SDS-page, blots loaded with equal amounts of protein were treated with antiserum to OsGBF1.

Figure 3.8. RepA pull-down assay showing potential interacting proteins in developing endosperm. Soluble proteins from developing endosperms were incubated with RepA agarose beads as described in Materials and Methods. A. Coomassie Blue-stained SDS-PAGE separation of nuclear-enriched proteins adsorbed by His-RepA. Lanes 1-4 show molecular weight standards, RepA-agarose flow-through, 1M NaCl eluant from RepA agarose, and Ni2+ flow through, respectively. (B) Immunoblot analysis of samples in A with HmgA-antisera. Lanes 1-3 are Ni2+ agarose flow through, RepA-agarose

A. 1 2 3 4



B. 1 2 3



three proteins were recovered following the interaction of nuclear proteins with RepA. Each of these bands migrated at positions similar to major chromosomal proteins and are presumably histones. Immunoblot analyses of the eluant also detected the presence of HmgI/Y, a non-histone major chromosomal protein (Fig. 3.8B).

Discussion

A role for RRB inactivation in the plant cell cycle and endocycle initiation

These results demonstrate a role for RRBs in the plant cell cycle. ZmRb1, but not a mutant defective in LxCxE-binding could inhibit cell division (Table 3.2). Opposition of native RRB by ectopic expression of RepA was sufficient to induce endoreduplication in maize callus cells (Fig. 3.1), and overcome a G1/G0 block in tobacco BY-2 cells (Table 3.1). Furthermore, concomitant expression of ZmRb1 and RepA had no effect on cell cycle progression in tobacco cells after 24h, suggesting that these two proteins act in opposition. Endoreduplication in RepA treatments was dependent on stable accumulation of RepA. Conversely, calli from RepA treatments that did not endoreduplicate failed to accumulate RepA. Despite evidence for some degree of endoreduplication in these calli (Fig. 3.1), they retained morphogenic competence and regenerated fertile diploid plants (Gordon-Kamm et al., 2002). This may reflect that only some of the cells endoreduplicated; polyploid cells divided more slowly; only differentiating cells within the callus were prone to endoreduplication; or polyploid cells were inefficiently recruited into differentiating shoot meristems. The latter two of these hypotheses are consistent with the observation that over-expression of the E2F/Dp1

proteins, typically repressed by RRBs, lead to increased endoreduplication only in differentiating cells within the arabidopsis leaf (De Veylder et al., 2002; Rossignol et al., 2002).

In animal systems, mitotic cells can be sensitized to enter an endoreduplication cell cycle through the loss of RRB function, either by genetic ablation of the expressed RRBs or by inactivation by viral RRB-binding proteins such as the human papillomavirus E7 protein (Niculescu et al., 1998). However, these cells require the co-expression of CDK-inhibitors to induce endoreduplication. Expression of RepA, a protein analogous to the viral LxCxE-containing oncoproteins, was sufficient to induce endocycles in cultured diploid cells of maize. Thus, cultured animal and plant cells may share a necessary condition for the induction of endoreduplication, i.e. abrogation of RRB function; however, they may differ in the conditions sufficient for endoreduplication, i.e. animal cells additionally require CDK inhibition.

We hypothesized that RRB modification is an important step in the induction of endocycles in normal plant tissues. In maize endosperm, for example, RRB becomes phosphorylated and presumably is inactive (Grafi et al., 1996). If RRB becomes inactive in endoreduplicating endosperm cells and RepA induces endocycles by disrupting RRB functions, RepA expression could have no effect on endopolyploidy levels in endosperm. If RepA alters the plant cell cycle via DNA-binding and -transactivation independent of RRB, RepA expression might increase endoreduplication in maize endosperm. However, we found in endosperm that RepA accumulation at low and high levels did not influence the degree of endopolyploidy (Table 3.3). While it is possible that RepA does not affect

endopolyploidy because it or its target is inactive in starchy endosperm cells, data from several experiments suggest otherwise. For example, RepA from transgenic endosperm interacted with GST-ZmRb1 *in vitro,* and immunoblots of total non-zeins with ZmRb1 antiserum immunodecorated proteins that showed increased concentration and altered mobility in GluB1:RepA transgenic kernels (data not shown). Also, the degree of RepA expression in the endosperm was associated with the severity of altered storage protein accumulation, further demonstrating that RepA possessed some activity capable of altering gene expression. Thus, our finding that RepA induced endoreduplication in mitotic tissue, but did not influence endopolyploidy in endosperm cells, is consistent with RRB serving as a negative regulator of S-phase. Phosphorylated RRB, such as that detected in 14-DAP endosperm (Grafi et al., 1996), may be unable to inhibit S-phase initiation and/or progression, rendering endosperm endocycles insensitive to RepA.

RepA expression disrupts endosperm storage functions in a dose-responsive manner

The phenotypic abnormalities of maize endosperm accumulating RepA protein suggest an alteration in transcriptional regulation. Kernels from the highest RepA-expressing transformants were smaller than wildtype, and wrinkled with a chalky endosperm at maturity (Fig. 3.4 and Table 3.4). Storage protein accumulation was generally reduced, as many zeins did not accumulate to wild-type levels. The 22-kDa α and 27-kDa γ-zeins were the most severely affected storage proteins, while the 16-kDa γ- and 15-kDa β-zeins were only slightly affected in the highest RepA-accumulating

transformant (Fig. 3.5). Similar results were observed for the b-32 RIP. An ABA-regulated G-box binding factor (de Vetten and Ferl, 1995; Nakagawa et al., 1996) was also down regulated in high RepA-expressing endosperms (Fig. 3.6), implicating this growth regulator in the observed phenotypes. In contrast, eEF1A, which is expressed at high levels throughout endosperm development, was unaffected by RepA accumulation. Additionally, the Cdc2 protein, which is expressed at slightly higher levels early in endosperm development, was relatively unaffected, and showed a slight increase in accumulation in the highest RepA-expressing endosperms.

Less starch was recovered from developing endosperms expressing GluB1:RepA. Nuclear extracts were not occluded with starch and zein extracts were pale rather than yellow, suggesting either inhibition of carotenoid biosynthesis or a paucity of amyloplast membranes due to a reduction in starch biosynthesis (data not shown). The levels of enzymes involved in starch synthesis were also lower in the highest RepA-expressing transformants. However, the rather minor decreases in the intensity of staining in immunoblots calls into question whether the decreases in the enzymes monitored in this study can account for the decrease in starch accumulation.

These data demonstrate that RepA represses the expression of genes typically up-regulated during starchy endosperm differentiation, while some genes expressed constitutively are relatively unaffected. Proteins with storage functions were synthesized in RepA-accumulating endosperm, but were reduced in concentration, suggesting that RepA interferes with a process required for the accumulation of these proteins to high levels, but not starchy endosperm cell differentiation, per se. If RepA suppresses

differentiation to create a permissive environment for viral replication, we might expect a qualitative phenotype. However, if RepA were to interfere with a signal that is required for high level gene expression in endosperm, we might expect the phenotype observed.

ABA is an important growth regulator for fruit maturation, and it plays a role in endosperm development in maize. Moderate applications of exogenous ABA positively affect both zein and starch synthesis (Muller et al., 1997; Ahmadi and Baker, 1999). The decrease in GBF1 accumulation (Fig. 3.7) implies an interference with ABA-regulated gene expression and ABA-mediated endosperm differentiation and could explain the phenotypes observed. However, a more detailed analysis of the effects of ABA on maize starchy endosperm cells and storage functions are required to clarify these findings.

The dosage dependence of the defects in endosperm development due to RepA expression suggest they are RRB independent. The amount of RepA accumulated in transgenic endosperms is likely to be many fold greater than endogenous RRBs, which were detected only after affinity purification (Grafi et al., 1996). Given the affinity of RepA for RRB, even line 11 (phenotypically normal) endosperms are unlikely to contain appreciable amounts of RRB that is not bound to RepA. Thus, the dose-dependant effects of RepA on starch and storage protein synthesis, visible in the high RepA-expressing transformantss, are more likely mediated by an RRB-independent effect of RepA.

RepA has been demonstrated to interact with two related transcription factors, GRAB1 and GRAB2 (Xie et al., 1999). The RRB-independent effects of RepA expression in endosperm could be mediated by these transcription factors. GRAB1 and

GRAB2 are of unknown function in plant cells, but they could play important roles in endosperm development. However, the maize genes in the public databases most similar to GRABs are not found in maize seed libraries (data not shown). Further research will be required to determine if these or similar factors are important to endosperm development and storage gene expression.

We attempted to identify endosperm proteins that interacted with RepA. While no biochemical identification of the three major bands was made, they migrated at positions identical to those of histones. In addition to these proteins, positive identification was made of HmgI/Y, and the three major RepA-interacting bands and HmgI/Y were removed from the flow through of the column (Fig. 3.8). This observation suggests an additional mechanism for RepA action independent of RRB. Besides RRB-binding, RepA could mediate changes in chromatin organization by binding proteins that modulate DNA architecture. A number of transcription factors regulating storage gene functions in cereal endosperm are known to be sensitive to the ratio of Hmg to linker-histone proteins (Schultz et al., 1996; Krohn et al., 2002). Hmg proteins bind the AT-rich enhancer element, TATA-, and CAAT-boxes of zein promoters (Grasser et al., 1990; Maier et al., 1990; Ponte et al., 1994; Guillen et al., 1998) and are implicated in high level transcription of these genes concomitant with endoreduplication. HmgI/Y is up-regulated, while linker histones are down-regulated, concomitant with DNA endoreduplication (Zhao and Grafi, 2000). Furthermore, as these proteins are extremely abundant, interference with these proteins may explain the RepA-dosage sensitivity of the endosperm phenotypes. The creation of endosperm-specific RRB knockouts and the

delimitation of domains within RepA required to induce these effects would be necessary to clarify these observations and test the mechanisms by which RepA influences storage gene expression in maize endosperm.

Materials and Methods

Tobacco Cultures and Transformation

Nicotiana tabacum L. cv. Bright Yellow 2 (BY2) suspension cultures were used for transient evaluations of RepA and ZmRb1 expression on cell division. Suspension cells were grown in a medium comprised of Murashige and Skoog (Murashige and Skoog, 1962) salts (Life Technologies, Inc., Grand island, NY), 100 mg/L inositol, 1 mg/L thiamine, 180 mg/L KH2PO4, 30 g/L sucrose, and 2 mg/L 2,4-D, subcultured every 7-10 days, and grown on a gyratory shaker at 150 RPM, 24°C in the dark. Three, six or nine days after subculturing, cells were pipetted onto solidified agar medium and left in the dark for 24 hours prior to bombardment. Plasmid DNA was precipitated on gold particles by the addition of PEG and $CaCl_2$ as previously described (Lowe et al., 1995). Bombardment was performed using a BioRad PDS-1000 helium gun at 650 PSI of helium, the chamber evacuated to 25 inches Hg, with 8 cm distance between the stopping plate and petri dish. All cells were shot once with 500 ng gold and 0.5 μg DNA. All constructs were derivatives of pRTL2 which contains, from 5' to 3' of the gene, a duplicated 35S enhancer, the 35S promoter, the tobacco etch virus omega-leader, multiple cloning site and 35S terminator (Carrington et al., 1990). All bombardments utilized a 35S:GFP expression cassette. Treatments consisted of 35S:RepA,

35S:ZmRb1$^{341-866}$, 35S:ZmRb1^{706G} or combinations thereof. 24h after bombardment, the cells were monitored for GFP expression and cell division by epi-fluorescence microscopy. The total number of GFP-expressing foci and whether these GFP-expressing foci were comprised of single or multiple cells were recorded. The data from the controls and treatments was then tested for significant differences by Students t-test (Student, 1907) as modified for testing proportions (Wonnacott and Wonnacott, 1990).

Plant material and production of RepA transgenic maize

pHP3630, which contains the maize 27-kDa γ-zein promoter, a multiple cloning site and the γ-zein terminator, was the vector plasmid used for construction of pGluB1:RepA (Coleman et al., 1997). The WDV RepA gene was obtained by PCR amplification of pGEM-RepA (Grafi et al., 1996). The sense primer (5'GGGCCATGGCCAT GGCCTCTTC ATCTGCACCCA 3') contains an *NcoI* site and in frame ATG start codon, and the antisense primer (5' CCCGGATCCGGATCCCTAGAG ACCTTG CCCAGGAAG 3') contains a *BamHI* site. The amplified DNA fragment (0.8 kb) was subcloned into the *NcoI/BamHI* site of pHP3630. The rice Glutelin B1 promoter (Takaiwa et al., 1991) was obtained by PCR amplification with a sense primer (5'AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCT 3') containing an *XbaI* site and an antisense primer (5' GGGCCATGGTGTCGATCGGGTTCTTCTGCGCT TTAA-3') containing an *NcoI* site and an ATG start codon. The amplified DNA fragment (1.3kb) was subcloned into the *XbaI/NcoI* site of pHP3630-RepA to create the plasmid pGluB1:RepA. The Nos:RepA and Ubi:RepA constructs for constitutive

expression of RepA and the BAR cassette conferring resistance to BASTA and bialaphos were described previously (Gordon-Kamm et al., 2002). The RepA constructs were introduced into scutellar cells of the Hi-II genotype along with the BAR cassette. Herbicide resistant calli were maintained on selection media, and fertile plants were regenerated essentially as described previously (Gordon-Kamm et al., 2002).

Fertile plants were recurrently backcrossed using the B73 inbred line as the ear parent. PCR analysis of GluB1:RepA transgenic plants was carried out with RepA oligonucleotide primers used in vector construction using DNA extracted from one g of lyophilized young leaf tissue. Integration of the target genes into the maize genome was analyzed by Southern hybridization using the 0.8 kb RepA coding sequence as probe. For DNA blot analysis, 10 μg of total DNA was digested with NcoI, separated by 0.8% agarose gel electrophoresis, transferred to a nylon membrane, and hybridized with a ^{32}P labeled RepA probe (Sambrook et al., 1989).

Production of polyclonal antiserum to WDV RepA

Eight mg of GST-tagged RepA (Grafi et al., 1996) was purified from *E. coli* lysate by glutathione-agarose affinity chromatography (Frangioni and Neel, 1993). Multiple protein extracts were pooled and concentrated by anion exchange chromatography with a Hi-Trap Q column (Pharmacia, Location). Proteins were separated by SDS-PAGE, visualized by nickel negative staining, and the band consisting of GST-RepA excised (Harlow and Lane, 1988). HTI Biosciences (Ramona, CA) was contracted for the immunization of two rabbits with GST-RepA protein in Freund's complete adjuvant; a total of four one mg injections were given to each rabbit. Serum

collected from immunized animals, before and after injection, was tested for reaction with maize extracts, purified GST, and purified histidine-tagged RepA (see below). The crude antiserum was diluted 1:5000 for use in immunoblots.

Flow cytometric analyses

Actively growing calli or dissected endosperms of developing kernels were analyzed by flow cytometry and immunoblot analysis for RepA protein. Kernels were obtained at 18-DAP from greenhouse-grown ears that segregated 1:1 for the GluB1:RepA transgene; this permitted the analysis of transgenic and non-transgenic sibs developing on the same plant. Maize tissue was homogenized by chopping with a razor blade in modified PARTEC buffer and nuclei prepared as described previously (Dilkes et al., 2002). Data from no fewer than 10,000 nuclei were obtained on a PARTEC CA III flow analzer (Partec; Hamburg, Germany). The remainder of the tissue homogenate was analyzed by immunoblotting as follows. Membranes and proteins were solubilized by the addition of 1/10 vol of 10% (w/v) deoxycholate. After clearing by centrifugation, protein was precipitated by addition of 1 vol of 20% (w/v) trichloroacetic acid, pelleted by centrifugation, washed with acetone, resuspended in 8 M urea and analyzed for protein content by the Bradford method (Bradford, 1976). For each sample, equal quantities of protein were separated by SDS-PAGE and tested for the presence of RepA by immunoblot analysis with the anti-GST-RepA antiserum.

Flow cytometric histograms were analyzed using the WinMDI software package (V. 2.8, Joe Trotter personal communication). The statistical outputs from manually gated histograms were imported into the Excel spreadsheet program (Microsoft, Redland

WA). Mean ploidy levels and the percent of endoreduplicated cells were calculated from the output from each histogram, as described previously (Dilkes et al., 2002). Pair-wise Student's t-tests were utilized to determine if endoreduplication in endosperms accumulating RepA protein was significantly different from non-transgenic sibs (Student, 1907).

Analysis of proteins in developing endosperms

Endosperms were dissected from 18-DAP BC2 kernels, placed in weighed microfuge tubes, and 400 µl of zein solubilization buffer (SDS, Borate, 2-ME) was added (Wallace et al., 1990). Tissue was homogenized in microfuge tubes using a plastic pestle; extraction was enhanced by incubating the homogenate at 37 C on a rotary shaker at 225 RPM for 8-16 h. Separation of alcohol soluble (zein) and alcohol-insoluble (non-zein) proteins proceeded as described by Wallace et al. (1990). Non-zein pellets were washed three times with 70% ethanol, resuspended in 8 M urea, 10 mM Tris (pH 7.5), and protein quantified by the Bradford method (Bradford, 1976). Zein proteins were separated by SDS-PAGE in 12.5% gels, transferred to nitrocellulose and immunoreacted with rabbit polyclonal anti-α-zein, anti-27-kDa γ-zein, anti-16-kDa γ-zein, and anti-15-kDa β-zein antiserum (Woo et al., 2001; Hunter et al., 2002). Non-zein blots were immunoreacted with rabbit polyclonal anti-GST-RepA, anti-G-Box-binding-factor 1 (Kosugi and Ohashi, 1997), anti-Shrunken1, anti-elongation factor 1-α (Sun et al., 1999), anti-ZmCdc2a (Colasanti et al., 1993), anti-B32 (Bass et al., 1992), anti ZmRb1 (Huntley et al., 1998), and anti-ADP-glucose-pyrophosphorylase (Giroux and Hannah, 1994)

antibodies. Goat anti-rabbit alkaline phosphatase conjugates (Jackson Immunoresearch Laboratories Inc., West Grove PA) were used for the indirect detection of the antibodies by NBT/BCIP colorimetric reaction (Harlow and Lane, 1988).

Protein pull down assays

The wheat dwarf virus RepA coding sequence was subcloned from pACT2-RepA (Grafi et al., 1996) into pQE60 (Qiagen, Chattsworth, CA), using the *NcoI* and *BglII* restriction enzymes to generate a carboxy-terminal histidine tagged RepA. Recombinant protein was produced in *E. coli* strains DH5-alpha or BL21-CodonPlus-RIL (Stratagene, Torrey Pines CA) and induced by 1mM IPTG at 30 C. Cells were harvested by centrifugation and resuspended in imidazole buffered saline (40mM Imidazole, 0.5% Triton X-100, 300mM NaCl, pH 8.3) supplemented with 5 mg/ml lysozyme. Complete lysis and reduced lysate viscosity were effected by two 30 s sonication pulses using a cell disruptor. Cellular debris was pelleted by centrifugation at 15,000 g for 20 min before loading extracts on iminodiacetic acid Sepharose Fast Flow (Amersham Biosciences, Sunnyvale CA) saturated with Ni^{2+}. Columns were washed with at least five column volumes of IBS with 0.1% Triton-X100 and eluted with 200 mM imidazole in IBS. Eluants were split into aliquots for re-adsorption onto affinity matrices or adsorption onto immobilized target proteins.

Kernel extracts were separated into soluble and chromatin-associated proteins similar to the procedure described for germinating seedlings (Lechner et al., 2000). Briefly, kernels were chopped to a fine meal in a steel Waring blender under liquid nitrogen. Frozen meal was suspended in chromatin isolation buffer (4ml/g; 20mM Tris

pH 7.5, 5 mM $MgCl_2$, 5mM KCl, 0.25 M sucrose, 0.1% Triton, and 40% glycerol) and filtered through cheesecloth. Filtrate was pelleted gently by centrifugation at 600 RPM in an SS-34 rotor (Sorvall, Ashville VA) and the supernatant removed and re-centrifuged at 15,000 g for 15 minutes. This supernatant was regarded as the soluble protein fraction from maize kernels. The 600 RPM pellet was gently resuspended and washed in chromatin isolation buffer twice before nuclei were disrupted and proteins solubilized by the addition of either 1M KCl or 1M NaCl modified IBS. Insoluble material was pelleted by centrifugation at 15,000 g for 30 minutes, and the resulting supernatant was regarded as the chromatin-enriched fraction, although it also contained proteins from other organelles. Extracts were pre-adsorbed on Iminodiacetic Acid Sepharose loaded with $NiCl_2$. The flow through from these columns was passed over the RepA-containing resin, washed with at least five column volumes of IBS and eluted with increasing concentration of NaCl. Adsorption of proteins, and retention of RepA on the affinity matrix, were assessed by SDS-PAGE and Coomassie Blue staining or immunoblot analysis of column eluants with an anti-HmgI/Y (Spiker, 1984), a monoclonal ZmRb1 antibody (Grafi et al., 1996), a polyclonal ZmRb1 antibody (Huntley et al., 1998), or anti-GST-RepA polyclonal antiserum.

Statement of collaboration

The Ubi:RepA and Nos:RepA transgenes and transgenic calli were generated by Keith Lowe at Pioneer Hi-Bred, Johnston, IA. The GluB1:RepA transgene and transgenic maize lines were generated by Yan Liu at the University of Arizona. Southern

blotting of GluB1:RepA transgenic maize and storage protein immunoblot reactions were also carried out by Yan Liu.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Investigation of characters correlated with endoreduplication and correlation of characters within endoreduplication

The results obtained studying natural variation in endosperm endopolyploidy do not suggest that endoreduplication contributes to an increase in kernel size, at least in these crosses. A three-fold difference in endoreduplication at 19-DAP was observed in the (B73 x Sg18) x B73 BC1 and BC2 generations. Despite this difference, no dramatic alterations in endosperm size were seen between kernels. This seems to fly in the face of earlier reports of the positive correlation of endopolyploidy and seed size (Brunori et al., 1993; Lemontey et al., 2000), as well as the purported link between increases in endoreduplication and metabolic output (D'Amato, 1984). These reports appear to argue that "more is better" and would result in larger kernels. Additionally, the genotypes with smaller kernels, namely the popcorns, had the highest degrees of endoreduplication (Fig 2.1). While this may be a peculiarity of popcorn germplasm, it impacts the interpretation of the role of endoreduplication in endosperm development.

The apparent association of smaller size, or no difference in size, with increased endopolyploidy in these crosses could be explained by the following predictions for endosperm growth. Either endoreduplication and cell division is reciprocally affected or

the relationship between ploidy and cell size are altered such that increases in endopolyploidy have no effect on cell size. As cell size tends to be roughly equivalent within ploidy classes between closely related organisms (Flemming et al., 2000), it seems more likely that a decrease in cell number was responsible for the increases in endoreduplication at 19-DAP. A possible mechanism for this is suggested by the data in Fig. 2.2; Sg18 and (B73 x Sg18) x B73 endosperms manifest significant increases in endopolyploidy by 13-DAP, as compared to B73. A hypothesis consistent with this, and a reduction in cell division, is that Sg18 alleles condition endosperm cells to adopt the endoreduplication cell cycle earlier in development. This could result in fewer cells and an increase in the E6P value. Later in development one would expect, all other things being equal, endosperms that endoreduplicated earlier to show higher E3P and E6P values. This is similar to what occurs in kernels subjected to heat stress early in development, which exhibit a reduction in cell divisions in favor of earlier endoreduplication. At mid development (11- to 13-DAP), endosperms from stress-treated kernels exhibit higher degrees of endopolyploidy (Engelen-Eigles et al., 2001). Unlike the genotypic variability in endopolyploidy, heat stress early in development additionally shortens the endoreduplication phase of endosperm growth, resulting in no increase or lower endopolyploidy by 18-DAP (Engelen-Eigles et al., 2000, 2001).

Evaluating the relationship between cell number and endopolyploidy will require an investigation into characters correlated with the degree of endopolyploidy. With proper controls, cell numbers can be estimated flow-cytometrically while simultaneously measuring endopolyploidy. Isolated endosperm nuclei from BC1 ears segregating for

differences in endopolyploidy could be spiked with a known quantity of chicken red blood cell nuclei prior to analysis. The ratio of chicken red blood cell nuclei to maize nuclei in a flow cytometric histogram would provide an estimate of the total number of cells isolated from the endosperm. The same flow cytometric histogram used to calculate relative nuclear numbers, could be used to estimate the %E, E6P, and E3P values for an individual endosperm. Estimates of nuclear number and the three measures of endopolyploidy could then be subjected to an analysis for phenotypic correlation. If the lack of change in endosperm size was the result of reciprocal regulation of endoreduplication and cell division, the correlation between nuclear number and endopolyploidy should be strongly negative. Performing the analysis using a sufficient set of crosses could be used to partition any phenotypic covariance, positive or negative, into genetic components.

Of all the germplasm tested, significant differences in %E were only detected between B73 and Mo17. The failure to detect significant genetic components for %E in the popcorn by B73 3^2 populations (Tables 2.5, 2.6 and 2.7) may be due to a lack of differences for this trait in these genotypes. No significant variance components were detected for %E in B73 by Mo17 as well, suggesting that it is sporophytically or cytoplasmically inherited. However, a problem with these interpretations is provided by the significant heritability of %E detected by the regression of BC2 on BC1 (B73 x Sg18) x B73 values and observations within inbred lines. There was a two-fold variation in %E in the BC1, and the mean %E of the BC2 was well predicted by the BC1 %E values (Table 2.10). Thus, despite the lack of any significant variance components in the

analysis of the Sg18 x B73 3^2 factorial-population, the zygotic contribution to %E was heritable in the parent-offspring regression experiment. In fact, %E showed the highest narrow-sense heritability of the three traits in the parent-offspring regression experiment. Within inbred lines the %E and E6P displayed a high phenotypic correlation, suggesting that they are environmentally co-regulated.

The failure to detect differences in %E utilizing a 3^2 factorial design, and subsequent finding of %E heritability in the parent-offspring regression, may be explained if endosperm size influences the %E value. As the volume of a solid decreases, if the proportion of the total volume comprised by the shell is to stay the same, the shell must decrease in thickness. The %E value is the number of 3C cells, concentrated at the periphery of the endosperm, divided by the total number of cells. Thus, the number of cell files contributing to this shell or the number of cells required to span an equal surface area must have decreased in the smaller endosperm genotype with similar %E. An identical %E value in these two kernel types requires a smaller or narrower "shell" of cells with triploid nuclei. This could be envisioned to have taken place either by transverse cellular expansion of the same number of cell files (a cell size *increase* in smaller endosperms), or distributing ploidy within a sharper boundary at the edge of the endosperm. In the (B73 x Sg18) x B73 materials used for parent-offspring regression, kernels were not visibly different in size within an ear. Thus, the two-fold differences in %E between BC1 kernels were not subject to endosperm size effects and "became" heritable. Reanalyzing the data presented in Chapter 2 to correct for this would require determining the average size of endosperms in each generation at 19-DAP, the

size of cells at the periphery, and/or the number of cell files that fail to endoreduplicate for each genotype. This would also determine if cell size was altered in the popcorn germplasm. A scaling factor could then be applied to the data, or the average number of cells between the aleurone and the first endoreduplicated cell could be analyzed directly. The latter may be a more developmentally relevant measure of the extent of endoreduplication in the endosperm, where endopolyploidy is spatially segregated. Regardless, in the absence of a scaling factor, a negative association between increased endopolyploidy and kernel size results in a more conservative estimation of E3P and %E components, and may explain the failure of the 3^2 factorial design to identify variability, and genetic components for these traits in some populations.

Despite my hypothesis that endoreduplication and cell number confound an interpretation of endoreduplication's effect on endosperm size, a positive role in cellular growth is still possible. Based on the correlations between ploidy and metabolic activity per cell, it is expected that endopolyploidy contributes to the level of gene expression per cell in polysomatic tissues (see Chapter 1). If endoreduplication is responsible for limiting cell numbers, but still contributes positively to cellular growth, both endopolyploidy and cell number should be required for optimal regression of endosperm weight by estimates of cell cycle parameters. It has been proposed that endoreduplication is important for storage protein accumulation and high metabolic activity (D'Amato, 1964, 1984; Grafi, 1998; Larkins et al., 2001). Endoreduplication and phosphorylation of the chromosomal protein HmgI/Y, a positive regulator of zein gene promoters, are temporally correlated (Zhao and Grafi, 2000). If a link between endoreduplication and

storage protein accumulation is mechanistic, one would expect the two to be positively correlated. For example, the protein content in g per kernel and/or g protein per total weight should be higher in individuals with greater degrees of endopolyploidy. If research into maize endosperm endoreduplication is to be continued on the grounds that it impacts seed growth and development and endosperm is to be cited as a tissue in which endopolyploidy impacts growth, clearly a test of this is in order.

Endoreduplication and the relationship to parent-of-origin effects

A large body of literature has been devoted to parent-of-origin genetic effects on the endosperm of maize and other species. In plants, altering the dosage of either the male or female contributions to the endosperm, e.g. via interploidy crosses, leads to alterations in endosperm and seed development . In maize, 2n x 4n interploidy crosses, and the reciprocal, are lethal (Cooper and Brink, 1945; Cooper, 1951; Charlton et al., 1995). The seeds produced by interploidy crosses produce defective endosperms, and it is the genotype of the endosperm that controls seed abortion (Lin, 1982; Lin, 1984). In crosses in which an excess of paternal genomes is contributed, the tetraploid (2:2 m:p) endosperms fail to fill with starch and defects in growth are visible by 10 DAP (Cooper and Brink, 1945; Brink and Cooper, 1947; Cooper, 1951; Charlton et al., 1995). Cellular differentiation is affected such that the cells of the basal-endosperm transfer layer die at 12-DAP following a failure to cellularize. Maternal excess pentaploid endosperms (4:1) grow relatively normally until 16-DAP. Seeds from maternal excess crosses are plump, smaller than diploid maize kernels, and defective in germination (Cooper, 1951; Charlton et al., 1995). The effect of increased and decreased dosage of individual chromosomes

and chromosomal segments via BA-translocations has also been investigated. In these cases, a group of hypothesized endosperm dosage factors appears to disrupt endosperm development. In most cases, deficiency of these factors from the pollen parent leads to a decrease in endosperm size, and simultaneous increases in maternal dosage enhance the size defect (Birchler, 1993), suggesting that the dosage of particular genomic segments relative to the total genome is a critical feature of balancing the contributions to endosperm.

Parent-of-origin effects have also been identified for mutants affecting endosperm development. A number of mutants display differential transmission through either germline, suggesting either that the effect the development of the gametophyte, affect meiotic drive, or are unequally expressed from the two parental copies in the progeny (Pan and Peterson, 1989; Huang and Sheridan, 1996; Klucher et al., 1996; Feldmann et al., 1997; Christensen et al., 1998; Howden et al., 1998; Mayer et al., 1999; Siddiqi et al., 2000; Springer et al., 2000; Evans and Kermicle, 2001; Grini et al., 2002; Pischke et al., 2002). Among the maternally-expressed mutants of arabidopsis a subset have been identified with fertilization independent seed development (Kinoshita et al., 1999; Kiyosue et al., 1999; Luo et al., 1999; Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000). Of this group of mutants, the *mea*, *fie*, *fis* mutant endosperms show phenotypes similar to those caused by paternal excess interploidy crosses. Development of endosperms inheriting these alleles via the egg is characterized by a prolonged proliferative stage and defects in cellularization (Ohad et al., 1996; Kiyosue et al., 1999; Vinkenoog et al., 2000). Cloning of the genes responsible for the phenotypes has

determined that all share sequence similarity with suspected modulators of chromatin-organization-mediated gene expression. Additionally, maternal transmission of a MetI antisense transgene suppresses interploidy effects in tetraploid (2m:2p) arabidopsis endosperms and paternal transmission of *DDM1* or *METI*-antisense can suppress some of the maternal effect mutations or allow complementation by a paternally-inherited copy (Vielle-Calzada et al., 1999; Adams et al., 2000; Vinkenoog et al., 2000; Yadegari et al., 2000). So far, the methods employed in maize have not provided the resolution necessary to determine if the effects of altered genomic dosage can be manipulated by alleles of particular imprinted genes, such as those identified in arabidopsis, or is due only to genomic-scale effects. My study, similarly, cannot be used to claim demonstration of imprinted gene expression on endosperm cell cycles. Genetic analyses of A1-6 by B73 and Sg18 by B73 3^2 factorial designs detected significant genetic components of the variance, with allelic effects exerted by the maternal contribution to the endosperm, a situation only allowed if segregation for genomic loci or segments is allowed. This could be consistent with genomic segments encompassing many genes, persistent effects of the female gametophyte on endosperm development, or imprinted gene expression.

The detection of parent-of-origin-specific expression for some of the endoreduplication traits suggests that endoreduplication, or something related to it, has been under fitness selection (McVean and Hurst, 1997; Hurst and McVean, 1998; Spencer et al., 1998; Haig and Wilkins, 2000). Unfortunately, endoreduplication in maize endosperm correlates temporally and spatially with a number of events, such as the

onset of storage product accumulation, which could be hypothesized to affect later embryogenesis and seedling growth. The measurement of these temporally correlated processes in a population of known variability for endoreduplication may allow some or all of them to be eliminated as associated with endoreduplication.

In some interspecific crosses, failures in endosperm development enforce a species barrier. Efforts to manipulate the endosperm barrier have led to the development of a hypothetical endosperm balance number (EBN). By generating autopolyploids, closely related species that were previously inter-sterile can be crossed efficiently. The EBN is the number of genomes from one species necessary to balance, i.e. produce fertile seeds, in a cross with another (Katsiotis et al., 1995). The EBN derived from different crosses is often the same, such that if three species are used one can predict the viability of the first and second by the EBN each has in crosses with the third (Ehlenfeldt and Ortiz, 1995; Katsiotis et al., 1995; Carputo et al., 1997; Masuelli, 2001; Carputo et al., 2003). EBN has been hypothesized to be the result of the expression level or activity of imprinted genes critical to endosperm development, and may be the interspecific manifestation of the action of the same genes that contribute to the requirement of endosperm for ploidy balance in interploidy intraspecific crosses (Birchler, 1993). As an example, crosses between diploid maize and diploid *Tripsacum dactyloides* are extremely inefficient. If tetraploid *Tripsacum* and diploid maize are crossed, the efficiency is substantially greater. Thus, it would appear that maize has a greater EBN than *T. dactyloides*. In both cases, it is primarily a defect in endosperm development that ensues, as endosperms fail to fill with starch and collapse while embryos grow if isolated from

developing kernels and placed on agar-solidified media (Kindiger and Beckett, 1992; Grimanelli et al., 1997).

A number of economic, evolutionary and genetic models have been hypothesized to account for the sensitivity of endosperm development, and early organismal growth in general, to balanced parental genetic contribution. All share the postulate that genes affecting the dosage sensitivity of endosperm development are sensitive to the number of copies, and activity, of rate-limiting regulators of gene expression (Haig and Westoby, 1991; Birchler, 1993; Ehlenfeldt and Ortiz, 1995; Gregory and Hebert, 1999; Birchler et al., 2001; Baroux et al., 2002). Each also suggests that an observation of parent-of-origin effects suggest that the trait measured has been, or is downstream of genes that have been, selected on the basis of effective dose (Haig and Westoby, 1989, 1991; Birchler, 1993; Hurst and McVean, 1998; Spencer et al., 1998). Imprinting, or parent-of-origin-specific expression, is simply one mechanism to alter the dosage of a particular gene. Fortunately, these models for the regulation of genomic balance make predictions about the behavior of a trait displaying maternal allelic effects, such as endopolyploidy. If endosperm-expressed dosage sensitive regulators control endopolyploidy in these crosses, we expect endoreduplication to respond dramatically to other alterations in gene dosage, such as unbalanced BA translocations, interploidy crosses, and crosses with related species and subspecies, such as the various other members of *Zea* and *Tripsacum*. Furthermore, we might expect inheritance of alleles affecting high or low endoreduplication to impact the fertility, or severity of disruption in endosperm development, in interploidy and interspecies crosses.

Some evidence suggests that genomic balance requirements are altered in the more endopolyploid popcorn genotypes and the potential connection warrants further investigation. Firstly the behavior of the EBN is different in popcorns with higher endopolyploidy. The popcorn inbreds Sg1533, Sg18 and A1-6, but not the lesser endoreduplicated Kp58k, are highly fecund ear parents for diploid maize by tetraploid *T. dactyloides* and diploid maize by diploid *T. dactyloides* crosses (Kindiger and Beckett, 1992). This suggests that the maternal EBN of popcorns with high endoreduplication is different from other stocks. Either parental balance less important for endosperm development, or the EBN has changed such that it more closely matches the EBN of *Tripsacum*. The latter seems less likely as both 2n x 4n and 2n x 2n interspecific crosses were improved.

A number of experiments utilizing the materials generated and described in Chapter 2 could be used to test the predictions outlined above. If the maternal effects on endopolyploidy are due to a change in dosage sensitive regulators, the B73 by Sg18 backcross progeny which have been analyzed for endopolyploidy in endosperm should also posses a similar variability for interspecific and interploidy cross effects. Crosses could be used to determine if the maternal expression of genes affecting endoreduplication in endosperm was due to a reduction in maternal-side effects or requirements for balance. Many possible outcomes of the experiments can be envisioned, however the possibility of validating the relationship between EBN and interploidy effects make it a worthwhile experiment even if endoreduplication shows no association. If the presence and dosage of shared factors explains endosperm-based fertility barriers in

maize, interploidy effects (Birchler, 1993) and higher degrees of endoreduplication in maize endosperm, A1-6 or Sg18 by B73 BC1 and F1s should be more fecund ear parents than B73 in crosses with *T. dactyloides*. Furthermore, fecundity in BC1 x *T. dactyloides* crosses, as measured by progeny viability and/or size, should correlate with the degree of endopolyploidy measured for the BC1 endosperm. Similarly, popcorn should be a more fertile ear parent, or display weaker or delayed symptoms, when used in interploidy crosses. Endopolyploidy of the BC1 endosperms should predict the severity of interploidy effects, and the viability of progeny from BC1 x tetraploid crosses. These types of experiments could provide a link or refute an association between intraspecific-intraploidy parent-of-origin inheritance, the effects of interploidy crosses, and interspecific cross effects in the endosperm. Even a negative result, ruling out the genes affecting endoreduplication as a contributing factor to the EBN and allied impacts of genomic imprinting, would be valuable. If the EBN and interploidy phenomena are allied, however, the selection of maize stocks for the increased fertility with *T. dactyloides* could benefit both the introgression of traits from *T. dactyloides* into maize germplasm and attempts to induce apomixis in maize. Indeed, if popcorn germplasm affects fertility with *T. dactyloides* via a decreased requirement for endosperm balance, the exploitation of this could move maize closer to an apomictic reproductive habit.

QTL mapping of genes affecting endoreduplication in maize endosperm

QTL mapping experiments for the three endoreduplication traits, and testing the inheritance of QTL for maternal expression could be used to localize the genes responsible, validate the mode of inheritance suggested by the study in Chapter 2, and

attempt to identify the molecular nature of the allelic variation responsible for increased endoreduplication in these crosses. The materials generated by a QTL mapping experiment could also be used to better assess the genetic correlation between traits, as shared alleles should co-localize. Lastly, the materials generated in a QTL mapping experiment could be used to test any of the hypotheses regarding correlated characters proposed in the previous two sections. The identification of individual genes and gene products responsible for maternal zygotic control of endoreduplication, if such can be identified, is the only way to link imprinted gene expression (in the molecular sense) to the parent-of-origin-specific allele effects seen in A1-6 x B73 and Sg18 x B73. This is especially true, given the inability of the quantitative genetic analysis to distinguish gametophytic effects from parent-of-origin effects on gene expression within the developing endosperm.

The choice of materials for QTL mapping may be largely a matter of taste. However, at least four technical points impact the choice of material for QTL mapping the three endopolyploidy traits. Firstly, mapping should proceed utilizing a cross in which significant, and the corresponding, mean and variance components were identified. Secondly, if validation and localization of genes affecting parent-of-origin-specific effects is a goal, then the mapping should proceed in a cross in which significant variance and mean components consistent with zygotic maternal inheritance were observed. Depending on the crossing design employed, the cleanest results will be obtained a cross with less maternal sporophytic influence, as this will confound the analysis when different ears and later generations are to be compared. The confounding effects of

sporophytic maternal control of trait mean values will require a greater number of repetitions and generations to map or control for it. Thirdly, the observations of increased error, little genetic influence, and confounding effects of endosperm size on %E suggest that this trait may be refractory to further analysis by similar methods to those employed in Chapter 2. Considering this, crosses in which %E and endosperm size displayed reciprocal differences between the two parents would lead to the least information and artificial deflation of %E and E3P differences, as may have occurred in the analyses of the factorial design experiments in Chapter 2. If BC1 generation material were to be used for QTL mapping, size concerns could largely be ignored, allowing %E and E3P to be subjected to QTL mapping. However, as no information is currently available about the mode of inheritance for %E, the best model to apply to a mapping population is difficult to determine. In a BC1 population additive gene action resulting in differences between kernels could be due to maternal, triploid, or diploid zygotic inheritance. Due to segregation occurring in only one of two parents, mapping would proceed essentially as for haploids, but would not distinguish between any of these inheritance modes. Lastly, a potential validation population already exists for a B73 x Sg18 mapping population in the form of the reciprocal backcross progeny (Table 2.9) which have been measured for endopolyploidy. The progeny of these plants could be used to validate the effects and mode of inheritance for large effect QTL, and provide material for fine mapping, much like a recombinant-inbred population (Mackay, 2001). Unfortunately, the small number of BC1 individuals founding this population limits its utility (Hill, 1998).

As the detection of parent-of-origin effects has the widest potential impact from a basic research standpoint, it seems that the Sg18 and A1-6 by B73 crosses, which identified significant variance in maternal genetic contributions to both the E3P and E6P, would be the best choices. QTL mapping is a time consuming and costly process, and thus it is probably only feasible to carry out a mapping experiment using one of these two crosses. The existence of BC2 (Table 2.9) and higher (data not shown) back-cross generations for B73 x Sg18 may make this cross technically superior. Major QTL could be verified in backcross progeny derived from these generations, provided they are present in the population, and a more precise estimate of allele effects and map position determined. Furthermore, the identification of QTL affecting increased endopolyploidy in these materials would allow, with marker association testing, a more robust and specific test of the inheritance mode for QTL controlling these traits. Thus, this material truly constitutes a validation or rejection of the inheritance modes proposed by the 3^2 factorial analysis. However, the B73 x A1-6 cross provides the best material considering the first three technical concerns (Tables 2.5, 2.7 and 2.8) and the size of the B73 x Sg18 backcross population mitigates its utility. Thus, I favor mapping in an A1-6 x B73 population. This cross identified both paternal and maternal effects, had the least error of the four, and did not provide support for maternal sporophytic effects (though none can be ruled out). These results suggest that the magnitude of QTL required for detection will be lower. If fewer confounding factors need to be considered and differences between sporophytes do not exert any genetic influence on endoreduplication, then a multi-generation population could be used without the problems of maternal effect co-

linearity. Lastly, the E6P differences are greater A1-6 and B73 than any of the other crosses. As this is the trait which displayed the strongest maternal zygotic effects in the popcorn by B73 populations, a QTL analysis of E6P in A1-6 x B73 should produce the most robust conclusions.

The phenotypes of arabidopsis maternal effect mutations suggest the maize orthologs as possible candidate genes affecting endoreduplication in endosperm. Maternally-expressed, paternally silent allelic effects on endoreduplication could be seen as the opposite phenotype of loss of function at *Fie*, *Fis*, or *Mea*. Mutations in the maternally expressed *Fie* and *Mea* both lead to a prolonged mitotic phase of development in endosperm (Ohad et al., 1996; Kiyosue et al., 1999; Vinkenoog et al., 2000). While the effects of alleles of the maize orthologs on endosperm development have not been demonstrated, some have been cloned and their expression in endosperm determined (Springer et al., 2002) and maternal expression and paternal imprinting reported (Lai and Messing, 2001). Mapping of related genes in maize, and tests for co-localization with QTL affecting endoreduplication in the endosperm, would be required to associate these genes with the phenotype observed in Chapter 2.

Application of gametophytic inheritance models to previous studies

Before we leave the subject of the quantitative genetic analysis presented in Chapter 2, it is important to discuss perhaps the best evidence for parent-of-origin-specific effects on endosperm storage functions. A number of genetic studies, including QTL have demonstrated that seed and endosperm traits are strongly influenced by the maternal parent (Shi et al., 1997; Alonso-Blanco et al., 1999; Chen and Zhu, 1999; Shi et

al., 1999; Shi et al., 1999; Shi et al., 2000). Some studies utilized populations in which maternal sporophytic effects and maternal gametophytic effects would have identical influence on trait means or variance, such as the diallele design (Shaw and Waser, 1994) and QTL mapping with recombinant inbreds. One area for further experimentation is the extension of the genetic models presented in Chapter 2 (Tables 2.1 and 2.2) to the predictions for mapping populations, such as those used in previous studies. Indeed, prior to initiating a QTL mapping project, the theory required must worked out to allow for proper experimental design. Some crossing designs, such as those used to analyze grain quality in rice (Shi et al., 2000), allowed for the estimation of sporophytic and cytoplasmic components and may be properly designed to distinguish between sporophytic and gametophytic (zygotic) maternal effects. Despite this, the authors conclude that maternal genetic control is the result of the influence of the genotype of the maternal sporophyte and cytoplasm (Shi et al., 2000). The identification of differences in variance within generations and the diagnostic "breakout" variance in the second generation in the raw data collected in some of these studies may detect of parent-of-origin effects on these traits if the appropriate mathematical models are applied. Additionally, conclusions of maternal sporophytic or cytoplasmic effects may have been premature, as they are often co-linear with maternal zygotic effects when they cannot be distinguished. A test for the presence of these effects, and correction of the theoretical models employed to analyze the data may reduce, or remove, the presumed effect of sporophytic effects or add zygotic maternal genetic components to the necessary explanatory variables.

The impact of parent-of-origin effects on endosperm storage functions could be substantial. If maternal zygotic inheritance controls nutritional quality, selection for improved nutritional quality assuming a sporophytic mode of inheritance will lead to increased trait mean values. However, selection would be more efficient, and require fewer kernels from fewer generations to achieve the same increase in trait mean values, if the trait were under gametophytic inheritance and selected accordingly. Furthermore, the hypothesis that maternal-specific gene expression is more substantial and important than previously thought, as suggested by a recent and controversial study (Vielle-Calzada et al., 2000), could find validation and even application this way. Traditional breeding methods have resulted in two new varieties of protein-content-enhance maize this year. These varieties join the *o2*-regulated high lysine and Top-cross high oil maize varieties in a marketplace that has an increasing share of specialty corns. If parent-of-origin effects impact nutritional quality, application of this knowledge to further improve these lines could be accomplished in the very near term. Observations of maternal-specific expression and demethylation of zein genes suggest that parental imprinting may affect storage protein accumulation in maize (Lund et al., 1995).

Mechanism of RepA effects on the cell cycle

While natural variation could be exploited to both identify and select for increased endoreduplication in maize endosperm (Fig. 2.1 and Table 2.10), expression of the viral cell cycle regulator, RepA, did not alter endoreduplication in maize endosperm (Fig 3.2 and Table 3.4). However, RepA expression induced maize callus to endoreduplicate (Fig. 3.1) and increased cell division in tobacco BY-2 cells (Tables 3.1-3.2). As

endopolyploidy in endosperm cells is manipulable, the failure of RepA to alter endopolyploidy suggests that it interferes with cell cycle regulators that are not rate limiting steps in endosperm endoreduplication. Earlier findings of RRB phosphorylation and high level RRB-kinase activity in these cells (Grafi and Larkins, 1995; Grafi et al., 1996) suggestive of RRB-inactivation, are consistent with the failure of RepA to induce increased endopolyploidy. However, the RRB-dependence of RepA-promoted endocycles in callus and cell cycle stimulation in tobacco BY-2 cells has not been determined.

Further experimentation is necessary to demonstrate, or rule out RRB-dependence for cell cycle stimulation by RepA. The most obvious solution is to repeat the callus and BY-2 experiments of Chapter 3 using a point mutant of RepA that disrupts the LxCxE motif and prevents binding to RRBs (Grafi et al., 1996; Xie et al., 1996; Ach et al., 1997; Huntley et al., 1998). If an RRB-binding mutant of RepA induces endocycles in calli or stimulates cell division in tobacco BY-2 cells, then RepA must mediate these effects by an RRB-independent mechanism. If it does not, this strongly suggests that RRB-binding is necessary, but it does not allow one to presume RRB binding to be sufficient for these cell cycle effects. RepA is known to have a number of activities, including DNA binding and nicking, oligomerization and binding to GRAB transcription factors (Horvath et al., 1998; Xie et al., 1999; Gutierrez, 2000; Hanley-Bowdoin et al., 2000; Missich et al., 2000) all or none of which may be necessary for cell cycle stimulation. While no evidence that these functions exert an influence on the cell cycle is available, any and all activities of RepA are formally possible contributing factors. Lastly, if RepA stimulates

endocycles via RRB-inactivation, overexpression of RRB proteins in maize callus should oppose endoreduplication.

In animal cells, endoreduplication can be accomplished by the concomitant inhibition of RRB and oscillation of CDK activity (Bates et al., 1998; MacAuley et al., 1998; Niculescu et al., 1998; Jiang et al., 2000). CDK oscillations can be accomplished in a myriad of ways, but this is often done via cyclin abundance (Sauer et al., 1995) or oscillations in the activity of CDK inhibitor proteins (Hattori et al., 2000). Animal CKIs are rapidly degraded following phosphorylation by CDKs, and this relationship can be exploited to form a CDK oscillator (Morisaki et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999). If the plant cell cycle is similarly regulated, co-expression of a CKI in RepA-expressing calli could lead to extensive endoreduplication.

Transfection of animal cell cultures is a highly efficient technique that allows transgenes to be delivered to a large percentage treated cells. In plants, transgenes are typically expressed in only a subset of treated cells. For this reason, extensively endoreduplicating cells are likely to be overwhelmed by mitotically active cells in maize calli. Furthermore, the maize CKIs are expected to block cell proliferation and thereby negatively select for transgenic cells. Thus, only a small fraction of the cells analyzed in a flow cytometric analysis would contain, or express, the CKI-treatment transgenes. Inducible expression systems could be adapted to permit the delay of CKI expression, and subsequent mitotic arrest, until homogeneously CKI^+ $RepA^+$ callus was available. However, a simple transfection based assay, as follows, could also be used. First, a few lines of RepA-expressing calli should be generated. Subcultures of these calli could be

made and tested for RepA accumulation to ensure that the callus was homogeneously $RepA^+$. Similar to the experiments I performed with BY-2 tobacco cells, bombardment with a GFP expression construct and co-bombardment with GFP and CKI expression cassettes could then be carried out on RepA and control calli. Following DAPI staining, measurements of nuclear diameter and microphotometric measurements of fluorescence intensity could provide estimates of endopolyploidy. If the animal model extends to plants, fluorescent cells from $RepA^+$ calli co-bombarded with GFP and CKI cassettes should have a higher ploidy than GFP-only controls after a few days of culture. This experiment could also demonstrate cell cycle inhibitory activity for the CKI, as control calli co-bombarded with GFP and CKI cassettes should exhibit cell cycle arrest.

While RepA expression had significant cell cycle-stimulatory effects on two types of cultured cells, it had no effect on the cell cycles of starchy endosperm cells. This differential cell cycle effect may be due to the fact that the starchy endosperm, unlike callus, is composed of differentiated and highly determined cells. Transgenic calli and endosperms expressing RepA were evaluated for endopolyploidy, but the vegetative somatic cells of plants regenerated from calli constitutively expressing RepA were not (Gordon-Kamm et al., 2002). Cursory visual examination did not reveal any major morphological defects in RepA-expressing plants. However, a more careful flow cytometric and microscopic analysis of tissues might do so. If some cell types are limited in their capacity for endoreduplication by RRB proteins, I would expect to see increased levels of polysomaty in a subset of tissues, and these tissues should contain active (e.g. unphosphorylated) RRB protein in wild-type plants. However, if positional cues limit

endoreduplication regardless of RRB status, I would expect to find no, or very little, increase in endopolyploidy in any plant tissue, regardless of RRB phosphorylation status.

Mechanism of storage protein down-regulation

The influence of RepA on endosperm storage functions is perhaps the most perplexing observation in this study. The next experiment to further our understanding of the changes in storage product accumulation as a consequence of RepA expression should be an investigation of the affected gene products at the level of mRNA accumulation. RRB and RepA are both known transcriptional regulators, and it is expected that all of the downregulated proteins identified in Chapter 3 would also exhibit a decrease in mRNA abundance. Second, a delimitation of the domains within RepA necessary to mediate these effects is needed. The failure to influence endoreduplication and the proposed RRB inactivation in maize endosperm during endopolyploidy, suggests that some function, other than RRB-binding, is important for the observed effects of GluB1:RepA on storage product accumulation. However, RRB proteins do influence non-cell cycle regulated gene expression in a phosphorylation-independent manner (Pradhan and Kim, 2002). Additionally, binding of DNA directly, nicking DNA, influencing GRAB-related transcription factors, or associating with chromosomal proteins, by RepA may or may not be related to the changes in gene expression in endosperm (Horvath et al., 1998; Xie et al., 1999; Hanley-Bowdoin et al., 2000; Missich et al., 2000). The use of RepA mutants defective in any of the activities outlined above could be analyzed for both endoreduplication in callus and endosperm gene expression

effects if promoter and terminator sequences for the transgenes were chosen for expression in both callus and endosperm. Following the production of transgenic calli for cell cycle analysis, plants could be regenerated and the endosperms tested for effects on storage product accumulation. As the expression of wild-type RepA in maize endosperm produces a visible phenotype (i.e. small wrinkled kernels), screening of events could be rather rapid.

A number of lines of evidence suggest that RepA is a transcriptional regulator. RepA expression is necessary for high-level transcription of the virion sense strand in some cell types (Gooding et al., 1999; Munoz-Martin et al., 2003). Stimulation of viral promoters has been shown to be dependant on the RRB-binding site (Munoz-Martin et al., 2003). In addition, RepA has been demonstrated to interact with two related transcription factors, GRAB1 and GRAB2 (Xie et al., 1999). This interaction is mediated by a distinct domain in RepA from the RRB binding site, but it may require the RepA dimerization domain (Horvath et al., 1998; Xie et al., 1999). GRAB1 and GRAB2 are of unknown function in plant cells, but they are members of a small plant-specific transcription factor family identified as modulators of plant form (Aida et al., 1997; Aida et al., 1999; Ishida et al., 2000; Takada et al., 2001; Daimon et al., 2003) and mediators of auxin signal transduction (Xie et al., 2000; Xie et al., 2002). Auxin is known to promote both the cell cycle and zein expression in maize endosperm (Lur and Setter, 1993, 1993), suggesting that an explanation of RepA effects via auxin signaling will be complicated and require differential effects on the two phenomena. Examination of cDNA libraries for the closest maize relatives of the wheat GRAB1 and GRAB2 proteins

revealed that neither of the maize genes were represented by cDNAs from endosperm libraries. A more direct investigation of the expression of these genes via RT-PCR would be better able to exclude them from the list of candidates for RepA modulation of gene expression in endosperm.

RepA also interfered with the accumulation of the GBF1 transcription factor, which is both downstream of and a likely transducer of ABA signals (de Vetten and Ferl, 1995; Nakagawa et al., 1996). While it is possible that the GBF1 transcription factor is important for endosperm-specific gene expression (e.g. the zeins genes), RepA is likely to have interfered with a step upstream of GBF1 action. Currently, the relationship of ABA signal transduction to zein gene regulation is not clear. One study reports a minor increase in zein gene expression following exogenous application of ABA to cultured whole kernels (Muller et al., 1997). Another study shows no influence of ABA on zein accumulation (Ober et al., 1991).

If RepA affects gene product accumulation via interference with ABA signal transduction, a number of predictions can be made. Firstly, exogenous ABA application to RepA transgenic material could restore zein accumulation. Second application of ABA-inhibitors should disrupt zein gene expression. If the above two experiments return positive results in then becomes necessary to determine if GBF1, or downstream targets of GBF1, are important for zein accumulation. Co delivery of Zein:GUS fusions (e.g.(Thompson et al., 1990) and GBF1 via particle bombardment of dissected endosperms could be used to test this hypothesis. If GBF1 regulates zein gene

expression, co-delivery of GBF1 and Zein:GUS expression cassettes should result in higher levels of GUS accumulation that the Zein:GUS cassette alone.

An additional potential mechanism for RepA-mediated effects on seed storage functions exists. The PKL protein, a Snf2-like DNA helicase from arabidopsis, encodes an LxCxE interaction motif which would compete with RepA for RRB binding. PKL mutants display GA-dependant phenotypes, and misregulation of genes important for embryo storage functions (Ogas et al., 1997; Ogas et al., 1999). In suspension cultured maize endosperm cells, GA application leads to an increase in protein content and fresh weight (Cao and Shannon, 1997). However, no information is currently available regarding a role for GA in the regulation of storage proteins in intact endosperms. Tests of a hypothetical involvement of PKL in endosperm-specific gene expression and/or RRB-mediated changes in gene expression would be more involved. In vitro interaction tests of RRB with PKL should determine whether or not the LxCxE encodes an RRB-interaction motif or not. If PKL is a genuine RRB interactor, RepA could be tested for it's ability to dissociate the RRB-PKL complex. PKL is an upstream regulator of LEC1 (Ogas et al., 1999), which is important for embryo storage protein gene expression (Lotan et al., 1998; Vicient et al., 2000). A role for LEC1 or LEC1-like genes in endosperm development or zein regulation has not been investigated, and the differences between the regulation of embryo and endosperm storage functions do not suggest a link between the two. However, these genes are a part of the CAAT-box transcription factor important for activation of CAAT-box containing promoters (Lotan et al., 1998; Lee et al., 2003). As

some zeins promoters contain CAAT-boxes (Grasser et al., 1990; Maier et al., 1990; Song et al., 2001) a link is possible.

An additional possible mechanism for transcriptional control may be via major chromosomal proteins. At least three intensely staining proteins were identified by affinity chromatography of nuclear extracts of endosperms with immobilized recombinant RepA. While none of these proteins have been positively identified, they migrate at the same relative molecular mass as histones. Additionally the HmgI/Y proteins, which do not co-migrate with any of the intensely staining bands were also found to bind RepA. HmgI/Y proteins promote zein gene transcription (Grasser et al., 1990; Maier et al., 1990; Ponte et al., 1994; Zhao and Grafi, 2000; Krohn et al., 2002) suggesting that a disruption in HmgI/Y function could affect storage product accumulation. The reduction in GBF1 accumulation may also be an effect of HmgI/Y activity, as transcription factors involved in binding promoter elements within GBF1 show altered in vitro DNA binding and transactivation capacities at different Hmg to Histone H1 ratios (Nakagawa et al., 1996; Schultz et al., 1996). At this time, it is unknown if RepA binds directly to HmgI/Y, and what domains of RepA are required for this association. An investigation into the HmgI/Y binding requirements of RepA and the molecular nature of the histone-like proteins in the eluant from RepA-agarose will be required to begin to address the potential of a direct role for RepA in alterations to chromatin structure.

REFERENCES

Ach RA, Durfee T, Miller AB, Taranto P, Hanley-Bowdoin L, Zambryski PC, Gruissem W (1997) RRB1 and RRB2 encode maize retinoblastoma-related proteins that interact with a plant D-type cyclin and geminivirus replication protein. Mol Cell Biol 17: 5077-5086

Adams S, Vinkenoog R, Spielman M, Dickinson HG, Scott RJ (2000) Parent-of-origin effects on seed development in Arabidopsis thaliana require DNA methylation. Development 127: 2493-2502.

Ahmadi A, Baker DA (1999) Effects of abscisic acid (ABA) on grain filling processes in wheat. Plant Growth Regulation 28: 187-197

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. Plant Cell 9: 841-857.

Aida M, Ishida T, Tasaka M (1999) Shoot apical meristem and cotyledon formation during Arabidopsis embryogenesis: interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. Development 126: 1563-1570.

Ait-Si-Ali S, Polesskaya A, Filleur S, Ferreira R, Duquet A, Robin P, Vervish A, Trouche D, Cabon F, Harel-Bellan A (2000) CBP/p300 histone acetyl-transferase activity is important for the G1/S transition. Oncogene 19: 2430-2437

Allard S, Utley RT, Savard J, Clarke A, Grant P, Brandl CJ, Pillus L, Workman JL, Cote J (1999) NuA4, an essential transcription adaptor/histone H4 acetyltransferase complex containing Esa1p and the ATM-related cofactor Tra1p. Embo J 18: 5108-5119

Alleman M, Doctor J (2000) Genomic imprinting in plants: observations and evolutionary implications. Plant Mol Biol 43: 147-161.

Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Koornneef M (1999) Natural allelic variation at seed size loci in relation to other life history traits of Arabidopsis thaliana. Proc Natl Acad Sci U S A 96: 4710-4717

Aparicio OM, Stout AM, Bell SP (1999) Differential assembly of Cdc45p and DNA polymerases at early and late origins of DNA replication. Proc Natl Acad Sci U S A 96: 9130-9135

Aparicio OM, Weinstein DM, Bell SP (1997) Components and dynamics of DNA replication complexes in S. cerevisiae: redistribution of MCM proteins and Cdc45p during S phase. Cell 91: 59-69

Artlip TS, Madison JT, Setter TL (1995) Water-deficit in developing endosperm of maize - cell-division and nuclear-DNA endoreduplication. Plant Cell Environ 18: 1034-1040

Ascenzi R, Ingram JL, Massel M, Thompson WF, Spiker S, Weissinger AK (2001) The role of cell differentiation state and HMG-I/Y in the expression of transgenes flanked by matrix attachment regions. Transgenic Res 10: 465-470.

Ayaydin F, Vissi E, Meszaros T, Miskolczi P, Kovacs I, Feher A, Dombradi V, Erdodi F, Gergely P, Dudits D (2000) Inhibition of serine/threonine-specific protein phosphatases causes premature activation of cdc2MsF kinase at G2/M transition and early mitotic microtubule organisation in alfalfa. Plant J 23: 85-96.

Baldin V, Ducommun B (1995) Subcellular localisation of human wee1 kinase is regulated during the cell cycle. J Cell Sci 108: 2425-2432.

Baroux C, Spillane C, Grossniklaus U (2002) Genomic imprinting during seed development. Adv Genet 46: 165-214

Barrieu F, Chaumont F, Chrispeels MJ (1998) High expression of the tonoplast aquaporin ZmTIP1 in epidermal and conducting tissues of maize. Plant Physiol 117: 1153-1163

Bass HW, Webster C, OBrian GR, Roberts JK, Boston RS (1992) A maize ribosome-inactivating protein is controlled by the transcriptional activator Opaque-2. Plant Cell 4: 225-234

Bates S, Ryan KM, Phillips AC, Vousden KH (1998) Cell cycle arrest and DNA endoreduplication following p21(Waf1/Cip1) expression. Oncogene 17: 1691-1703

Bell MH, Halford NG, Ormrod JC, Francis D (1993) Tobacco plants transformed with cdc25, a mitotic inducer gene from fission yeast. Plant Mol Biol 23: 445-451.

Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. Annu Rev Biochem 71: 333-374

Ben-Israel H, Kleinberger T (2002) Adenovirus and cell cycle control. Front Biosci 7: d1369-1395

Ben-Israel H, Kleinberger T (2002) Adenovirus and cell cycle control. Front Biosci 7: d1369-1395.

Berger F (1999) Endosperm development. Curr Opin Plant Biol 2: 28-32

Berta G, Fusconi A, Sampo S, Lingua G, Perticone S, Repetto O (2000) Polyploidy in tomato roots as affected by arbuscular mycorrhizal colonization. Plant Soil 226: 37-44

Bethke PC, Swanson SJ, Hillmer S, Jones RL (1998) From storage compartment to lytic organelle: The metamorphosis of the aleurone protein storage vacuole. 82: 399-412

Birchler JA (1993) Dosage analysis of maize endosperm development. Annu Rev Genet 27: 181-204

Birchler JA, Bhadra U, Bhadra MP, Auger DL (2001) Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndromes, and quantitative traits. Dev Biol 234: 275-288.

Blow JJ, Laskey RA (1988) A role for the nuclear envelope in controlling DNA replication within the cell cycle. Nature 332: 546-548

Blow JJ, Sheehan MA, Watson JV, Laskey RA (1989) Nuclear structure and the control of DNA replication in the Xenopus embryo. J Cell Sci Suppl 12: 183-195

Bogre L, Zwerger K, Meskiene I, Binarova P, Csizmadia V, Planck C, Wagner E, Hirt H, Heberle-Bors E (1997) The cdc2Ms kinase Is differently regulated in the cytoplasm and in the nucleus. Plant Physiol 113: 841-852.

Bonello JF, Opsahl-Ferstad HG, Perez P, Dumas C, Rogowsky PM (2000) Esr genes show different levels of expression in the same region of maize endosperm. Gene 246: 219-227

Bonello JF, Sevilla-Lecoq S, Berne A, Risueno MC, Dumas C, Rogowsky PM (2002) Esr proteins are secreted by the cells of the embryo surrounding region. J Exp Bot 53: 1559-1568

Boniotti MB, Gutierrez C (2001) A cell-cycle-regulated kinase activity phosphorylates plant retinoblastoma protein and contains, in Arabidopsis, a CDKA/cyclin D complex. Plant J 28: 341-350

Bosco G, Du W, Orr-Weaver TL (2001) DNA replication control through interaction of E2F-RB and the origin recognition complex. Nat Cell Biol 3: 289-295

Boyer CD, Daniels RR, Shannon JC (1976) Abnormal starch granule formation in Zea mays L. endosperms possessing the amylose-extender mutant. Crop Sci 16: 298-301

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391: 597-601

Brehm A, Miska EA, McCance DJ, Reid JL, Bannister AJ, Kouzarides T (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature 391: 597-601.

Brink RA, Cooper DC (1947) The endosperm in seed development. Bot Rev 13: 479-541

Brown RC, Lemmon BE (2001) The cytoskeleton and spatial control of cytokinesis in the plant life cycle. Protoplasma 215: 35-49

Brown RC, Lemmon BE, Olsen OA (1994) Endosperm development in barley: microtubule involvement in the morphogenetic pathway. Plant Cell 6: 1241-1252.

Brunori A, Forino LMC, Frediani M, Ruberti F (1993) Cell number and polyploidy in the starchy endosperm of Triticum aestivum in relation to seed weight. J Genet and Breed 47: 217-220

Bryans C, Smith DL (1985) Endopolyploidy, cell-volume and nuclear volume interrelationships in cotyledons of the leguminosae. Ann Bot 56: 225-237

Cao H, Shannon JC (1997) Effect of gibberellin on growth, protein secretion, and starch accumulation in maize endosperm suspension cells. 16: 137-140

Capesius I, Stohr M (1974) Endopolyploidy during elongation growth of hypocotyl of Sinapis-alba. Protoplasma 82: 147-153

Carlini LE, Ketudat M, Parsons RL, Prabhakar S, Schmidt RJ, Guiltinan MJ (1999) The maize EmBP-1 orthologue differentially regulates opaque2-dependent gene expression in yeast and cultured maize endosperm cells. Plant Mol Biol 41: 339-349.

Carputo D, Barone A, Cardi T, Sebastiano A, Frusciante L, Peloquin SJ (1997) Endosperm balance number manipulation for direct in vivo germplasm

introgression to potato from a sexually isolated relative (Solanum commersonii Dun.). Proc Natl Acad Sci U S A 94: 12013-12017

Carputo D, Frusciante L, Peloquin SJ (2003) The role of 2n gametes and endosperm balance number in the origin and evolution of polyploids in the tuber-bearing solanums. Genetics 163: 287-294

Carrington JC, Freed DD, Oh CS (1990) Expression of Potyviral Polyproteins in Transgenic Plants Reveals 3 Proteolytic Activities Required For Complete Processing. Embo J 9: 1347-1353

Carvalheira G, Guerra M (1998) The polytene chromosomes of tapetal cells in the anther of some Vigna Savi cultivars and species. Cytobios 94: 161-168

Castellano MM, del Pozo JC, Ramirez-Parra E, Brown S, Gutierrez C (2001) Expression and stability of Arabidopsis CDC6 are associated with endoreplication. Plant Cell 13: 2671-2686.

Cavallini A, Baroncelli S, Lercari B, Cionini G, Rocca M, Damato F (1995) Effect of light and gibberellic-acid on chromosome endoreduplication in leaf epidermis of Triticum-durum Desf. Protoplasma 186: 57-62

Cavallini A, Natali L, Balconi C, Rizzi E, Motto M, Cionini G, Damato F (1995) Chromosome endoreduplication in endosperm cells of two maize genotypes and their progenies. Protoplasma 189: 156-162

Cavallini A, Natali L, Balconi C, Rizzi E, Motto M, Cionini G, D'Amato F (1995) Chromosome endoreduplication in endosperm cells of two maize genotypes and their progenies. Protoplasma 189: 156-162

Cavallini A, Natali L, Cionini G, Balconi C, Damato F (1997) Inheritance of nuclear DNA content in leaf epidermal cells of Zea mays L. Theor Appl Genet 94: 782-787

Cavallini A, Natali L, Tonfoni R, Cionini G, Lercari B (2001) White and UV light effects on cell nuclei in the aurea genotype of Lycopersicum esculentum L. Cytobios 104: 83-98

Cebolla A, Vinardell JM, Kiss E, Olah B, Roudier F, Kondorosi A, Kondorosi E (1999) The mitotic inhibitor ccs52 is required for endoreduplication and ploidy-dependent cell enlargement in plants. Embo J 18: 4476-4484

Chaboute ME, Clement B, Philipps G (2002) S phase and meristem-specific expression of the tobacco RNR1b gene is mediated by an E2F element located in the 5' leader sequence. J Biol Chem 277: 17845-17851.

Chaboute ME, Clement B, Sekine M, Philipps G, Chaubet-Gigot N (2000) Cell cycle regulation of the tobacco ribonucleotide reductase small subunit gene is mediated by E2F-like elements. Plant Cell 12: 1987-2000.

Chan HM, Smith L, La Thangue NB (2001) Role of LXCXE motif-dependent interactions in the activity of the retinoblastoma protein. Oncogene 20: 6152-6163

Charles A, Tang X, Crouch E, Brody JS, Xiao ZX (2001) Retinoblastoma protein complexes with C/EBP proteins and activates C/EBP-mediated transcription. J Cell Biochem 83: 414-425

Charles A, Tang X, Crouch E, Brody JS, Xiao ZX (2001) Retinoblastoma protein complexes with C/EBP proteins and activates C/EBP-mediated transcription. J Cell Biochem 83: 414-425.

Charlton WL, Keen CL, Merriman C, Lynch P, Greenland AJ, Dickinson HG (1995) Endosperm development in Zea mays - implication of gametic imprinting and paternal excess in regulation of transfer layer development. Development 121: 3089-3097

Cheikh N, Jones RJ (1994) Disruption of maize kernel growth and development by heat-stress - role of cytokinin abscisic-acid balance. Plant Physiol 106: 45-51

Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC, Nevins JR (1992) Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc Natl Acad Sci U S A 89: 4549-4553

Chen JG, Zhu J (1999) Genetic effects and genotype x environment interactions for cooking quality traits in Indica-japonica crosses of rice (Oryza sativa L.). Euphytica 109: 9-15

Chittenden T, Livingston DM, Kaelin WG, Jr. (1991) RB associates with an E2F-like, sequence-specific DNA-binding protein. Cold Spring Harb Symp Quant Biol 56: 187-195

Chittenden T, Livingston DM, Kaelin WG, Jr. (1991) The T/E1A-binding domain of the retinoblastoma product can interact selectively with a sequence-specific DNA-binding protein. Cell 65: 1073-1082

Chong JP, Mahbubani HM, Khoo CY, Blow JJ (1995) Purification of an MCM-containing complex as a component of the DNA replication licensing system. Nature 375: 418-421

Christensen CA, Subramanian S, Drews GN (1998) Identification of gametophytic mutations affecting female gametophyte development in Arabidopsis. Dev Biol 202: 136-151.

Cleary AL, Fowke LC, Wang H, John PCL (2002) The effect of ICK1, a plant cyclin-dependent kinase inhibitor, on mitosis in living plant cells. Plant Cell Reports 20: 814-820

Clore AM, Larkins BA (1998) Protein quality and its potential relationship to the cytoskeleton in maize endosperm. Journal of Plant Physiology 152: 630-635

Clutter M, Brady T, Walbot V, Sussex I (1974) Macromolecular-synthesis during plant embryogeny - cellular rates of RNA-synthesis in diploid and polytene cells in bean embryos. J Cell Biol 63: 1097-1102

Cockcroft CE, den Boer BG, Healy JM, Murray JA (2000) Cyclin D control of growth rate in plants. Nature 405: 575-579

Cockcroft CE, den Boer BG, Healy JM, Murray JA (2000) Cyclin D control of growth rate in plants. Nature 405: 575-579.

Colasanti J, Cho SO, Wick S, Sundaresan V (1993) Localization of the functional p34(Cdc2) homolog of maize in root-tip and stomatal complex cells - association with predicted division sites. Plant Cell 5: 1101-1111

Colasanti J, Cho SO, Wick S, Sundaresan V (1993) Localization of the functional p34cdc2 homolog of maize in root tip and stomatal complex cells: association with predicted division sites. Plant Cell 5: 1101-1111.

Colasanti J, Tyers M, Sundaresan V (1991) Isolation and characterization of cDNA clones encoding a functional p34cdc2 homologue from Zea mays. Proc Natl Acad Sci U S A 88: 3377-3381.

Coleman CE, Clore AM, Ranch JP, Higgins R, Lopes MA, Larkins BA (1997) Expression of a mutant alpha-zein creates the floury2 phenotype in transgenic maize. Proc Natl Acad Sci U S A 94: 7094-7097

Coleman CE, Clore AM, Ranch JP, Higgins R, Lopes MA, Larkins BA (1997) Expression of a mutant alpha-zein creates the floury2 phenotype in transgenic maize. Proc Natl Acad Sci U S A 94: 7094-7097.

Collin S, Fernandez-Lobato M, Gooding PS, Mullineaux PM, Fenoll C (1996) The two nonstructural proteins from wheat dwarf virus involved in viral gene expression and replication are retinoblastoma-binding proteins. Virology 219: 324-329

Commuri PD, Jones RJ (1999) Ultrastructural characterization of maize (Zea mays L.) kernels exposed to high temperature during endosperm cell division. Plant Cell Environ 22: 375-385

Commuri PD, Jones RJ (2001) High temperatures during endosperm cell division in maize: A genotypic comparison under in vitro and field conditions. Crop Sci 41: 1122-1130

Condeelis J (1995) Elongation-factor 1-alpha, translation and the cytoskeleton. Trends in Biochemical Sciences 20: 169-170

Cook JG, Park CH, Burke TW, Leone G, DeGregori J, Engel A, Nevins JR (2002) Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. Proc Natl Acad Sci U S A 99: 1347-1352

Cooper DC (1951) Caryopsis development following matings between diploid and tetraploid strains of Zea mays. Am J Bot 38: 702-708

Cooper DC, Brink RA (1945) Seed collapse following matings between diploid and tetrapolid races of Lycopersicon-pimpinellifolium. Genetics 30: 302-303

Correa-Bordes J, Nurse P (1995) p25rum1 orders S phase and mitosis by acting as an inhibitor of the p34cdc2 mitotic kinase. Cell 83: 1001-1009

Cross FR (1991) CLN- and CDC28-dependent stimulation of CLN1 and CLN2 RNA levels: implications for regulation by alpha-factor and by cell cycle progression. Cold Spring Harb Symp Quant Biol 56: 1-8

Dahl M, Meskiene I, Bogre L, Ha DT, Swoboda I, Hubmann R, Hirt H, Heberle-Bors E (1995) The D-type alfalfa cyclin gene cycMs4 complements G1 cyclin-deficient yeast and is induced in the G1 phase of the cell cycle. Plant Cell 7: 1847-1857.

Daimon Y, Takabe K, Tasaka M (2003) The CUP-SHAPED COTYLEDON genes promote adventitious shoot formation on calli. Plant Cell Physiol 44: 113-121.

D'Amato F (1964) Endopolyploidy as factor in plant tissue development. Caryologia 17: 317-325

D'Amato F (1977) Nuclear cytology in relation to development. Cambridge University Press, Cambridge ; New York

D'Amato F (1984) Role of polyploidy in reproductive organs and tissues. *In* BM Johri, ed, Embryology of Angiosperms. Springer-Verlag, New York, pp 519-566

Datar SA, Jacobs HW, de la Cruz AF, Lehner CF, Edgar BA (2000) The Drosophila cyclin D-Cdk4 complex promotes cellular growth. Embo J 19: 4543-4554

de Jager SM, Menges M, Bauer UM, Murra JA (2001) Arabidopsis E2F1 binds a sequence present in the promoter of S-phase-regulated gene AtCDC6 and is a member of a multigene family with differential activities. Plant Mol Biol 47: 555-568.

de la Paz Sanchez M, Torres A, Boniotti MB, Gutierrez C, Vazquez-Ramo JM (2002) PCNA protein associates to Cdk-A type protein kinases in germinating maize. Plant Mol Biol 50: 167-175.

de Nooij JC, Letendre MA, Hariharan IK (1996) A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during Drosophila embryogenesis. Cell 87: 1237-1247.

De Smedt V, Poulhe R, Cayla X, Dessauge F, Karaiskou A, Jessus C, Ozon R (2002) Thr-161 phosphorylation of monomeric Cdc2. Regulation by protein phosphatase 2C in Xenopus oocytes. J Biol Chem 277: 28592-28600

de Vetten NC, Ferl RJ (1995) Characterization of a maize G-box binding factor that is induced by hypoxia. Plant J 7: 589-601

de Vetten NC, Ferl RJ (1995) Characterization of a maize G-box binding factor that is induced by hypoxia. Plant J 7: 589-601.

De Veylder L, Beeckman T, Beemster GT, de Almeida Engler J, Ormenese S, Maes S, Naudts M, Van Der Schueren E, Jacqmard A, Engler G, Inze D (2002) Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J 21: 1360-1368

De Veylder L, Beeckman T, Beemster GT, de Almeida Engler J, Ormenese S, Maes S, Naudts M, Van Der Schueren E, Jacqmard A, Engler G, Inze D (2002) Control of proliferation, endoreduplication and differentiation by the Arabidopsis E2Fa-DPa transcription factor. EMBO J 21: 1360-1368.

De Veylder L, Beeckman T, Beemster GTS, Krols L, Terras P, Landrieu I, Van der Schueren E, Maes S, Naudts M, Inze D (2001) Functional analysis of cyclin-dependent kinase inhibitors of Arabidopsis. Plant Cell 13: 1653-1667

De Veylder L, Beemster GTS, Beeckman T, Inze D (2001) CKS1At overexpression in Arabidopsis thaliana inhibits growth by reducing meristem size and inhibiting cell-cycle progression. Plant J 25: 617-626

Demchenko NP (1994) The completion of cell-proliferation and growth in wheat radicle. Plant Soil 167: 43-49

Deng C, Zhang P, Harper JW, Elledge SJ, Leder P (1995) Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 82: 675-684.

Dilkes BP, Dante RA, Coelho C, Larkins BA (2002) Genetic analyses of endoreduplication in Zea mays endosperm. evidence of sporophytic and zygotic maternal control. Genetics 160: 1163-1177.

Dilkes BP, Dante RA, Coelho C, Larkins BA (2002) Genetic analyses of endoreduplication in Zea mays endosperm: Evidence of sporophytic and zygotic maternal control. Genetics 160: 1163-1177

Doan DN, Linnestad C, Olsen OA (1996) Isolation of molecular markers from the barley endosperm coenocyte and the surrounding nucellus cell layers. Plant Mol Biol 31: 877-886

Doerner P, Jorgensen JE, You R, Steppuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. Nature 380: 520-523

Doerner P, Jorgensen JE, You R, Steppuhn J, Lamb C (1996) Control of root growth and development by cyclin expression. Nature 380: 520-523.

Donnelly PM, Bonetta D, Tsukaya H, Dengler RE, Dengler NG (1999) Cell cycling and cell enlargement in developing leaves of Arabidopsis. Dev Biol 215: 407-419.

Donovan S, Diffley JF (1996) Replication origins in eukaroytes. Curr Opin Genet Dev 6: 203-207

Donovan S, Harwood J, Drury LS, Diffley JF (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. Proc Natl Acad Sci U S A 94: 5611-5616

Du W, Dyson N (1999) The role of RBF in the introduction of G1 regulation during Drosophila embryogenesis. Embo J 18: 916-925

Du W, Vidal M, Xie JE, Dyson N (1996) RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in Drosophila. Genes Dev 10: 1206-1218

Dunaief JL, Strober BE, Guha S, Khavari PA, Alin K, Luban J, Begemann M, Crabtree GR, Goff SP (1994) The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. Cell 79: 119-130.

Duronio RJ, Ofarrell PH (1994) Developmental control of a G(1)-S transcriptional program in drosophila. Development 120: 1503-1515

Durso NA, Cyr RJ (1994) Beyond translation - elongation factor-1-alpha and the cytoskeleton. Protoplasma 180: 99-105

Dutcher SK, Hartwell LH (1983) Genes that act before conjugation to prepare the Saccharomyces cerevisiae nucleus for caryogamy. Cell 33: 203-210.

Dvorak J (1968) Endopolyploidy in roots of Rye secale cereale L. Biol Plant 10: 112-&

Dynlacht BD (1997) Regulation of transcription by proteins that control the cell cycle. Nature 389: 149-152.

Dyson N (1994) pRB, p107 and the regulation of the E2F transcription factor. J Cell Sci Suppl 18: 81-87

Dyson N (1998) The regulation of E2F by pRB-family proteins. Genes Dev 12: 2245-2262

Edgar BA, Orr-Weaver TL (2001) Endoreplication cell cycles: More for less. Cell 105: 297-306

Edmonds BT (1993) Abp50 - an actin-binding elongation factor-1-alpha from dictyostelium-discoideum. Journal of Cellular Biochemistry 52: 134-139

Egelkrout EM, Robertson D, Hanley-Bowdoin L (2001) Proliferating cell nuclear antigen transcription is repressed through an E2F consensus element and activated by geminivirus infection in mature leaves. Plant Cell 13: 1437-1452

Ehlenfeldt MK, Ortiz R (1995) Evidence on the nature and origins of endosperm dosage requirements in Solanum and other angiosperm genera. Sex Plant Reprod 8: 189-196

Elledge SJ (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274: 1664-1672.

Endicott JA, Noble ME, Tucker JA (1999) Cyclin-dependent kinases: inhibition and substrate recognition. Curr Opin Struct Biol 9: 738-744.

Engelen-Eigles G, Jones RJ, Phillips RL (2000) DNA endoreduplication in maize endosperm cells: the effect of exposure to short-term high temperature. Plant Cell Environ 23: 657-663

Engelen-Eigles G, Jones RJ, Phillips RL (2001) DNA endoreduplication in maize endosperm cells is reduced by high temperature during the mitotic phase. Crop Sci 41: 1114-1121

Engler JD, De Vleesschauwer V, Burssens S, Celenza JL, Inze D, Van Montagu M, Engler G, Gheysen G (1999) Molecular markers and cell cycle inhibitors show the importance of cell cycle progression in nematode-induced galls and syncytia. Plant Cell 11: 793-807

Evans LS, Vanthof J (1975) Is polyploidy necessary for tissue differentiation in higher-plants. Am J Bot 62: 1060-1064

Evans MM, Kermicle JL (2001) Interaction between maternal effect and zygotic effect mutations during maize seed development. Genetics 159: 303-315.

Ezhevsky SA, Ho A, Becker-Hapak M, Davis PK, Dowdy SF (2001) Differential regulation of retinoblastoma tumor suppressor protein by G(1) cyclin-dependent kinase complexes in vivo. Mol Cell Biol 21: 4773-4784

Ezhevsky SA, Nagahara H, Vocero-Akbani AM, Gius DR, Wei MC, Dowdy SF (1997) Hypo-phosphorylation of the retinoblastoma protein (pRb) by cyclin D:Cdk4/6 complexes results in active pRb. Proc Natl Acad Sci U S A 94: 10699-10704

Falconer DS, Mackay TFC (1996) Introduction to quantitative genetics, Ed 4th. Longman, Essex, England

Feldmann KA, Coury DA, Christianson ML (1997) Exceptional segregation of a selectable marker (KanR) in Arabidopsis identifies genes important for gametophytic growth and development. Genetics 147: 1411-1422.

Felker FC, Shannon JC (1978) Movement of sugars into corn (Zea-mays-L) kernels - anatomical and microautoradiographic study. 61: 71-71

Felker FC, Shannon JC (1980) Movement of C-14-labeled assimilates into kernels of Zea-mays-L .3. an anatomical examination and micro-auto-radiographic study of assimilate transfer. 65: 864-870

Ferreira PC, Hemerly AS, Van Montagu M, Inze D (1993) A protein phosphatase 1 from Arabidopsis thaliana restores temperature sensitivity of a Schizosaccharomyces pombe cdc25ts/wee1- double mutant. Plant J 4: 81-87.

Ferreira R, Magnaghi-Jaulin L, Robin P, Harel-Bellan A, Trouche D (1998) The three members of the pocket proteins family share the ability to repress E2F activity through recruitment of a histone deacetylase. Proc Natl Acad Sci U S A 95: 10493-10498

Findeisen M, El-Denary M, Kapitza T, Graf R, Strausfeld U (1999) Cyclin A-dependent kinase activity affects chromatin binding of ORC, Cdc6, and MCM in egg extracts of Xenopus laevis. Eur J Biochem 264: 415-426

Flemming AJ, Shen ZZ, Cunha A, Emmons SW, Leroi AM (2000) Somatic polyploidization and cellular proliferation drive body size evolution in nematodes. Proc Natl Acad Sci U S A 97: 5285-5290

Flores-Rozas H, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell M, Hurwitz J (1994) Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc Natl Acad Sci U S A 91: 8655-8659

Fobert PR, Gaudin V, Lunness P, Coen ES, Doonan JH (1996) Distinct classes of cdc2-related genes are differentially expressed during the cell division cycle in plants. Plant Cell 8: 1465-1476.

Foiani M, Ferrari M, Liberi G, Lopes M, Lucca C, Marini F, Pellicioli A, Falconi MM, Plevani P (1998) S-phase DNA damage checkpoint in budding yeast. Biol Chem 379: 1019-1023.

Foolad MR, Jones RA (1992) Models to estimate maternally controlled genetic-variation in quantitative seed characters. Theor Appl Genet 83: 360-366

Fotedar R, Fitzgerald P, Rousselle T, Cannella D, Doree M, Messier H, Fotedar A (1996) p21 contains independent binding sites for cyclin and cdk2: both sites are required to inhibit cdk2 kinase activity. Oncogene 12: 2155-2164

Foucher F, Kondorosi E (2000) Cell cycle regulation in the course of nodule organogenesis in Medicago. Plant MolBiol 43: 773-786

Frangioni JV, Neel BG (1993) Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Anal Biochem 210: 179-187.

Fujii K, Kawano M, Kako S (1999) Effects of benzyladenine and alpha - Naphthaleneacetic acid on cell division and nuclear DNA contents in outer tissue of Cymbidium explants cultured in vitro. J Jpn Soc Hortic Sci 68: 41-48

Furstenthal L, Kaiser BK, Swanson C, Jackson PK (2001) Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. J Cell Biol 152: 1267-1278

Galbraith DW, Harkins KR, Knapp S (1991) Systemic endopolyploidy in Arabidopsis-thaliana. Plant Physiol 96: 985-989

Galitski T, Saldanha AJ, Styles CA, Lander ES, Fink GR (1999) Ploidy regulation of gene expression. Science 285: 251-254.

Garcia P, Cales C (1996) Endoreplication in megakaryoblastic cell lines is accompanied by sustained expression of G1/S cyclins and downregulation of cdc25C. Oncogene 13: 695-703

Gendreau E, Hofte H, Grandjean O, Brown S, Traas J (1998) Phytochrome controls the number of endoreduplication cycles in the Arabidopsis thaliana hypocotyl. Plant J 13: 221-230

Gendreau E, Orbovic V, Hofte H, Traas J (1999) Gibberellin and ethylene control endoreduplication levels in the Arabidopsis thaliana hypocotyl. Planta 209: 513-516

Gendreau E, Traas J, Desnos T, Grandjean O, Caboche M, Hofte H (1997) Cellular basis of hypocotyl growth in Arabidopsis thaliana. Plant Physiol 114: 295-305

Gibbs E, Pan ZQ, Niu H, Hurwitz J (1996) Studies on the in vitro phosphorylation of HSSB-p34 and -p107 by cyclin- dependent kinases. Cyclin-substrate interactions dictate the efficiency of phosphorylation. J Biol Chem 271: 22847-22854.

Gilbert SF (2000) Developmental biology, Ed 6th. Sinauer Associates, Sunderland, Mass.

Gilissen LJW, Vanstaveren MJ, Creemersmolenaar J, Verhoeven HA (1993) Development of polysomaty in seedlings and plants of Cucumis- sativus L. Plant Sci 91: 171-179

Gilissen LJW, vanStaveren MJ, Hakkert JC, Smulders MJM (1996) Competence for regeneration during tobacco internodal development - Involvement of plant age, cell elongation stage, and degree of polysomaty. Plant Physiol 111: 1243-1250

Gillespie PJ, Li A, Blow JJ (2001) Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins. BMC Biochem 2: 15

Giroux MJ, Hannah LC (1994) ADP-glucose pyrophosphorylase in shrunken-2 and brittle-2 mutants of maize. Mol Gen Genet 243: 400-408

Glotzer M (1995) Cell cycle. The only way out of mitosis. Curr Biol 5: 970-972.

Glotzer M, Murray AW, Kirschner MW (1991) Cyclin is degraded by the ubiquitin pathway. Nature 349: 132-138.

Glover DV, Mertz ET (1987) Corn. *In* RA Olson, KJ Frey, eds, Nutritional quality of cereal grains: genetic and agronomic improvement. American Society for Agronomy, Soil Science Society of America, Crop Science Society of America, Madison, WI, pp 184-307

Gomez E, Royo J, Guo Y, Thompson R, Hueros G (2002) Establishment of cereal endosperm expression domains: identification and properties of a maize transfer cell-specific transcription factor, ZmMRP-1. Plant Cell 14: 599-610

Gooding PS, Batty NP, Goldsbrough AP, Mullineaux PM (1999) Plant cell-directed control of virion sense gene expression in wheat dwarf virus. Nucleic Acids Res 27: 1709-1718

Gordon-Kamm W, Dilkes BP, Lowe K, Hoerster G, Sun X, Ross M, Church L, Bunde C, Farrell J, Hill P, Maddock S, Snyder J, Sykes L, Li Z, Woo YM, Bidney D, Larkins BA (2002) Stimulation of the cell cycle and maize transformation by disruption of the plant retinoblastoma pathway. Proc Natl Acad Sci U S A 16: 16

Grafi G (1998) Cell cycle regulation of DNA replication: the endoreduplication perspective. Exp Cell Res 244: 372-378.

Grafi G, Burnett RJ, Helentjaris T, Larkins BA, DeCaprio JA, Sellers WR, Kaelin WG (1996) A maize cDNA encoding a member of the retinoblastoma protein family: Involvement in endoreduplication. Proc Natl Acad Sci U S A 93: 8962-8967

Grafi G, Burnett RJ, Helentjaris T, Larkins BA, DeCaprio JA, Sellers WR, Kaelin WG, Jr. (1996) A maize cDNA encoding a member of the retinoblastoma protein family: involvement in endoreduplication. Proc Natl Acad Sci U S A 93: 8962-8967

Grafi G, Larkins BA (1995) Endoreduplication in maize endosperm - involvement of M-phase- promoting factor inhibition and induction of S-phase-related kinases. Science 269: 1262-1264

Grafi G, Larkins BA (1995) Endoreduplication in maize endosperm - involvement of M-phase-promoting factor inhibition and induction of S-phase-related kinases. Science 269: 1262-1264

Granier C, Tardieu F (1998) Spatial and temporal analyses of expansion and cell cycle in sunflower leaves. A common pattern of development for all zones of a leaf and different leaves of a plant. Plant Physiol 116: 991-1001.

Grasser KD, Maier UG, Haass MM, Feix G (1990) Maize high mobility group proteins bind to CCAAT and TATA boxes of a zein gene promoter. J Biol Chem 265: 4185-4188.

Grasser KD, Maier UG, Haass MM, Feix G (1990) Maize high mobility group proteins bind to CCAAT and TATA boxes of a zein gene promoter. J Biol Chem 265: 4185-4188

Gregory TR, Hebert PDN (1999) The modulation of DNA content: proximate causes and ultimate consequences. Genome Res 9: 317-324

Grimanelli D, Hernandez M, Perotti E, Savidan Y (1997) Dosage effects in the endosperm of diplosporous apomictic Tripsacum (Poaceae). Sex Plant Reprod 10: 279-282

Grini PE, Jurgens G, Hulskamp M (2002) Embryo and endosperm development is disrupted in the female gametophytic capulet mutants of Arabidopsis. Genetics 162: 1911-1925

Guadagno TM, Newport JW (1996) Cdk2 kinase is required for entry into mitosis as a positive regulator of Cdc2-cyclin B kinase activity. Cell 84: 73-82.

Guillen P, Debon RM, Grasser KD, Harrsch P, Grimm R, Ponte I, Palau J (1998) Isolation and characterization of a 28-kDa HMG-like protein that binds to A/T-rich distal promoter regions of zein genes. Plant Science 135: 31-38

Guo M, Davis D, Birchler JA (1996) Dosage effects on gene expression in a maize ploidy series. Genetics 142: 1349-1355.

Gutierrez C (2000) DNA replication and cell cycle in plants: learning from geminiviruses. EMBO J 19: 792-799

Gutierrez C (2000) Geminiviruses and the plant cell cycle. Plant Mol Biol 43: 763-772

Haber JE (1992) Mating-type gene switching in Saccharomyces cerevisiae. Trends Genet 8: 446-452.

Haig D, Westoby M (1989) Parent-Specific Gene-Expression and the Triploid Endosperm. Am Nat 134: 147-155

Haig D, Westoby M (1991) Genomic imprinting in endosperm - its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. Philos Trans R Soc Lond Ser B-Biol Sci 333: 1-13

Haig D, Wilkins JF (2000) Genomic imprinting, sibling solidairity and the logic of collective action. Philos Trans R Soc Lond B Biol Sci 355: 1593-1597.

Hanley-Bowdoin L, Settlage SB, Orozco BM, Nagar S, Robertson D (2000) Geminiviruses: models for plant DNA replication, transcription, and cell cycle regulation. Crit Rev Biochem Mol Biol 35: 105-140

Hansen K, Farkas T, Lukas J, Holm K, Ronnstrand L, Bartek J (2001) Phosphorylation-dependent and -independent functions of p130 cooperate to evoke a sustained G(1) block. EMBO Journal 20: 422-432

Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98: 859-869

Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC (1999) Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. Cell 98: 859-869.

Harlow E, Lane D (1988) Antibodies: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY

Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L, Swindell E, et al. (1995) Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell 6: 387-400.

Hartwell L (1992) Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell 71: 543-546

Hartwell LH, Kastan MB (1994) Cell cycle control and cancer. Science 266: 1821-1828

Hata S, Kouchi H, Suzuka I, Ishii T (1991) Isolation and characterization of cDNA clones for plant cyclins. EMBO J 10: 2681-2688.

Hattori N, Davies TC, Anson-Cartwright L, Cross JC (2000) Periodic expression of the cyclin-dependent kinase inhibitor p57(Kip2) in trophoblast giant cells defines a G2-like gap phase of the endocycle. Mol Biol Cell 11: 1037-1045

Hauser PJ, Agrawal D, Chu B, Pledger WJ (1997) p107 and p130 associated cyclin A has altered substrate specificity. J Biol Chem 272: 22954-22959.

Hayles J, Fisher D, Woollard A, Nurse P (1994) Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex. Cell 78: 813-822

Hayman BI (1960) Maximum likelihood estimation of genetic components of variation. Biometrics 16: 369-381

Healy JM, Menges M, Doonan JH, Murray JA (2001) The Arabidopsis D-type cyclins CycD2 and CycD3 both interact in vivo with the PSTAIRE cyclin-dependent kinase Cdc2a but are differentially controlled. J Biol Chem 276: 7041-7047.

Hemerly A, Bergounioux C, Van Montagu M, Inze D, Ferreira P (1992) Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from Arabidopsis thaliana. Proc Natl Acad Sci U S A 89: 3295-3299

Hemerly A, Engler Jde A, Bergounioux C, Van Montagu M, Engler G, Inze D, Ferreira P (1995) Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. EMBO J 14: 3925-3936

Hemerly AS, Ferreira P, de Almeida Engler J, Van Montagu M, Engler G, Inze D (1993) cdc2a expression in Arabidopsis is linked with competence for cell division. Plant Cell 5: 1711-1723

Hemerly AS, Ferreira PC, Van Montagu M, Engler G, Inze D (2000) Cell division events are essential for embryo patterning and morphogenesis: studies on dominant-negative cdc2aAt mutants of arabidopsis. Plant J 23: 123-130

Herrera RE, Chen F, Weinberg RA (1996) Increased histone H1 phosphorylation and relaxed chromatin structure in Rb-deficient fibroblasts. Proc Natl Acad Sci U S A 93: 11510-11515

Higashi H, Suzuki-Takahashi I, Taya Y, Segawa K, Nishimura S, Kitagawa M (1995) Differences in substrate specificity between Cdk2-cyclin A and Cdk2- cyclin E in vitro. Biochem Biophys Res Commun 216: 520-525.

Hill WG (1998) Selection with recurrent backcrossing to develop congenic lines for quantitative trait loci analysis. Genetics 148: 1341-1352

Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inze D, Beeckman T (2002) Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell 14: 2339-2351.

Hindley J, Phear G, Stein M, Beach D (1987) Sucl+ encodes a predicted 13-kilodalton protein that is essential for cell viability and is directly involved in the division cycle of Schizosaccharomyces pombe. Mol Cell Biol 7: 504-511.

Hirt H (1996) In and out of the plant cell cycle. Plant Mol Biol 31: 459-464.

Hirt H, Mink M, Pfosser M, Bogre L, Gyorgyey J, Jonak C, Gartner A, Dudits D, Heberle-Bors E (1992) Alfalfa cyclins: differential expression during the cell cycle and in plant organs. Plant Cell 4: 1531-1538.

Hollung K, Espelund M, Schou K, Jakobsen KS (1997) Developmental, stress and ABA modulation of mRNA levels for bZip transcription factors and Vp1 in barley embryos and embryo-derived suspension cultures. Plant Mol Biol 35: 561-571

Holmes JK, Solomon MJ (1996) A predictive scale for evaluating cyclin-dependent kinase substrates. A comparison of p34cdc2 and p33cdk2. J Biol Chem 271: 25240-25246.

Horton LE, Templeton DJ (1997) The cyclin box and C-terminus of cyclins A and E specify CDK activation and substrate specificity. Oncogene 14: 491-498.

Horvath GV, Pettko-Szandtner A, Nikovics K, Bilgin M, Boulton M, Davies JW, Gutierrez C, Dudits D (1998) Prediction of functional regions of the maize streak virus replication-associated proteins by protein-protein interaction analysis. Plant Mol Biol 38: 699-712

Howden R, Park SK, Moore JM, Orme J, Grossniklaus U, Twell D (1998) Selection of T-DNA-tagged male and female gametophytic mutants by segregation distortion in Arabidopsis. Genetics 149: 621-631.

Hsieh J, Liu J, Kostas SA, Chang C, Sternberg PW, Fire A (1999) The RING finger/B-box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in Caenorhabditis elegans. Genes Dev 13: 2958-2970.

Hsieh J, Liu J, Kostas SA, Chang C, Sternberg PW, Fire A (1999) The RING finger/B-box factor TAM-1 and a retinoblastoma-like protein LIN-35 modulate context-dependent gene silencing in Caenorhabditis elegans. Genes Dev 13: 2958-2970

Hua XH, Newport J (1998) Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. J Cell Biol 140: 271-281

Huang BQ, Sheridan WF (1996) Embryo Sac Development in the Maize indeterminate gametophyte1 Mutant: Abnormal Nuclear Behavior and Defective Microtubule Organization. Plant Cell 8: 1391-1407.

Hueros G, Gomez E, Cheikh N, Edwards J, Weldon M, Salamini F, Thompson RD (1999) Identification of a promoter sequence from the BETL1 gene cluster able to confer transfer-cell-specific expression in transgenic maize. Plant Physiol 121: 1143-1152

Hueros G, Royo J, Maitz M, Salamini F, Thompson RD (1999) Evidence for factors regulating transfer cell-specific expression in maize endosperm. Plant Mol Biol 41: 403-414

Hueros G, Varotto S, Salamini F, Thompson RD (1995) Molecular characterization of BET1, a gene expressed in the endosperm transfer cells of maize. Plant Cell 7: 747-757

Hunter BG, Beatty MK, Singletary GW, Hamaker BR, Dilkes BP, Larkins BA, Jung R (2002) Maize opaque endosperm mutations create extensive changes in patterns of gene expression. Plant Cell 14: XX-XX

Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J, Makker J, Walker E, Jackman M, Xie Q, Bannister AJ, Kouzarides T, Gutierrez C, Doonan JH, Murray JA (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. Plant Mol Biol 37: 155-169.

Huntley R, Healy S, Freeman D, Lavender P, de Jager S, Greenwood J, Makker J, Walker E, Jackman M, Xie Q, Bannister AJ, Kouzarides T, Gutierrez C, Doonan JH, Murray JA (1998) The maize retinoblastoma protein homologue ZmRb-1 is regulated during leaf development and displays conserved interactions with G1/S regulators and plant cyclin D (CycD) proteins. Plant Mol Biol 37: 155-169

Hurst LD, McVean GT (1998) Do we understand the evolution of genomic imprinting? Curr Opin Genet Dev 8: 701-708.

Hush J, Wu LP, John PCL, Hepler LH, Hepler PK (1996) Plant mitosis promoting factor disassembles the microtubule preprophase band and accelerates prophase progression in Tradescantia. Cell Biol Int 20: 275-287

Igarashi M, Nagata A, Jinno S, Suto K, Okayama H (1991) Wee1(+)-like gene in human cells. Nature 353: 80-83.

Illidge TM, Cragg MS, Fringes B, Olive P, Erenpreisa JA (2000) Polyploid giant cells provide a survival mechanism for p53 mutant cells after DNA damage. 24: 621-633

Ishida T, Aida M, Takada S, Tasaka M (2000) Involvement of CUP-SHAPED COTYLEDON genes in gynoecium and ovule development in Arabidopsis thaliana. Plant Cell Physiol 41: 60-67.

Ishimi Y (1997) A DNA helicase activity is associated with an MCM4, -6, and -7 protein complex. J Biol Chem 272: 24508-24513

Jacqmard A, De Veylder L, Segers G, Engler JD, Bernier G, Van Montagu M, Inze D (1999) Expression of CKS1At in Arabidopsis thaliana indicates a role for the protein in both the mitotic and the endoreduplication cycle. Planta 207: 496-504

Jallepalli PV, Brown GW, Muzi-Falconi M, Tien D, Kelly TJ (1997) Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. Genes Dev 11: 2767-2779.

Jasinski S, Riou-Khamlichi C, Roche O, Perennes C, Bergounioux C, Glab N (2002) The CDK inhibitor NtKIS1a is involved in plant development, endoreduplication and restores normal development of cyclin D3; 1- overexpressing plants. J Cell Sci 115: 973-982.

Jiang W, Wells NJ, Hunter T (1999) Multistep regulation of DNA replication by Cdk phosphorylation of HsCdc6. Proc Natl Acad Sci U S A 96: 6193-6198

Jiang Z, Liang P, Leng R, Guo Z, Liu Y, Liu XD, Bubnic S, Keating A, Murray D, Goss P, Zacksenhaus E (2000) E2F1 and p53 are dispensable, whereas p21(Waf1/Cip1) cooperates with Rb to restrict endoreduplication and apoptosis during skeletal myogenesis. Dev Biol 227: 28-41

John PC (1984) Control of the cell division cycle in Chlamydomonas. Microbiol Sci 1: 96-101

John PC, Sek FJ, Lee MG (1989) A homolog of the cell cycle control protein p34cdc2 participates in the division cycle of Chlamydomonas, and a similar protein is detectable in higher plants and remote taxa. Plant Cell 1: 1185-1193

Johnson DG (2000) The paradox of E2F1: oncogene and tumor suppressor gene. Mol Carcinog 27: 151-157.

Johnson DG, Schwarz JK, Cress WD, Nevins JR (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature 365: 349-352.

Jones RJ, Schreiber BMN, Roessler JA (1996) Kernel sink capacity in maize: genotypic and maternal regulation. Crop Sci 36: 301-306

Joubes J, Chevalier C, Dudits D, Heberle-Bors E, Inze D, Umeda M, Renaudi JP (2000) CDK-related protein kinases in plants. Plant Mol Biol 43: 607-620.

Kaelin WG, Jr., Krek W, Sellers WR, DeCaprio JA, Ajchenbaum F, Fuchs CS, Chittenden T, Li Y, Farnham PJ, Blanar MA, et al. (1992) Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell 70: 351-364

Kaiser P, Moncollin V, Clarke DJ, Watson MH, Bertolaet BL, Reed SI, Bailly E (1999) Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets. Genes Dev 13: 1190-1202

Karaiskou A, Jessus C, Brassac T, Ozon R (1999) Phosphatase 2A and polo kinase, two antagonistic regulators of cdc25 activation and MPF auto-amplification. J Cell Sci 112 (Pt 21): 3747-3756

Katsiotis A, Hanneman RE, Forsberg RA (1995) Endosperm balance number and the polar-nuclei activation hypotheses for endosperm development in interspecific crosses of solanaceae and gramineae, respectively. Theor Appl Genet 91: 848-855

Kausch AP, Horner HT (1984) Increased nuclear DNA content in raphide crystal idioblasts during development in Vanilla planifolia L. (Orchidaceae). Eur J Cell Biol 33: 7-12

Kelly BL, Wolfe KG, Roberts JM (1998) Identification of a substrate-targeting domain in cyclin E necessary for phosphorylation of the retinoblastoma protein. Proc Natl Acad Sci U S A 95: 2535-2540.

Kennedy BK, Barbie DA, Classon M, Dyson N, Harlow E (2000) Nuclear organization of DNA replication in primary mammalian cells. Genes Dev 14: 2855-2868

Khan SH, Wahl GM (1998) p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G1 arrest. Cancer Res 58: 396-401.

Kiesselbach TA (1922) Corn investigations. Nebr St Res Bul 20: 1-151

Kiesselbach TA (1949) The structure and reproduction of corn. Nebr Agric Exp Stn Ann Rep 161: 1-96

Kindiger B, Beckett JB (1992) Popcorn germplasm as a parental source for maize X Tripsacum dactyloides hybridization. Maydica 37: 245-249

King RW, Deshaies RJ, Peters JM, Kirschner MW (1996) How proteolysis drives the cell cycle. Science 274: 1652-1659.

King RW, Glotzer M, Kirschner MW (1996) Mutagenic analysis of the destruction signal of mitotic cyclins and structural characterization of ubiquitinated intermediates. Mol Biol Cell 7: 1343-1357.

Kingham KI, Duckett JG, Glyn MCP, Leitch AR (1995) Nuclear differentiation in the filamentous caulonema of the moss Funaria hygrometrica. New Phytol 131: 543-556

Kinoshita T, Yadegari R, Harada JJ, Goldberg RB, Fischer RL (1999) Imprinting of the MEDEA polycomb gene in the Arabidopsis endosperm. Plant Cell 11: 1945-1952

Kitagawa M, Higashi H, Jung HK, Suzuki-Takahashi I, Ikeda M, Tamai K, Kato J, Segawa K, Yoshida E, Nishimura S, Taya Y (1996) The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. Embo J 15: 7060-7069.

Kiyosue T, Ohad N, Yadegari R, Hannon M, Dinneny J, Wells D, Katz A, Margossian L, Harada JJ, Goldberg RB, Fischer RL (1999) Control of fertilization-independent endosperm development by the MEDEA polycomb gene in Arabidopsis. Proc Natl Acad Sci U S A 96: 4186-4191

Klemm RD, Austin RJ, Bell SP (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell 88: 493-502

Klemm RD, Bell SP (2001) ATP bound to the origin recognition complex is important for preRC formation. Proc Natl Acad Sci U S A 98: 8361-8367

Klucher KM, Chow H, Reiser L, Fischer RL (1996) The AINTEGUMENTA gene of Arabidopsis required for ovule and female gametophyte development is related to the floral homeotic gene APETALA2. Plant Cell 8: 137-153.

Knudsen ES, Buckmaster C, Chen TT, Feramisco JR, Wang JY (1998) Inhibition of DNA synthesis by RB: effects on G1/S transition and S-phase progression. Genes Dev 12: 2278-2292

Kondorosi E, Roudier F, Gendreau E (2000) Plant cell-size control: growing by ploidy? Curr Opin Plant Biol 3: 488-492

Kosugi S, Ohashi Y (1997) PCF1 and PCF2 specifically bind to cis elements in the rice proliferating cell nuclear antigen gene. Plant Cell 9: 1607-1619.

Kosugi S, Ohashi Y (2002) E2F sites that can interact with E2F proteins cloned from rice are required for meristematic tissue-specific expression of rice and tobacco proliferating cell nuclear antigen promoters. Plant J 29: 45-59.

Kosugi S, Ohashi Y (2002) Interaction of the Arabidopsis E2F and DP proteins confers their concomitant nuclear translocation and transactivation. Plant Physiol 128: 833-843.

Kovesdi I, Reichel R, Nevins JR (1987) Role of an adenovirus E2 promoter binding factor in E1A-mediated coordinate gene control. Proc Natl Acad Sci U S A 84: 2180-2184

Kowles RV, McMullen MD, Yerk G, Phillips RL, Kraemer S, Srienc F (1992) Endosperm mitotic-activity and endoreduplication in maize affected by defective kernel mutations. Genome 35: 68-77

Kowles RV, Phillips RL (1985) DNA amplification patterns in maize endosperm nuclei during kernel development. Proc Natl Acad Sci U S A 82: 7010-7014

Kowles RV, Srienc F, Phillips RL (1990) Endoreduplication of nuclear-DNA in the developing maize endosperm. Dev Genet 11: 125-132

Kowles RV, Yerk GL, Haas KM, Phillips RL (1997) Maternal effects influencing DNA endoreduplication in developing endosperm of Zea mays. Genome 40: 798-805

Kranz E, von Wiegen P, Quader H, Lorz H (1998) Endosperm development after fusion of isolated, single maize sperm and central cells in vitro. Plant Cell 10: 511-524

Krek W, Nigg EA (1992) Cell cycle regulation of vertebrate p34cdc2 activity: identification of Thr161 as an essential in vivo phosphorylation site. New Biol 4: 323-329.

Krek W, Xu G, Livingston DM (1995) Cyclin A-kinase regulation of E2F-1 DNA binding function underlies suppression of an S phase checkpoint. Cell 83: 1149-1158.

Krohn NM, Yanagisawa S, Grasser KD (2002) Specificity of the stimulatory interaction between chromosomal HMGB proteins and the transcription factor Dof2 and its negative regulation by protein kinase CK2-mediated phosphorylation. J Biol Chem 277: 32438-32444.

Krude T, Jackman M, Pines J, Laskey RA (1997) Cyclin/Cdk-dependent initiation of DNA replication in a human cell-free system. Cell 88: 109-119

Kudo N, Kimura Y (2001) Flow cytometric evidence for endopolyploidy in seedlings of some Brassica species. Theor Appl Genet 102: 104-110

Kudo N, Kimura Y (2002) Nuclear DNA endoreduplication during petal development in cabbage: relationship between ploidy levels and cell size. J Exp Bot 53: 1017-1023

Kumagai A, Dunphy WG (1991) The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. Cell 64: 903-914.

Lacy S, Whyte P (1997) Identification of a p130 domain mediating interactions with cyclin A/cdk 2 and cyclin E/cdk 2 complexes. Oncogene 14: 2395-2406.

Ladenstein R, Ambros IM, Potschger U, Amann G, Urban C, Fink FM, Schmitt K, Jones R, Slociak M, Schilling F, Ritter J, Berthold F, Gadner H, Ambros PF (2001) Prognostic significance of DNA di-tetraploidy in neuroblastoma. 36: 83-92

Lagunes-Espinoza LD, Huyghe C, Bousseau D, Barre P, Papineau J (2000) Endoreduplication occurs during pod wall development in temperate grain legumes. Ann Bot 86: 185-190

Lai J, Messing J (2001) A pair of putative fie genes and it parent of origin dependant allelic expression in maize. *In* Maize Genetics Conference Abstracts, Vol 43, p p39

Lane ME, Sauer K, Wallace K, Jan YN, Lehner CF, Vaessin H (1996) Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during Drosophila development. Cell 87: 1225-1235.

Lang SE, McMahon SB, Cole MD, Hearing P (2001) E2F transcriptional activation requires TRRAP and GCN5 cofactors. J Biol Chem 276: 32627-32634

Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo Y, Liu Y (2001) Investigating the hows and whys of DNA endoreduplication. J Exp Bot 52: 183-192.

Laurie JD, Zhang G, McGann LE, Gordon-Kamm WJ, Cass DD (1999) A novel technique for the partial isolation of maize embryo sacs and subsequent regeneration of plants. In Vitro Cell Dev-Pl 35: 320-325

Lechner T, Lusser A, Pipal A, Brosch G, Loidl A, Goralik-Schramel M, Sendra R, Wegener S, Walton JD, Loidl P (2000) RPD3-type histone deacetylases in maize embryos. Biochemistry 39: 1683-1692.

Lee DG, Makhov AM, Klemm RD, Griffith JD, Bell SP (2000) Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. Embo J 19: 4774-4782

Lee H, Fischer RL, Goldberg RB, Harada JJ (2003) Arabidopsis LEAFY COTYLEDON1 represents a functionally specialized subunit of the CCAAT binding transcription factor. Proc Natl Acad Sci U S A 100: 2152-2156.

Lehner CF, Lane ME (1997) Cell cycle regulators in Drosophila: downstream and part of developmental decisions. J Cell Sci 110: 523-528

Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK (1997) Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. Genes Dev 11: 3365-3374

Leise W, 3rd, Mueller PR (2002) Multiple Cdk1 inhibitory kinases regulate the cell cycle during development. Dev Biol 249: 156-173.

Lemontey C, Mousset-Declas C, Munier-Jolain N, Boutin JP (2000) Maternal genotype influences pea seed size by controlling both mitotic activity during early embryogenesis and final endoreduplication level/cotyledon cell size in mature seed. J Exp Bot 51: 167-175

Lending CR, Larkins BA (1989) Changes in the zein composition of protein bodies during maize endosperm development. Plant Cell 1: 1011-1023

Lin B (1984) Ploidy barrier to endosperm development in maize. Genetics 107: 103-115

Lin BY (1982) Association of endosperm reduction with parental imprinting in maize. Genetics 100: 475-486

Liu G, Edmonds BT, Condeelis J (1996) pH, EF-1 alpha and the cytoskeleton. Trends in Cell Biology 6: 168-171

Liu L, Saunders K, Thomas CL, Davies JW, Stanley J (1999) Bean yellow dwarf virus RepA, but not rep, binds to maize retinoblastoma protein, and the virus tolerates mutations in the consensus binding motif. Virology 256: 270-279

Lopes MA, Coleman CE, Kodrzycki R, Lending CR, Larkins BA (1994) Synthesis of an unusual alpha-zein protein is correlated with the phenotypic effects of the floury2 mutation in maize. Mol Gen Genet 245: 537-547

Lopes MA, Larkins BA (1993) Endosperm origin, development, and function. Plant Cell 5: 1383-1399

Lopez-Girona A, Mondesert O, Leatherwood J, Russell P (1998) Negative regulation of Cdc18 DNA replication protein by Cdc2. Mol Biol Cell 9: 63-73

Lotan T, Ohto M, Yee KM, West MA, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ (1998) Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. Cell 93: 1195-1205.

Lowe K, Bowen B, Hoerster G, Ross M, Bond D, Pierce D, Gordon-Kamm B (1995) Germline Transformation of Maize Following Manipulation of Chimeric Shoot Meristems. Bio-Technol 13: 677-682

Lu X, Horvitz HR (1998) lin-35 and lin-53, two genes that antagonize a C. elegans Ras pathway, encode proteins similar to Rb and its binding protein RbAp48. Cell 95: 981-991.

Lui H, Wang H, Delong C, Fowke LC, Crosby WL, Fobert PR (2000) The Arabidopsis Cdc2a-interacting protein ICK2 is structurally related to ICK1 and is a potent inhibitor of cyclin-dependent kinase activity in vitro. Plant J 21: 379-385.

Lund G, Ciceri P, Viotti A (1995) Maternal-specific demethylation and expression of specific alleles of zein genes in the endosperm of Zea mays L. Plant J 8: 571-581

Luo M, Bilodeau P, Dennis ES, Peacock WJ, Chaudhury A (2000) Expression and parent-of-origin effects for FIS2, MEA, and FIE in the endosperm and embryo of developing Arabidopsis seeds. Proc Natl Acad Sci U S A 97: 10637-10642

Luo M, Bilodeau P, Koltunow A, Dennis ES, Peacock WJ, Chaudhury AM (1999) Genes controlling fertilization-independent seed development in Arabidopsis thaliana. Proc Natl Acad Sci U S A 96: 296-301

Luo RX, Postigo AA, Dean DC (1998) Rb interacts with histone deacetylase to repress transcription. Cell 92: 463-473.

Lur HS, Setter TL (1993) Endosperm development of maize defective-kernel (Dek) mutants - auxin and cytokinin levels. Ann Bot 72: 1-6

Lur HS, Setter TL (1993) Role of auxin in maize endosperm development - timing of nuclear-DNA endoreduplication, zein expression, and cytokinin. Plant Physiol 103: 273-280

Luszczek D, Swierczynska J, Bohdanowicz J (2000) Polyploidization of suspensor basal cell in Triglochin maritimum L. (Juncaginaceae). Acta Biol Crac Ser Bot 42: 131-137

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland, Mass.

MacAuley A, Cross JC, Werb Z (1998) Reprogramming the cell cycle for endoreduplication in rodent trophoblast cells. Mol Biol Cell 9: 795-807

Mackay TFC (2001) The genetic architecture of quantitative traits. Annu Rev Genet 35: 303-339

Magnaghi-Jaulin L, Groisman R, Naguibneva I, Robin P, Lorain S, Le Villain JP, Troalen F, Trouche D, Harel-Bellan A (1998) Retinoblastoma protein represses transcription by recruiting a histone deacetylase. Nature 391: 601-605.

Magyar Z, Meszaros T, Miskolczi P, Deak M, Feher A, Brown S, Kondorosi E, Athanasiadis A, Pongor S, Bilgin M, Bako L, Koncz C, Dudits D (1997) Cell cycle phase specificity of putative cyclin-dependent kinase variants in synchronized alfalfa cells. Plant Cell 9: 223-235.

Maier UG, Grasser KD, Haass MM, Feix G (1990) Multiple proteins bind to the P2 promoter region of the zein gene pMS1 of maize. Mol Gen Genet 221: 164-170.

Malinowski S, Maszewski J (1994) DNA endoreduplication, RNA and protein synthesis during growth and development of the antheridial basal cell in Chara vulgaris L. Folia Histochem Cytobiol 32: 137-142.

Mambelli S, Setter TL (1998) Inhibition of maize endosperm cell division and endoreduplication by exogenously applied abscisic acid. Physiol Plant 104: 266-272

Markey MP, Angus SP, Strobeck MW, Williams SL, Gunawardena RW, Aronow BJ, Knudsen ES (2002) Unbiased analysis of RB-mediated transcriptional repression identifies novel targets and distinctions from E2F action. Cancer Res 62: 6587-6597

Marks MD, Lindell JS, Larkins BA (1985) Quantitative analysis of the accumulation of Zein mRNA during maize endosperm development. J Biol Chem 260: 16445-16450.

Martin-Castellanos C, Edgar BA (2002) A characterization of the effects of Dpp signaling on cell growth and proliferation in the Drosophila wing. Development 129: 1003-1013

Masuda T, Mimura S, Takisawa H (2003) CDK- and Cdc45-dependent priming of the MCM complex on chromatin during S-phase in Xenopus egg extracts: possible activation of MCM helicase by association with Cdc45. Genes Cells 8: 145-161

Masuelli RW (2001) Mitotic-cycle time and the development of embryo and endosperm in compatible and incompatible crosses in tuber-bearing Solanum species. Genome 44: 426-431

Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, Sherr CJ (1992) Identification and properties of an atypical catalytic subunit (p34PSK- J3/cdk4) for mammalian D type G1 cyclins. Cell 71: 323-334.

Mayer U, Herzog U, Berger F, Inze D, Jurgens G (1999) Mutations in the pilz group genes disrupt the microtubule cytoskeleton and uncouple cell cycle progression from cell division in Arabidopsis embryo and endosperm. Eur J Cell Biol 78: 100-108.

McKibbin RS, Halford NG, Francis D (1998) Expression of fission yeast cdc25 alters the frequency of lateral root formation in transgenic tobacco. Plant Mol Biol 36: 601-612.

McVean GT, Hurst LD (1997) Molecular evolution of imprinted genes: no evidence for antagonistic coevolution. Proc R Soc Lond B Biol Sci 264: 739-746.

Meijer M, Murray JAH (2000) The role and regulation of D-type cyclins in the plant cell cycle. Plant Mol Biol 43: 621-633.

Melaragno JE, Mehrotra B, Coleman AW (1993) Relationship between endopolyploidy and cell-size in epidermal tissue of arabidopsis. Plant Cell 5: 1661-1668

Mena M, Vicente-Carbajosa J, Schmidt RJ, Carbonero P (1998) An endosperm-specific DOF protein from barley, highly conserved in wheat, binds to and activates transcription from the prolamin-box of a native B-hordein promoter in barley endosperm. Plant J 16: 53-62.

Mendenhall MD (1993) An inhibitor of p34CDC28 protein kinase activity from Saccharomyces cerevisiae. Science 259: 216-219.

Mendenhall MD, al-Jumaily W, Nugroho TT (1995) The Cdc28 inhibitor p40SIC1. Prog Cell Cycle Res 1: 173-185

Menges M, Murray JA (2002) Synchronous Arabidopsis suspension cultures for analysis of cell-cycle gene activity. Plant J 30: 203-212.

Meskiene I, Bogre L, Dahl M, Pirck M, Ha DT, Swoboda I, Heberle-Bors E, Ammerer G, Hirt H (1995) cycMs3, a novel B-type alfalfa cyclin gene, is induced in the G0-to-G1 transition of the cell cycle. Plant Cell 7: 759-771.

Meszaros T, Miskolczi P, Ayaydin F, Pettko-Szandtner A, Peres A, Magyar Z, Horvath GV, Bako L, Feher A, Dudits D (2000) Multiple cyclin-dependent kinase complexes and phosphatases control G2/M progression in alfalfa cells. Plant Mol Biol 43: 595-605.

Meyer CA, Jacobs HW, Datar SA, Du W, Edgar BA, Lehner CF (2000) Drosophila Cdk4 is required for normal growth and is dispensable for cell cycle progression. Embo J 19: 4533-4542

Michael WM, Newport J (1998) Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. Science 282: 1886-1889.

Mishiba KI, Okamoto T, Mii M (2001) Increasing ploidy level in cell suspension cultures of Doritaenopsis by exogenous application of 2,4- dichlorophenoxyacetic acid. Physiol Plant 112: 142-148

Missich R, Ramirez-Parra E, Gutierrez C (2000) Relationship of oligomerization to DNA binding of Wheat dwarf virus RepA and Rep proteins. Virology 273: 178-188

Montagnoli A, Fiore F, Eytan E, Carrano AC, Draetta GF, Hershko A, Pagano M (1999) Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. Genes Dev 13: 1181-1189.

Moran E (1988) A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A products. Nature 334: 168-170

Moran E (1993) Interaction of adenoviral proteins with pRB and p53. Faseb J 7: 880-885

Moreno S, Nurse P (1994) Regulation of progression through the G1 phase of the cell cycle by the rum1+ gene. Nature 367: 236-242

Morgan DO (1995) Principles of CDK regulation. Nature 374: 131-134.

Morgan DO (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 13: 261-291

Morisaki H, Fujimoto A, Ando A, Nagata Y, Ikeda K, Nakanishi M (1997) Cell cycle-dependent phosphorylation of p27 cyclin-dependent kinase (Cdk) inhibitor by cyclin E/Cdk2. Biochem Biophys Res Commun 240: 386-390.

Motorin YA, Wolfson AD, Orlovsky AF, Gladilin KL (1988) Mammalian valyl-transfer RNA-synthetase forms a complex with the first elongation-factor. Febs Letters 238: 262-264

Muchardt C, Yaniv M (2001) When the SWI/SNF complex remodels...the cell cycle. Oncogene 20: 3067-3075.

Mueller PR, Coleman TR, Dunphy WG (1995) Cell cycle regulation of a Xenopus Wee1-like kinase. Mol Biol Cell 6: 119-134.

Mueller PR, Coleman TR, Kumagai A, Dunphy WG (1995) Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 270: 86-90.

Muller M, Dues G, Balconi C, Salamini F, Thompson RD (1997) Nitrogen and hormonal responsiveness of the 22 kDa alpha-zein and b-32 genes in maize endosperm is displayed in the absence of the transcriptional regulator Opaque-2. Plant Journal 12: 281-291

Muller M, Dues G, Balconi C, Salamini F, Thompson RD (1997) Nitrogen and hormonal responsiveness of the 22 kDa alpha-zein and b-32 genes in maize endosperm is displayed in the absence of the transcriptional regulator Opaque-2. Plant J 12: 281-291.

Munoz-Martin A, Collin S, Herreros E, Mullineaux PM, Fernandez-Lobato M, Fenoll C (2003) Regulation of MSV and WDV virion-sense promoters by WDV nonstructural proteins: a role for their retinoblastoma protein-binding motifs. Virology 306: 313-323

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497

Myers PN, Setter TL, Madison JT, Thompson JF (1990) Abscisic-acid inhibition of endosperm cell-division in cultured maize kernels. Plant Physiol 94: 1330-1336

Myers PN, Setter TL, Madison JT, Thompson JF (1992) Endosperm cell-division in maize kernels cultured at 3 levels of water potential. Plant Physiol 99: 1051-1056

Nagata T, Nemoto Y, S. H (1992) Tobacco BY-2 cell line as the He-La cell in the cell biology of higher plants. Int Rev Cytol 132: 1-30

Nagl W (1976) DNA endoreduplication and polyteny understood as evolutionary strategies. Nature 261: 614-615

Nakagami H, Kawamura K, Sugisaka K, Sekine M, Shinmyo A (2002) Phosphorylation of retinoblastoma-related protein by the cyclin D/cyclin-dependent kinase complex is activated at the G1/S-phase transition in tobacco. Plant Cell 14: 1847-1857

Nakagami H, Sekine M, Murakami H, Shinmyo A (1999) Tobacco retinoblastoma-related protein phosphorylated by a distinct cyclin-dependent kinase complex with Cdc2/cyclin D in vitro. Plant J 18: 243-252

Nakagawa H, Ohmiya K, Hattori T (1996) A rice bZIP protein, designated OSBZ8, is rapidly induced by abscisic acid. Plant J 9: 217-227.

Nakata PA, Okita TW (1996) Cis-elements important for the expression of the ADP-glucose pyrophosphorylase small-subunit are located both upstream and downstream from its structural gene. Mol Gen Genet 250: 581-592

Nasmyth K (1996) Viewpoint: putting the cell cycle in order. Science 274: 1643-1645.

Nasmyth K, Dirick L (1991) The role of SWI4 and SWI6 in the activity of G1 cyclins in yeast. Cell 66: 995-1013.

Niculescu AB, 3rd, Chen X, Smeets M, Hengst L, Prives C, Reed SI (1998) Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol 18: 629-643.

Niculescu AB, Chen XB, Smeets M, Hengst L, Prives C, Reed SI (1998) Effects of p21(Cip1/Waf1) at both the G(1)/S and the G(2)/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Molecular and Cellular Biology 18: 629-643

Niculescu AB, Chen XB, Smeets M, Hengst L, Prives C, Reed SI (1998) Effects of p21(Cip1/Waf1) at both the G(1)/S and the G(2)/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication (vol 18, pg 629, 1998). Molecular and Cellular Biology 18: 1763-1763

Niebel A, Engler JD, Hemerly A, Ferreira P, Inze D, VanMontagu M, Gheysen G (1996) Induction of cdc2a and cyc1At expression in Arabidopsis thaliana during early phases of nematode-induced feeding cell formation. Plant J 10: 1037-1043

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, Kouzarides T (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412: 561-565

Nielsen SJ, Schneider R, Bauer UM, Bannister AJ, Morrison A, O'Carroll D, Firestein R, Cleary M, Jenuwein T, Herrera RE, Kouzarides T (2001) Rb targets histone H3 methylation and HP1 to promoters. Nature 412: 561-565

Nikovics K, Simidjieva J, Peres A, Ayaydin F, Pasternak T, Davies JW, Boulton MI, Dudits D, Horvath GV (2001) Cell-cycle, phase-specific activation of Maize streak virus promoters. Mol Plant Microbe Interact 14: 609-617

Novak B, Tyson JJ (1993) Numerical analysis of a comprehensive model of M-phase control in Xenopus oocyte extracts and intact embryos. J Cell Sci 106: 1153-1168.

Novak B, Tyson JJ (1997) Modeling the control of DNA replication in fission yeast. Proc Natl Acad Sci U S A 94: 9147-9152.

Nurse P (2000) A long twentieth century of the cell cycle and beyond. Cell 100: 71-78

Nurse P, Thuriaux P (1980) Regulatory genes controlling mitosis in the fission yeast Schizosaccharomyces pombe. Genetics 96: 627-637.

Ober ES, Setter TL, Madison JT, Thompson JF, Shapiro PS (1991) Influence of water deficit on maize endosperm development - enzyme-activities and RNA transcripts of starch and zein synthesis, abscisic-acid, and cell-division. Plant Physiol 97: 154-164

Ochatt SJ, Mousset-Declas C, Rancillac M (2000) Fertile pea plants regenerate from protoplasts when calluses have not undergone endoreduplication. Plant Sci 156: 177-183

Ogas J, Cheng JC, Sung ZR, Somerville C (1997) Cellular differentiation regulated by gibberellin in the Arabidopsis thaliana pickle mutant. Science 277: 91-94.

Ogas J, Kaufmann S, Henderson J, Somerville C (1999) PICKLE is a CHD3 chromatin-remodeling factor that regulates the transition from embryonic to vegetative development in Arabidopsis. Proc Natl Acad Sci U S A 96: 13839-13844.

Ohad N, Margossian L, Hsu YC, Williams C, Repetti P, Fischer RL (1996) A mutation that allows endosperm development without fertilization. Proc Natl Acad Sci U S A 93: 5319-5324

Olsen OA (2001) Endosperm development: cellularization and cell fate specification. Annu Rev Plant Physiol Plant Mol Biol 52: 233-267.

Olsen OA, Linnestad C, Nichols SE (1999) Developmental biology of the cereal endosperm. Trends Plant Sci 4: 253-257.

Olsen OA, Potter RH, Kalla R (1992) Histo-differentiation and molecular biology of developing cereal endosperm. Seed Science Research 2: 117-131

Opsahl-Ferstad HG, Le Deunff E, Dumas C, Rogowsky PM (1997) ZmEsr, a novel endosperm-specific gene expressed in a restricted region around the maize embryo. Plant J 12: 235-246

Otegui M, Staehelin LA (2000) Syncytial-type cell plates: a novel kind of cell plate involved in endosperm cellularization of Arabidopsis. Plant Cell 12: 933-947.

Pagano M, Draetta G, Jansen-Durr P (1992) Association of cdk2 kinase with the transcription factor E2F during S phase. Science 255: 1144-1147.

Pagano M, Durst M, Joswig S, Draetta G, Jansen-Durr P (1992) Binding of the human E2F transcription factor to the retinoblastoma protein but not to cyclin A is abolished in HPV-16-immortalized cells. Oncogene 7: 1681-1686.

Palmer KE, Rybicki EP (1998) The molecular biology of mastreviruses. Adv Virus Res 50: 183-234

Pan YB, Peterson PA (1989) Tagging of a maize gene involved in kernel development by an activated Uq transposable element. Mol Gen Genet 219: 324-327.

Parker LL, Sylvestre PJ, Byrnes MJ, 3rd, Liu F, Piwnica-Worms H (1995) Identification of a 95-kDa WEE1-like tyrosine kinase in HeLa cells. Proc Natl Acad Sci U S A 92: 9638-9642.

Pedersen TJ, Arwood LJ, Spiker S, Guiltinan MJ, Thompson WF (1991) High mobility group chromosomal proteins bind to AT-rich tracts flanking plant genes. Plant Mol Biol 16: 95-104.

Peeper DS, Parker LL, Ewen ME, Toebes M, Hall FL, Xu M, Zantema A, van der Eb AJ, Piwnica-Worms H (1993) A- and B-type cyclins differentially modulate substrate specificity of cyclin-cdk complexes. Embo J 12: 1947-1954.

Perazza D, Herzog M, Hulskamp M, Brown S, Dorne AM, Bonneville JM (1999) Trichome cell growth in Arabidopsis thaliana can be derepressed by mutations in at least five genes. Genetics 152: 461-476

Pischke MS, Jones LG, Otsuga D, Fernandez DE, Drews GN, Sussman MR (2002) An Arabidopsis histidine kinase is essential for megagametogenesis. Proc Natl Acad Sci U S A 99: 15800-15805.

Planchais S, Glab N, Trehin C, Perennes C, Bureau JM, Meijer L, Bergounioux C (1997) Roscovitine, a novel cyclin-dependent kinase inhibitor, characterizes restriction point and G2/M transition in tobacco BY-2 cell suspension. Plant J 12: 191-202

Poneleit CG, Egli DB (1983) Differences between reciprocal crosses of maize for kernel growth-characteristics. Crop Sci 23: 871-875

Ponte I, Guillen P, Debon RM, Reina M, Aragay A, Espel E, Di Fonzo N, Palau J (1994) Narrow A/T-rich zones present at the distal 5'-flanking sequences of the zein genes Zc1 and Zc2 bind a unique 30 kDa HMG-like protein. Plant Mol Biol 26: 1893-1906.

Pradhan S, Kim GD (2002) The retinoblastoma gene product interacts with maintenance human DNA (cytosine-5) methyltransferase and modulates its activity. EMBO J 21: 779-788.

Pritchard SG, Rogers HH, Prior SA, Peterson CM (1999) Elevated CO2 and plant structure: a review. Glob Change Biol 5: 807-837

Qin XQ, Chittenden T, Livingston DM, Kaelin WG, Jr. (1992) Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev 6: 953-964.

Qin XQ, Livingston DM, Kaelin WG, Jr., Adams PD (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A 91: 10918-10922.

Ramos GBA, Engler JD, Ferreira PCG, Hemerly AS (2001) DNA replication in plants: characterization of a cdc6 homologue from Arabidopsis thaliana. J Exp Bot 52: 2239-2240

Raychaudhuri P, Rooney R, Nevins JR (1987) Identification of an E1A-inducible cellular factor that interacts with regulatory sequences within the adenovirus E4 promoter. Embo J 6: 4073-4081

Reichheld JP, Chaubet N, Shen WH, Renaudin JP, Gigot C (1996) Multiple A-type cyclins express sequentially during the cell cycle in Nicotiana tabacum BY2 cells. Proc Natl Acad Sci U S A 93: 13819-13824.

Renaudin JP, Colasanti J, Rime H, Yuan Z, Sundaresan V (1994) Cloning of four cyclins from maize indicates that higher plants have three structurally distinct groups of mitotic cyclins. Proc Natl Acad Sci U S A 91: 7375-7379.

Renaudin JP, Doonan JH, Freeman D, Hashimoto J, Hirt H, Inze D, Jacobs T, Kouchi H, Rouze P, Sauter M, Savoure A, Sorrell DA, Sundaresan V, Murray JA (1996) Plant cyclins: a unified nomenclature for plant A-, B- and D-type cyclins based on sequence organization. Plant Mol Biol 32: 1003-1018.

Reski R, Faust M, Wang XH, Wehe M, Abel WO (1994) Genome analysis of the moss Physcomitrella patens (Hedw) Bsg. Mol Gen Genet 244: 352-359

Rhind N, Russell P (2000) Chk1 and Cds1: linchpins of the DNA damage and replication checkpoint pathways. J Cell Sci 113: 3889-3896.

Riou-Khamlichi C, Huntley R, Jacqmard A, Murray JA (1999) Cytokinin activation of Arabidopsis cell division through a D-type cyclin. Science 283: 1541-1544.

Ritchie SW, Hanway JJ, Benson GO (1997) How a corn plant develops. *In.* Iowa State University of Science and Technology, Ames, pp 1-21

Ronchi VN, Avanzi S, Damato F (1965) Chromosome endoreduplication (endopolyploidy) in pea root meristems induced by 8-azaguanine. Caryologia 18: 599

Rosiak M, Polit JT, Maszewski J (2002) Effects of 6-dimethylaminopurine, 2-aminopurine, olomoucine and sodium vanadate on DNA endoreduplication in primary roots of Pisum sativum. Biol Plant 45: 205-211

Rossi V, Locatelli S, Lanzanova C, Boniotti MB, Varotto S, Pipal A, Goralik-Schramel M, Lusser A, Gatz C, Gutierrez C, Motto M (2003) A maize histone deacetylase and retinoblastoma-related protein physically interact and cooperate in repressing gene transcription. Plant Mol Biol 51: 401-413

Rossi V, Varotto S, Locatelli S, Lanzanova C, Lauria M, Zanotti E, Hartings H, Motto M (2001) The maize WD-repeat gene ZmRbAp1 encodes a member of the MSI/RbAp sub-family and is differentially expressed during endosperm development. Mol Genet Genomics 265: 576-584

Rossignol P, Stevens R, Perennes C, Jasinski S, Cella R, Tremousaygue D, Bergounioux C (2002) AtE2F-a and AtDP-a, members of the E2F family of transcription factors, induce Arabidopsis leaf cells to re-enter S phase. Mol Genet Genomics 266: 995-1003.

Rowles A, Tada S, Blow JJ (1999) Changes in association of the Xenopus origin recognition complex with chromatin on licensing of replication origins. J Cell Sci 112 (Pt 12): 2011-2018

Russell DA, Fromm ME (1997) Tissue-specific expression in transgenic maize of four endosperm promoters from maize and rice. Transgenic Res 6: 157-168.

Russell P, Nurse P (1987) Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell 49: 559-567.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Sanchez I, Dynlacht BD (1996) Transcriptional control of the cell cycle. Curr Opin Cell Biol 8: 318-324.

Sauer K, Knoblich JA, Richardson H, Lehner CF (1995) Distinct modes of cyclin E/Cdc2c kinase regulation and S-phase control in mitotic endoreduplication cycles of drosophila embryogenesis. Genes Dev 9: 1327-1339

Sawicka Z (1991) Endoreduplication in ameboid anther tapetum of Arum-orientale Bieb. Acta Biol Crac Ser Bot 33: 45-53

Schalk HJ, Matzeit V, Schiller B, Schell J, Gronenborn B (1989) Wheat dwarf virus, a geminivirus of graminaceous plants needs splicing for replication. Embo J 8: 359-364

Scheid OM, Jakovleva L, Afsar K, Maluszynska J, Paszkowski J (1996) A change of ploidy can modify epigenetic silencing. Proc Natl Acad Sci U S A 93: 7114-7119

Schnittger A, Schobinger U, Stierhof YD, Hulskamp M (2002) Ectopic B-Type cyclin expression induces mitotic cycles in endoreduplicating Arabidopsis trichomes. Curr Biol 12: 415-420

Schub O, Rohaly G, Smith RW, Schneider A, Dehde S, Dornreiter I, Nasheuer HP (2001) Multiple phosphorylation sites of DNA polymerase alpha-primase cooperate to regulate the initiation of DNA replication in vitro. J Biol Chem 276: 38076-38083.

Schultz TF, Spiker S, Quatrano RS (1996) Histone H1 enhances the DNA binding activity of the transcription factor EmBP-1. J Biol Chem 271: 25742-25745.

Schuppler U, He PH, John PC, Munns R (1998) Effect of water stress on cell division and cell-division-cycle 2-like cell-cycle kinase activity in wheat leaves. Plant Physiol 117: 667-678.

Schweizer L, Yerkdavis GL, Phillips RL, Srienc F, Jones RJ (1995) Dynamics of maize endosperm development and DNA endoreduplication. Proc Natl Acad Sci U S A 92: 7070-7074

Scott RJ, Spielman M, Bailey J, Dickinson HG (1998) Parent-of-origin effects on seed development in Arabidopsis thaliana. Development 125: 3329-3341

Seka D, Cross HZ (1995) Xenia and maternal effects on maize agronomic traits at 3 plant densities. Crop Sci 35: 86-90

Seka D, Cross HZ (1995) Xenia and maternal effects on maize kernel development. Crop Sci 35: 80-85

Sellers WR, Novitch BG, Miyake S, Heith A, Otterson GA, Kaye FJ, Lassar AB, Kaelin WG, Jr. (1998) Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth. Genes Dev 12: 95-106.

Senior ML, Murphy JP, Goodman MM, Stuber CW (1998) Utility of SSRs for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci 38: 1088-1098

Shannon JC (1972) Movement of C-14-labeled assimilates into kernels of Zea-mays L .1. Pattern and rate of sugar movement. 49: 198-&

Shannon JC, Doughert.Ct (1972) Movement of C-14-labeled assimilates into kernels of Zea-mays L .2. Invertase activity of pedicel and placento-chalazal tissues. 49: 203-&

Shaul O, Mironov V, Burssens S, Van Montagu M, Inze D (1996) Two Arabidopsis cyclin promoters mediate distinctive transcriptional oscillation in synchronized tobacco BY-2 cells. Proc Natl Acad Sci U S A 93: 4868-4872.

Shaw RG, Waser NM (1994) Quantitative genetic interpretations of postpollination reproductive traits in plants. Am Nat 143: 617-635

Shen WH (2002) The plant E2F-Rb pathway and epigenetic control. Trends Plant Sci 7: 505-511

Sherwood RT (1995) Nuclear-DNA amount during sporogenesis and gametogenesis in sexual and aposporous buffelgrass. Sex Plant Reprod 8: 85-90

Shewry PR, Halford NG (2002) Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 53: 947-958

Shewry PR, Tatham AS (1990) The prolamin storage proteins of cereal seeds: structure and evolution. Biochem J 267: 1-12

Shi CH, Zhu J, Wu JG, Fan LJ (2000) Genetic and genotype x environment interaction effects from embryo, endosperm, cytoplasm and maternal plant for rice grain shape traits of indica rice. Field Crop Res 68: 191-198

Shi CH, Zhu J, Yang XO, Yu YG, Wu JG (1999) Genetic analysis for protein content in indica rice. Euphytica 107: 135-140

Shi CH, Zhu J, Zang RC, Chen GL (1997) Genetic and heterosis analysis for cooking quality traits of indica rice in different environments. Theor Appl Genet 95: 294-300

Shi CW, Zhu J, Wu JG, Yang XE, Yu YG (1999) Analysis of embryo, endosperm, cytoplasmic and maternal effects for heterosis of protein and lysine content in indica hybrid rice. Plant Breed 118: 574-576

Shiina N, Gotoh Y, Kubomura N, Iwamatsu A, Nishida E (1994) Microtubule severing by elongation-factor 1-alpha. Science 266: 282-285

Shivji MK, Grey SJ, Strausfeld UP, Wood RD, Blow JJ (1994) Cip1 inhibits DNA replication but not PCNA-dependent nucleotide excision-repair. Curr Biol 4: 1062-1068

Siddiqi I, Ganesh G, Grossniklaus U, Subbiah V (2000) The dyad gene is required for progression through female meiosis in Arabidopsis. Development 127: 197-207.

Sigrist SJ, Lehner CF (1997) Drosophila fizzy-related down-regulates mitotic cyclins and is required for cell proliferation arrest and entry into endocycles. Cell 90: 671-681

Simpson DJ (2001) Proteolytic degradation of cereal prolamins - the problem with proline. 161: 825-838

Smulders MJM, Ruskortekaas W, Gilissen LJW (1994) Development of polysomaty during differentiation in diploid and tetraploid tomato (Lycopersicon-esculentum) plants. Plant Sci 97: 53-60

Smulders MJM, Ruskortekaas W, Gilissen LJW (1995) Natural variation in patterns of polysomaty among individual tomato plants and their regenerated progeny. Plant Sci 106: 129-139

Snaith HA, Forsburg SL (1999) Rereplication phenomenon in fission yeast requires MCM proteins and other S phase genes. Genetics 152: 839-851

Song R, Llaca V, Linton E, Messing J (2001) Sequence, regulation, and evolution of the maize 22-kD alpha zein gene family. Genome Res 11: 1817-1825.

Soni R, Carmichael JP, Shah ZH, Murray JA (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7: 85-103.

Spencer HG, Feldman MW, Clark AG (1998) Genetic conflicts, multiple paternity and the evolution of genomic imprinting. Genetics 148: 893-904.

Spiker S (1984) High-mobility group chromosomal proteins of wheat. J Biol Chem 259: 12007-12013.

Spillane C, MacDougall C, Stock C, Kohler C, Vielle-Calzada JP, Nunes SM, Grossniklaus U, Goodrich J (2000) Interaction of the Arabidopsis polycomb group proteins FIE and MEA mediates their common phenotypes. Curr Biol 10: 1535-1538

Springer NM, Danilevskaya ON, Hermon P, Helentjaris TG, Phillips RL, Kaeppler HF, Kaeppler SM (2002) Sequence relationships, conserved domains and expression patterns for maize homologs of the polycomb group genes E(z), esc, and E(Pc). Plant Physiol 128: 1332-1345

Springer PS, Holding DR, Groover A, Yordan C, Martienssen RA (2000) The essential Mcm7 protein PROLIFERA is localized to the nucleus of dividing cells during the G(1) phase and is required maternally for early Arabidopsis development. Development 127: 1815-1822.

Staehling-Hampton K, Ciampa PJ, Brook A, Dyson N (1999) A genetic screen for modifiers of E2F in Drosophila melanogaster. Genetics 153: 275-287

Sterner JM, Dew-Knight S, Musahl C, Kornbluth S, Horowitz JM (1998) Negative regulation of DNA replication by the retinoblastoma protein is mediated by its association with MCM7. Mol Cell Biol 18: 2748-2757

Stevaux O, Dimova D, Frolov MV, Taylor-Harding B, Morris E, Dyson N (2002) Distinct mechanisms of E2F regulation by Drosophila RBF1 and RBF2. Embo J 21: 4927-4937

Stillman B (1996) Cell cycle control of DNA replication. Science 274: 1659-1664.

Stillman B, Bell SP, Dutta A, Marahrens Y (1992) DNA replication and the cell cycle. Ciba Found Symp 170: 147-156

Strausfeld UP, Howell M, Rempel R, Maller JL, Hunt T, Blow JJ (1994) Cip1 blocks the initiation of DNA replication in Xenopus extracts by inhibition of cyclin-dependent kinases. Curr Biol 4: 876-883

Strobeck MW, Knudsen KE, Fribourg AF, DeCristofaro MF, Weissman BE, Imbalzano AN, Knudsen ES (2000) BRG-1 is required for RB-mediated cell cycle arrest. Proc Natl Acad Sci U S A 97: 7748-7753.

Strobeck MW, Reisman DN, Gunawardena RW, Betz BL, Angus SP, Knudsen KE, Kowalik TF, Weissman BE, Knudsen ES (2002) Compensation of BRG-1 function by Brm: insight into the role of the core SWI-SNF subunits in retinoblastoma tumor suppressor signaling. J Biol Chem 277: 4782-4789.

Student (1907) On the error of counting with a haemacytometer. Biometrika 5: 351-360

Sun Y, Dilkes BP, Zhang C, Dante RA, Carneiro NP, Lowe KS, Jung R, Gordon-Kamm WJ, Larkins BA (1999) Characterization of maize (Zea mays L.) Wee1 and its activity in developing endosperm. Proc Natl Acad Sci U S A 96: 4180-4185.

Sun Y, Flannigan BA, Madison JT, Setter TL (1997) Alternative splicing of cyclin transcripts in maize endosperm. Gene 195: 167-175

Sun YJ, Carneiro N, Clore AM, Moro GL, Habben JE, Larkins BA (1997) Characterization of maize elongation factor 1A and its relationship to protein quality in the endosperm. Plant Physiology 115: 1101-1107

Sun YJ, Dilkes BP, Zhang CS, Dante RA, Carneiro NP, Lowe KS, Jung R, Gordon-Kamm WJ, Larkins BA (1999) Characterization of maize (Zea mays L.) Wee1 and its activity in developing endosperm. Proceedings of the National Academy of Sciences of the United States of America 96: 4180-4185

Sun YJ, Flannigan BA, Setter TL (1999) Regulation of endoreduplication in maize (Zea mays L.) endosperm. Isolation of a novel B1-type cyclin and its quantitative analysis. Plant MolBiol 41: 245-258

Surana U, Robitsch H, Price C, Schuster T, Fitch I, Futcher AB, Nasmyth K (1991) The role of CDC28 and cyclins during mitosis in the budding yeast S. cerevisiae. Cell 65: 145-161.

Swanson C, Ross J, Jackson PK (2000) Nuclear accumulation of cyclin E/Cdk2 triggers a concentration- dependent switch for the destruction of p27Xic1. Proc Natl Acad Sci U S A 97: 7796-7801.

Swenson KI, Borgese N, Pietrini G, Ruderman JV (1987) Three translationally regulated mRNAs are stored in the cytoplasm of clam oocytes. Dev Biol 123: 10-16.

Szymanski DB, Marks MD (1998) GLABROUS1 overexpression and TRIPTYCHON alter the cell cycle and trichome cell fate in Arabidopsis. Plant Cell 10: 2047-2062

Tada S, Chong JP, Mahbubani HM, Blow JJ (1999) The RLF-B component of the replication licensing system is distinct from Cdc6 and functions after Cdc6 binds to chromatin. Curr Biol 9: 211-214

Tada S, Li A, Maiorano D, Mechali M, Blow JJ (2001) Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol 3: 107-113

Takada S, Hibara K, Ishida T, Tasaka M (2001) The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development 128: 1127-1135.

Takaiwa F, Oono K, Kato A (1991) Analysis of the 5' flanking region responsible for the endosperm-specific expression of a rice glutelin chimeric gene in transgenic tobacco. Plant Mol Biol 16: 49-58.

Takemura M, Kitagawa T, Izuta S, Wasa J, Takai A, Akiyama T, Yoshida S (1997) Phosphorylated retinoblastoma protein stimulates DNA polymerase alpha. Oncogene 15: 2483-2492

Takemura M, Yamamoto T, Kitagawa M, Taya Y, Akiyama T, Asahara H, Linn S, Suzuki S, Tamai K, Yoshida S (2001) Stimulation of DNA polymerase alpha activity by cdk2-phosphorylated Rb protein. Biochem Biophys Res Commun 282: 984-990

Tanaka T, Knapp D, Nasmyth K (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell 90: 649-660

Tardieu F, Granier C (2000) Quantitative analysis of cell division in leaves: methods, developmental patterns and effects of environmental conditions. Plant Mol Biol 43: 555-567.

Thanos D, Du W, Maniatis T (1993) The high mobility group protein HMG I(Y) is an essential structural component of a virus-inducible enhancer complex. Cold Spring Harb Symp Quant Biol 58: 73-81

Thompson GA, Boston RS, Lyznik LA, Hodges TK, Larkins BA (1990) Analysis of promoter activity from an alpha-zein gene 5' flanking sequence in transient expression assays. Plant Mol Biol 15: 755-764.

Thompson RD, Hueros G, Becker H, Maitz M (2001) Development and functions of seed transfer cells. Plant Sci 160: 775-783

Tourret J, McKeon F (1996) Tyrosine kinases wee1 and mik1 as effectors of DNA replication checkpoint control. Prog Cell Cycle Res 2: 91-97

Traas J, Hulskamp M, Gendreau E, Hofte H (1998) Endoreduplication and development: rule without dividing? Curr Opin Plant Biol 1: 498-503

Trotter J (2000) WinMDI. *In*, Ed 2.8. The Scripps Research Institute, La Jolla, CA

Tyson JJ, Csikasz-Nagy A, Novak B (2002) The dynamics of cell cycle regulation. Bioessays 24: 1095-1109.

Umen JG, Goodenough UW (2001) Control of cell division by a retinoblastoma protein homolog in Chlamydomonas. Genes Dev 15: 1652-1661

Valente P, Tao WH, Verbelen JP (1998) Auxins and cytokinins control DNA endoreduplication and deduplication in single cells of tobacco. Plant Sci 134: 207-215

van den Heuvel S, Harlow E (1993) Distinct roles for cyclin-dependent kinases in cell cycle control. Science 262: 2050-2054.

Verdoodt B, Decordier I, Geleyns K, Cunha M, Cundari E, Kirsch-Volders M (1999) Induction of polyploidy and apoptosis after exposure to high concentrations of the spindle poison nocodazole. Mutagenesis 14: 513-520.

Vicente-Carbajosa J, Moose SP, Parsons RL, Schmidt RJ (1997) A maize zinc-finger protein binds the prolamin box in zein gene promoters and interacts with the basic leucine zipper transcriptional activator Opaque2. Proc Natl Acad Sci U S A 94: 7685-7690.

Vicient CM, Bies-Etheve N, Delseny M (2000) Changes in gene expression in the leafy cotyledon1 (lec1) and fusca3 (fus3) mutants of Arabidopsis thaliana L. J Exp Bot 51: 995-1003.

Vielle-Calzada JP, Baskar R, Grossniklaus U (2000) Delayed activation of the paternal genome during seed development. Nature 404: 91-94.

Vielle-Calzada JP, Thomas J, Spillane C, Coluccio A, Hoeppner MA, Grossniklaus U (1999) Maintenance of genomic imprinting at the Arabidopsis medea locus requires zygotic DDM1 activity. Genes Dev 13: 2971-2982

Vinkenoog R, Spielman M, Adams S, Fischer RL, Dickinson HG, Scott RJ (2000) Hypomethylation promotes autonomous endosperm development and rescues postfertilization lethality in fie mutants. Plant Cell 12: 2271-2282.

Vitrat N, Cohen-Solal K, Pique C, Le Couedic JP, Norol F, Larsen AK, Katz A, Vainchenker W, Debili N (1998) Endomitosis of human megakaryocytes are due to abortive mitosis. Blood 91: 3711-3723.

Vlach J, Hennecke S, Amati B (1997) Phosphorylation-dependent degradation of the cyclin-dependent kinase inhibitor p27. Embo J 16: 5334-5344.

Voitenleitner C, Rehfuess C, Hilmes M, O'Rear L, Liao PC, Gage DA, Ott R, Nasheuer HP, Fanning E (1999) Cell cycle-dependent regulation of human DNA polymerase alpha-primase activity by phosphorylation. Mol Cell Biol 19: 646-656

Walen KH (2002) The origin of transformed cells: studies of spontaneous and induced cell transformation in cell cultures from marsupials, a snail, and human amniocytes. 133: 45-54

Walker JD, Oppenheimer DG, Concienne J, Larkin JC (2000) SIAMESE, a gene controlling the endoreduplication cell cycle in Arabidopsis thaliana trichomes. Development 127: 3931-3940

Wallace JC, Lopes MA, Paiva E, Larkins BA (1990) New methods for extraction and quantitation of zeins reveal a high content of gamma-zein in modified opaque-2 maize. Plant Physiology 92: 191-196

Wang D, Russell JL, Johnson DG (2000) E2F4 and E2F1 have similar proliferative properties but different apoptotic and oncogenic properties in vivo. Mol Cell Biol 20: 3417-3424.

Wang H, Qi Q, Schorr P, Cutler AJ, Crosby WL, Fowke LC (1998) ICK1, a cyclin-dependent protein kinase inhibitor from Arabidopsis thaliana interacts with both

Cdc2a and CycD3, and its expression is induced by abscisic acid. Plant J 15: 501-510.

Wang H, Zhou Y, Gilmer S, Whitwill S, Fowke LC (2000) Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. Plant J 24: 613-623.

Wang X, Larkins BA (2001) Genetic analysis of amino acid accumulation in opaque-2 maize endosperm. Plant Physiol 125: 1766-1777

Wang X, Woo YM, Kim CS, Larkins BA (2001) Quantitative trait locus mapping of loci influencing elongation factor 1alpha content in maize endosperm. Plant Physiol 125: 1271-1282

Weinberg RA (1996) E2F and cell proliferation: a world turned upside down. Cell 85: 457-459.

Weinert T (1997) Yeast checkpoint controls and relevance to cancer. Cancer Surv 29: 109-132

Weinert T, Hartwell L (1989) Control of G2 delay by the rad9 gene of Saccharomyces cerevisiae. J Cell Sci Suppl 12: 145-148

Weinert TA, Kiser GL, Hartwell LH (1994) Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev 8: 652-665

Weiss A, Herzig A, Jacobs H, Lehner CF (1998) Continuous Cyclin E expression inhibits progression through endoreduplication cycles in Drosophila. Curr Biol 8: 239-242

Weschke W, Panitz R, Gubatz S, Wang Q, Radchuk R, Weber H, Wobus U (2003) The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. Plant J 33: 395-411

Weschke W, Panitz R, Sauer N, Wang Q, Neubohn B, Weber H, Wobus U (2000) Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. Plant J 21: 455-467

Wonnacott TH, Wonnacott RJ (1990) Introductory Statistics, Ed 5th. John Wiley and Sons, New York

Woo YM, Hu DW, Larkins BA, Jung R (2001) Genomics analysis of genes expressed in maize endosperm identifies novel seed proteins and clarifies patterns of zein gene expression. Plant Cell 13: 2297-2317.

Wright EA, Heckel T, Groenendijk J, Davies JW, Boulton MI (1997) Splicing features in maize streak virus virion- and complementary-sense gene expression. Plant J 12: 1285-1297

Wu CY, Washida H, Onodera Y, Harada K, Takaiwa F (2000) Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. Plant J 23: 415-421

Wu R, Lou XY, Ma CX, Wang X, Larkins BA, Casella G (2002) An improved genetic model generates high-resolution mapping of QTL for protein quality in maize endosperm. Proc Natl Acad Sci U S A 99: 11281-11286

Wu SM, Zhang P, Zeng XR, Zhang SJ, Mo J, Li BQ, Lee MY (1998) Characterization of the p125 subunit of human DNA polymerase delta and its deletion mutants. Interaction with cyclin-dependent kinase-cyclins. J Biol Chem 273: 9561-9569.

Wyrzykowska J, Pien S, Shen WH, Fleming AJ (2002) Manipulation of leaf shape by modulation of cell division. Development 129: 957-964.

Xiao B, Spencer J, Clements A, Ali-Khan N, Mittnacht S, Broceno C, Burghammer M, Perrakis A, Marmorstein R, Gamblin SJ (2003) Crystal structure of the retinoblastoma tumor suppressor protein bound to E2F and the molecular basis of its regulation. Proc Natl Acad Sci U S A 100: 2363-2368

Xie Q, Frugis G, Colgan D, Chua NH (2000) Arabidopsis NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. Genes Dev 14: 3024-3036.

Xie Q, Guo HS, Dallman G, Fang S, Weissman AM, Chua NH (2002) SINAT5 promotes ubiquitin-related degradation of NAC1 to attenuate auxin signals. Nature 419: 167-170.

Xie Q, Sanz-Burgos AP, Guo H, Garcia JA, Gutierrez C (1999) GRAB proteins, novel members of the NAC domain family, isolated by their interaction with a geminivirus protein. Plant Mol Biol 39: 647-656

Xie Q, Sanz-Burgos AP, Hannon GJ, Gutierrez C (1996) Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. Embo J 15: 4900-4908

Xie Q, Sanz-Burgos AP, Hannon GJ, Gutierrez C (1996) Plant cells contain a novel member of the retinoblastoma family of growth regulatory proteins. Embo J 15: 4900-4908.

Xie Q, Suarez-Lopez P, Gutierrez C (1995) Identification and analysis of a retinoblastoma binding motif in the replication protein of a plant DNA virus: requirement for efficient viral DNA replication. Embo J 14: 4073-4082

Xu M, Sheppard KA, Peng CY, Yee AS, Piwnica-Worms H (1994) Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol 14: 8420-8431.

Yadegari R, Kinoshita T, Lotan O, Cohen G, Katz A, Choi Y, Nakashima K, Harada JJ, Goldberg RB, Fischer RL, Ohad N (2000) Mutations in the FIE and MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell 12: 2367-2382

Yanagawa Y, Hasezawa S, Kumagai F, Oka M, Fujimuro M, Naito T, Makino T, Yokosawa H, Tanaka K, Komamine A, Hashimoto J, Sato T, Nakagawa H (2002) Cell-cycle dependent dynamic change of 26S proteasome distribution in tobacco BY-2 cells. Plant Cell Physiol 43: 604-613.

Yang F, Demma M, Warren V, Dharmawardhane S, Condeelis J (1990) Identification of an actin-binding protein from dictyostelium as elongation factor-1a. Nature 347: 494-496

Yee AS, Raychaudhuri P, Jakoi L, Nevins JR (1989) The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. Mol Cell Biol 9: 578-585

You Z, Ishimi Y, Masai H, Hanaoka F (2002) Roles of Mcm7 and Mcm4 subunits in the DNA helicase activity of the mouse Mcm4/6/7 complex. J Biol Chem 277: 42471-42479

You Z, Komamura Y, Ishimi Y (1999) Biochemical analysis of the intrinsic Mcm4-Mcm6-mcm7 DNA helicase activity. Mol Cell Biol 19: 8003-8015

Young TE, Gallie DR (1999) Analysis of programmed cell death in wheat endosperm reveals differences in endosperm development between cereals. Plant Mol Biol 39: 915-926.

Young TE, Gallie DR (2000) Programmed cell death during endosperm development. Plant Mol Biol 44: 283-301.

Young TE, Gallie DR (2000) Regulation of programmed cell death in maize endosperm by abscisic acid. Plant Mol Biol 42: 397-414.

Young TE, Gallie DR, DeMason DA (1997) Ethylene-mediated programmed cell death during maize endosperm development of wild-type and shrunken2 genotypes. Plant Physiol 115: 737-751.

Yunes JA, Vettore AL, da Silva MJ, Leite A, Arruda P (1998) Cooperative DNA binding and sequence discrimination by the Opaque2 bZIP factor. Plant Cell 10: 1941-1955

Zhang HS, Dean DC (2001) Rb-mediated chromatin structure regulation and transcriptional repression. Oncogene 20: 3134-3138.

Zhang HS, Gavin M, Dahiya A, Postigo AA, Ma D, Luo RX, Harbour JW, Dean DC (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. Cell 101: 79-89.

Zhang K, Letham DS, John PC (1996) Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. Planta 200: 2-12

Zhao J, Grafi G (2000) The high mobility group I/Y protein is hypophosphorylated in endoreduplicating maize endosperm cells and is involved in alleviating histone H1-mediated transcriptional repression. J Biol Chem 275: 27494-27499

Zhao J, Grafi G (2000) The high mobility group I/Y protein is hypophosphorylated in endoreduplicating maize endosperm cells and is involved in alleviating histone H1-mediated transcriptional repression. J Biol Chem 275: 27494-27499.

Zhu J, Weir BS (1994) Analysis of cytoplasmic and maternal effects .2. Genetic models for triploid endosperms. Theor Appl Genet 89: 160-166

Ziegler KE (2001) Popcorn. *In* AR Hallauer, ed, Specialty Corns. CRC press, Boca Raton, FL, pp 248-283