Molecular mechanism of imprinting of the maize endosperm gene fie1

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Molecular mechanism of imprinting of the maize endosperm gene *fie1*

by

Pedro Hermon-Cruz

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Genetics

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For the Major Program
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The main purpose of this study was to characterize the expression patterns of the maize fie genes and to identify possible modes of their regulation. Our study revealed that although the fie1 and fie2 genes in maize contain a 78% homology within their exonic regions, their promoter regions are complex and contain no similarity. The maize fie1 and fie2 genes also have different pattern of expression. fie1 has a highly regulated expression. fie1 transcription is detectable only in the endosperm at early stages of development. fie2 has a more ubiquitous expression, detectable in vegetative and reproductive tissues at all times of plant development. We showed that the fie1 gene is imprinted and that transcriptional silencing of the paternal allele takes place. We looked at DNA methylation as part of the imprinting mechanism that regulates fie1 expression. Our study showed that the fie1 gene and not the fie2 gene is methylated along the promoter and exonic regions of the gene. More importantly we showed that in the endosperm, where fie1 is expressed, only the silent paternal allele is methylated. In contrast, in tissues where the fie1 gene is not expressed, like leaves and embryo, both maternal and paternal alleles have high levels of methylation. We propose the following model of fie1 imprinting. Before fertilization, the fie1 maternal alleles are demethylated in the central cell of the female gametophyte, but remain methylated in the egg cell and in the male gametophyte. After fertilization, demethylation allows for expression of the maternal fie1 alleles in the endosperm. However, the paternal allele remains methylated in the endosperm and therefore silent and both paternal and maternal alleles also remain methylated and silent in the embryo.
GENERAL INTRODUCTION

Maize is one of the most important cereal crops in the world grown for human consumption as well as for animal feed and industrial purposes. Understanding the molecular mechanism and genes that regulate seed development are of significant importance because most of the nutritional and economic value of maize comes from the kernel. In maize and in higher plants the mature seed is the end result of sexual reproduction in a process known as double fertilization (Figure 1). During this process two sperm cells (1N) from a single pollen grain travel through the pollen tube to the ovule, where they enter the female gametophyte, or embryo sac, through the micropylar pole. The embryo sac is composed of 2 synergid cells (1N), the egg cell (1N), the central cell (2N) and the antipodal cells (1N) (Dumas and Mogensen, 1993; Ohad et al., 1996; Reiser and Fischer, 1993). Double fertilization takes place when one sperm cell fuses with the egg cell to give rise to the 2N embryo and the second sperm cell fuses with the central cell to give rise to the 3N endosperm. The endosperm is a determinate tissue and does not contribute genetic material to the next generation. Instead it surrounds and provides nutrients to the embryo through the accumulation of proteins and starch.

In maize the endosperm is present throughout seed development and helps support germination. The embryo and endosperm are genetically identical but differ in their ratio of maternal to paternal genomes. The embryo contains a 1:1 maternal to paternal ratio whereas the endosperm contains a 2:1 maternal to paternal ratio. Failure to maintain this genetic balance prevents endosperm development and gives rise to seed abortion (Leblanc et al., 2002; Scott et al., 1998).

In order to better understand the genetic mechanism that contributes to seed development in plants, female gametophytic mutations were identified in *Arabidopsis thaliana* (Chaudhury et al., 1997; Ohad et al., 1996; Ohad et al., 1999). One such mutation was the fertilization independent endosperm (*fie*) also known as fertilization independent seed (*fis*). The *fie* mutants show characteristics of seed development without fertilization including the elongation of the silique and initial signs of endosperm development. Three genes corresponding to the *fie* mutation were identified as FERTILIZATION INDEPENDENT SEED (*FIS1/MEDEA, FIS2* and *FIS3/FIE*) (Grossniklaus et al., 1998; Luo et al., 1999; Ohad
et al., 1999). These genes encode proteins that are members of the Polycomb group proteins (PcG) (Grossniklaus and Schneitz, 1998; Luo et al., 1999; Ohad et al., 1999). The Polycomb group proteins were first identified in Drosophila as repressors of homeotic genes, but members of the PcG family have also been identified in mammals, nematodes and plants (Kaneko-Ishino et al., 2003; Kohler and Grossniklaus, 2002; Pirrotta, 1997; Pirrotta, 2002).

We first reported that maize contains two putative homologues of the Drosophila extra sex combs (esc) and the Arabidopsis fertilization independent endosperm (FIE) (Springer et al., 2002). These two genes named fie1 and fie2 were discovered by a BLAST search of the Pioneer Hi-Bred proprietary database with the Drosophila esc and the Arabidopsis FIE sequences (Springer et al., 2002). fie1 was mapped to chromosome 4 (bin 5) and fie2 was mapped to chromosome 10 (bin 3). It appears that the fie1 and fie2 are duplicated genes, yet their expression patterns indicate different functions as well as different modes of regulation. Our study revealed that the maize fie1 and fie2 genes are 78% homologous within their exonic regions, yet their promoter regions showed no similarity. fie1 has a highly regulated expression pattern detectable only during early endosperm development, whereas fie2 is detectable in all analyzed tissues, during all times of development.

Differential gene expression is an important mechanism necessary for proper development in mammals and other organisms. Expression of imprinted genes is determined by the parent from which the allele was inherited and not by the specific allele. One mechanism involved in controlling imprinted genes is differential DNA methylation. In mammals differential methylation regions (DMRs) have been associated with imprinted genes (Kaneko-Ishino et al., 2003). DMRs may control gene expression by chromatin remodeling.

The fie1 gene was shown to be imprinted and the paternal allele transcriptionally silenced (Danilevskaya et al., 2003). Regulation of fie1 expression possibly involved methylation. Based on methylation sensitive restriction enzymes fie1 and not fie2 was shown to be methylated. More importantly we showed that in the endosperm where fie1 is expressed, the silenced paternal fie1 allele was highly methylated along the upstream region of the gene whereas the maternal allele has no detectable level of methylation. In other
tissues, like leaf and embryo, where *fie1* is not expressed, both maternal and paternal alleles showed high levels of methylation. Our findings indicate that the stable imprinting of *fie1* may be mediated by methylation of the upstream region of the gene. We proposed a model of *fie1* imprinting in which demethylation of the maternal alleles occur in the central cell some time before fertilization, allowing for the expression of the maternally transmitted genes, whereas the paternal allele remains methylated and silenced. Our model agrees with the recently proposed “one way control of imprinting” model presented by Kinoshita el. al., (2004). In this model, demethylation takes place in the central cell allowing expression of imprinted genes. However, due to the terminal nature of the endosperm methylation does not need to be reestablished in the next generation.

Because of the role the PcG genes play in development and the involvement of *FIE* in seed development in Arabidopsis, the study of the *fie* genes in maize represents a valuable lead in further understanding seed development in maize.

**Dissertation organization**

This dissertation is organized in four chapters using the format for journal papers. In chapter 1 (General introduction), I described previous work done in the model system *Arabidopsis thaliana*, with respect to the *FIE* genes. I also introduced some important concepts necessary for the understanding of the research. In chapter 2 (Duplicate *Fie* genes in maize: expression pattern and imprinting suggest distinct function), I analyzed the expression patterns of the maize *fie* genes. Chapter 2 was originally published in the journal *The Plant Cell* (Danilevskaya et al., 2003). However, in this dissertation this paper is not presented in the original format. To comply with the requirements for inclusion of a journal article in a dissertation, parts of the original paper were eliminated. The parts that were eliminated were contributed by other authors, and are not necessary to support my findings. Because I was not the primary writer of the original article, parts of the paper were rewritten, including the introduction as well as sections of the results and discussion. The data and their analysis in the section (Genomic structure of *fie* loci) were contributed by Olga Danilevskaya, who was the principal investigator. Because of my significant contribution to the original figures and materials and methods, these sections were not modified from the
original article. Chapter 3 (Imprinting of the maize endosperm gene fie1 is controlled by DNA methylation) is in the journal format and will be submitted for publication. In chapter 4 (General conclusion) I summarized my findings and pointed to some possible experiments that could help advance this area of study.

The work presented in this dissertation was done under the scientific supervision of Olga Danilevskaya, a research scientist at Pioneer Hi-Bred International and a collaborator in the Department of Genetics, Developmental and Cellular Biology (GDCB) at Iowa State University. The academic supervisor and major professor was Philip Becraft in the Department of Genetics, Developmental and Cellular Biology at Iowa State University.

References


Figure 1. Process of double fertilization in higher plants.

The male gametophyte, or pollen grain, contains two sperm cells, 1N each. Double fertilization occurs when one sperm fertilizes the egg cell (1N) giving rise to the 2N embryo and the second sperm cell fertilizes the central cell (2N) giving rise to the 3N endosperm.
Sperm cells

Vegetative cell

Male gametophyte (Pollen)

Antipodal Cells

Double Fertilization

3N endosperm

2N embryo

kernel

Female gametophyte (Embryo Sac)

Synergid cells

2N central cell

2N egg cell


DUPLICATED FIE GENES IN MAIZE: EXPRESSION PATTERN AND IMPRINTING SUGGEST DISTINCT FUNCTIONS

Modified from a paper published in The Plant Cell

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Abstract

The Arabidopsis FIE (fertilization independent endosperm) gene encodes a Polycomb group protein. FIE represses endosperm development in the central cell of the embryo sac until fertilization occurs. Two fie genes were identified in the maize genome and cloned. The fie1 and fie2 genes have similar exon/intron structures with significant homology throughout their coding regions. The fie1 gene is expressed exclusively in the endosperm during early development and is regulated by imprinting. The paternally inherited allele of fie1 has no detectable expression at any time during endosperm development. In contrast, the fie2 gene is constitutively expressed in all tissues throughout development. Unlike fie1, the fie2 gene is not imprinted in the endosperm. However, fie2 shows paternally delayed expression, with only the maternal allele expressing early in endosperm development, but both maternal and paternal alleles are expressed later. The expression pattern and paternal delay of fie2 expression is similar to the Arabidopsis FIE, suggesting that the fie2 is more likely to be a functional ortholog to the Arabidopsis FIE.

Introduction

Sexual reproduction in plants occurs by a process known as double fertilization. During double fertilization the female gametophyte, which consist of antipodal cells, synergid cells, a central cell and the egg cell, is fertilized by two sperm cells from one single pollen grain (Reiser and Fischer, 1993). During double fertilization, a sperm cell (1N)

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fertilizes the egg cell (1N) while the other sperm cell (1N) fertilizes the central cell (2N). This double fertilization process gives rise to a diploid (2N) embryo with a 1m:1p (maternal:paternal) genome contribution; and a triploid (3N) endosperm with a 2m:1p ratio.

In some plant species seed development can also occur without fertilization by a process known as apomixis. During apomictic reproduction the egg cell can develop into an embryo in the absence of fertilization. Thus the endosperm can develop nonautomously due to fertilization or autonomously without fertilization (Gallo-Meagher et al., 2001; Grimanelli et al., 2001). These developmental differences, between the embryo and endosperm during apomictic reproduction suggest that two separate processes may be involved in the regulation and initiation of development in the embryo and endosperm.

In order to understand the genetic mechanisms that regulate seed development in plants, researchers conducted mutagenesis screenings in *Arabidopsis thaliana* (Chaudhury et al., 1997; Ohad et al., 1996; Ohad et al., 1999). In these screenings three female gametophytic mutants were identified. These mutants are known as *fertilization independent endosperm (fie)* or *fertilization independent seed (fis)*. The fis mutants showed aspects of seed development without fertilization, including elongation of the silique and initiation of endosperm development. Three genes identified in the screens were: *FERTILIZATION INDEPENDENT SEED 1 (FIS1)*, also known as *MEDEA (MEA)*; *FERTILIZATION INDEPENDENT SEED 2 (FIS2)* and *FERTILIZATION INDEPENDENT SEED 3 (FIS3)* also known as *FERTILIZATION INDEPENDENT ENDOSPERM (FIE)* (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999). Proteins encoded by these three genes indicated that all genes were members of the Polycomb group (PcG). PcG proteins have been identified to be important for development in various organisms and work in complexes to repress transcription factors (Ng et al., 1997; Pirrotta, 1998).

MEA is a member of the Polycomb group proteins and is homologous to the Drosophila enhancer of zeste (Kaneko-Ishino et al., 2003; Kiyosue et al., 1999). MEA was also identified as a fertilization independent seed mutant and like FIE mutants it allows for the initiation of endosperm development without fertilization. MEA is expressed in both the embryo and endosperm, but in the endosperm MEA expression is regulated by imprinting
(Kinoshita et al., 1999; Luo et al., 2000; Vielle-Calzada et al., 1999; Xiao et al., 2003). In the endosperm the paternal MEA allele remains silent all-throughout development, but in other tissues including the embryo both maternal and paternal alleles are actively transcribed.

FIS2 encodes a C2H2 zing finger protein with homology to the Drosophila Polycomb protein Suppressor of zeste (Chaudhury et al., 2001; Luo et al., 1999). FIS2 also contain nuclear localization signals. FIS2 is expressed in the female gamotophyte before pollination and in the developing endosperm after pollination, where its expression is associated with the free endosperm nuclei (Luo et al., 2000). Like MEA, FIS2 is an imprinted gene, in which the paternal FIS2 allele remains silent.

The Arabidopsis FIE gene is expressed in the central cell before fertilization (Luo et al., 2000; Yadegari et al., 2000), and during early endosperm development after fertilization, but before cellularization. FIE showed a maternal effect in which the maternal allele is required for proper development regardless of the paternal allele (Chaudhury et al., 1997; Ohad et al., 1996; Yadegari et al., 2000). FIE has been classified as a Polycomb protein homologous to the Drosophila extra sex combs (ESC) (Ohad et al., 1999). Unlike MEA and FIS2, FIE is not imprinted in the endosperm. However, FIE shows a paternal delay of expression.

Yeast two-hybrid analysis have shown that FIE can interact with MEA (Chaudhury and Berger, 2001; Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000). However, no interaction has been shown between FIS2 and MEA or FIE (Chaudhury and Berger, 2001). The MEA/FIE complex has been shown to interact with the promoter region of the MADS-box gene PHERES1 (PHE1) (Kohler et al., 2003). In wild type seeds PHE1 is expressed after fertilization in the embryo and in the chalazal region of the endosperm. However, in fie and mea mutants PHE1 is expressed ectopically outside the chalazal region of the endosperm. The direct interaction of the MEA/FIE complex with PHE1 and the increased expression of PHE1 in fie and mea mutants suggest that the possible role of polycomb genes in plants may be to regulate transcription factors.

FIE also plays a role in the repression of flowering (Kinoshita et al., 2001). In fie mutants the vegetative state of the shoot apical meristem is diminished, giving rise to the
premature formation of floral-like structures. These floral-like structures are also formed ectopically on fie mutants. Low levels of FIE also exhibit loss of apical dominance as well as homeotic phenotypes (Katz et al., 2004). The involvement of FIE in flowering is in part due to its association with Curly leaf (CLF). CLF is a SET domain PcG protein involved in leaf and flower development (Goodrich et al., 1997). The formation of different FIE protein complexes in different plant tissues may be part of a mechanism used to regulate different developmental processes.

In our previous study we have shown that the Arabidopsis, FIE had high homology to several corn ESTs (Expressed Sequencing Tags) in the Pioneer Hi-Bred/Dupont maize EST database (Springer et al., 2002). Sequence analysis revealed two putative maize homologues to the Arabidopsis FIE designated fie1 and fie2. The role of the fie genes in maize has not yet been established. Here we report that the duplicated fie genes in maize have distinct expression patterns as well as different modes of regulation. Our study revealed that the maize fie1 and fie2 genes are 78% homologous within their exonic regions, yet their promoter regions showed no similarity. fie1 has a highly regulated expression pattern detectable only during early endosperm development, whereas fie2 is detectable in all analyzed tissues, at all times of seed development. We also showed that the fie1 gene is imprinted in the endosperm and that the paternal allele is transcriptionally silenced throughout endosperm development.

Results

Genomic structure of fie loci

To determine the genomic structure of the two maize fie genes, their genomic DNAs were cloned using previously identified ESTs (Springer et al., 2002). The inbred line Mo17 BAC library was screened with gene specific probes made from the 3' untranslated region (UTR) of fie1 and fie2 cDNAs (see Material and Methods). Genomic fragments of approximately 12-kb each were sub-cloned and sequenced. The intron-exon structure for each gene was determined by aligning genomic sequences of each gene with their corresponding cDNA (Figure 1A and B).
The coding regions of both fie genes are composed of 13 exons ranging in length from 65 to 125bp that are identical in size to each other and to the Arabidopsis FIE gene except the first and the last exons, where the initiation and termination of the transcription occurs. The fie exonic sequences show 78% homology with each other. The coding sequences are interrupted by 12 introns, which vary in length from 63bp to 1124bp and show no detectable sequence similarity. fie1 has an additional 384bp intron located in the 5'UTR, just 6 nucleotides upstream of the ATG codon.

The putative promoter region of fie1 may be positioned in a 780bp segment between the RNA transcription start (-550) and the long terminal repeat (-1330) of a RIRE retrotransposon. A 3.4-kb genomic fragment farther upstream (-1330 to -4711) is composed of several types of scrambled retrotransposon and MITE elements that have homology with genomic sequences at the bz2 locus (Fu et al., 2001).

The 5' region of fie2 has a complex structure. A dot-plot alignment of the 6-kb sequence between the fie2 transcription-start site and the MILT retroelement has revealed a complex pattern of repeats (Figure 1B and C). Sequences between -1161 and -3479 consist of three types of repeats, named A, B, and C. These repeats form a 2.6-kb structure with the following symmetry: A1-B1-C1-B2-A2. The B3 and C2 repeats are positioned again at -5328 to -6077, forming another cluster. Repeats A1-A2 are 550bp long and share 95% homology, B1-B2-B3 are 350-bp long with 94% homology, and C1-C2 are 420bp with 93% homology (Figure 1C). The repeats do not share any homology or features with any other known repetitive or transposable elements as judged by a Basic Local Alignment Search Tool (BLAST) search of GenBank. These repeats are organized in a unique configuration that may be a potential cis-regulating element of fie2. We estimate the putative basal fie2 promoter to be ~ 768-bp, if framed between -393 and -1162. This marks the transcription start site of the fie2 gene and the beginning of repeat A1.

The maize fie genes have distinct patterns of expression

Reverse transcription polymerase chain reaction (RT-PCR) was used to examine the expression patterns of the maize fie genes in a set of vegetative and reproductive tissues collected from the inbred line B73 (Figure 2). fie1 expression was not detected in vegetative
tissues (mature leaf, immature leaf, tassel, stem, silk and roots). In the reproductive tissues tested, *fie1* expression was detected only in developing kernels ~10 days after pollination (blister stage). *fie1* was not detected in the unfertilized ovule. *fie2* expression was detected in all analyzed tissues, vegetative and reproductive.

**Expression of *fie* genes in developing kernels**

Expression of *fie1* and *fie2* was analyzed in developing kernels by RT-PCR and Northern hybridization. Kernel tissue was collected at various days after pollination (DAP) from 3DAP to 20DAP. RT-PCR was performed for both the *fie1* and *fie2* genes (Figure 3A). *fie1* showed a time specific pattern of expression. Low basal levels of *fie1* was detected during the early stages of kernel development from ~3-5DAP. Higher levels of *fie1* expression was detected from ~9-18DAP with expression going down to basal levels after ~18DAP. In contrast, *fie2* showed a constant level of expression at all times of kernel development. There data were confirmed by Northern hybridization (Figure 3B). *fie1* was not expressed in the ovule but levels increased gradually after fertilization reaching maximum expression ~9DAP. The *fie2* gene had a constant level of expression but when compared to *fie1* the *fie2* expression level was lower.

To determine if the *fie1* and *fie2* mRNA detected in the developing kernel was coming from the embryo or the endosperm, RT-PCR was performed using RNA isolated from embryo or endosperm tissues of 16DAP kernels (Figure 3C). The data showed that *fie1* was only expressed in the endosperm and not in the embryo. *fie2* was expressed in both embryo and endosperm but with lower levels in the endosperm.

**Maternal and paternal *fie* allele expression during kernel development**

Expression analysis data showed that *fie1* is a highly regulated gene that expresses only during early endosperm development. Data from Arabidopsis indicate that only the maternal allele of *FIE* is required for proper seed development regardless of the paternal contribution, *fie* homozygous cannot be rescued by a wild type paternal allele (Luo et al.,
2000; Yadegari et al., 2000). Based on this information we analyzed the maternal and paternal mRNA contributions of *fie1* in the developing endosperm. An insertion/deletion (indel) was identified between the inbred lines B73 and Mo17. In Mo17 the *fie1* allele contains a 12bp deletion when compared to the B73 allele. This deletion is located in the last exon 96bp 5' of the TGA stop codon. Reverse and forward primers were designed around this indel to produce a 300bp RT-PCR product (see Methods). These primers amplified both the B73 and Mo17 alleles equally. The RT-PCR products produced by the Mo17 allele was 12bp smaller than the products from the B73 allele.

Reciprocal crosses were performed between B73 and Mo17 inbreds. Kernels were collected at 2, 5, 10, and 15 days after pollination for each of the reciprocal crosses. Unfertilized ovules and 11DAP kernels from self-pollinated plants were sampled as controls from both inbred lines. Total RNA was extracted from each of the samples collected and RT-PCR was performed. PCR fragments were separated on denaturing-HPLC (D-HPLC) columns using the WAVE system (Transgenomics, Omaha, NE). The samples were separated based on size. The smaller Mo17 allele elutes out of the column before the larger B73 allele. The results for 15DAP kernels from the reciprocal crosses are shown in Figure 4A and 4B. In a cross where Mo17 was used as a mother and B73 as a father only the Mo17 allele of *fie1* was detected (Figure 4A). In the reciprocal cross only the maternal B73 allele was detected (Figure 4B). Expression of the *fie1* paternal allele was not detected in any of our samples, only the maternal *fie1* product was found. Data were the same for all the developmental stages from 2-15DAP (early stages are not shown). These data demonstrated transcriptional silencing of the paternal *fie1* allele, which suggested that the *fie1* gene was regulated by imprinting.

RT-PCR was performed using the same samples but with primers that amplified a non-imprinted gene. This was done as a control to demonstrate that detection of bi-allelic expression was detectable in these samples using the WAVE system. RT-PCR was performed with primers designed around a B73/Mo17 indel and expression of both paternal and maternal alleles was detected in 15DAP kernels (Figure 4C). Parental expression of *fie1* was also analyzed using the same method in two Pioneer inbred lines SSS1 and NSS1. The
results also showed that the paternally derived \textit{fie1} allele was silenced in these backgrounds as seen in the B73/Mo17 reciprocal crosses (data not shown). These data confirms that the observed silencing of the paternal allele of \textit{fie1} is not allele specific but a general property of the gene and reflects the general regulation of this locus.

Expression of the paternal and maternal components was also done for \textit{fie2}. The \textit{fie2} gene contains a 185bp MITE insertion in the 3' UTR. This insertion is present in the B73 inbred line but not in the Mo17 allele (Figure 5A). The insertion is flanked by 14bp inverted repeats and a 5bp direct target duplication (Figure 5B). The B73 \textit{fie2} poly-A transcripts terminate in the middle of the MITE insertion. While the Mo17 \textit{fie2} poly-A transcripts terminate within the genomic sequence downstream of the MITE insertion site. The MITE sequence was used to design allele-specific primers to distinguish between B73 and Mo17 \textit{fie2} mRNAs (Figure 5A).

The forward primer (F) that recognizes both alleles was designed for exon 11th. Two allele specific reverse primers were designed for each inbred line. For B73, the reverse primer (R1) was located within the MITE sequence. Because this sequence was absent in the Mo17 allele, the combination of the (F) primer and the (R1) primer only amplified the B73 allele. Another reverse gene specific primer (R2) was designed for Mo17. The primer combinations were shown to be allele specific by RT-PCR using RNA isolated from ovules and kernels of self-pollinated inbred plants (Figure 5C). RT-PCR was performed on developing kernels at 2, 5, 10, and 15 days after pollination of reciprocal crosses between B73 and Mo17, as it was done for \textit{fie1}. \textit{fie2} maternal allele expression was detected at all stages in both reciprocal crosses. The paternally derived RT-PCR product appears faintly at 5DAP but increases in intensity in 10DAP and 15DAP kernels. Thus, the \textit{fie2} paternal allele shows delayed activation, but not complete silencing. Delayed expression of the paternal allele has also been shown for the \textit{Arabidopsis FIE} gene (Luo et. al., 2000; Yadegari et. al., 2000).
Discussion

The different temporal and spatial expression of fie1 and fie2 suggest different biological functions.

Previously, we reported that the maize genome contains two orthologs of the Arabisopsis FIE gene, fie1 and fie2 (Springer et al., 2002). These two genes were identified based on their homology to the Arabidopsis FIE. We analyzed and compared the genomic sequences of fie1 and fie2 and found that both genes have the same number of exons and that most of the homology between the two genes comes from the coding region (Figure 1). However, the intragenic sequences do not share significant homology. Differences were found within the promoter regions of both genes. The fie1 promoter contains various repeats that are absent in the fie2 promoter region. Although the high level of homology along the coding region of the two genes may indicate similar function, differences in their spatial and temporal expression seem to suggest otherwise.

Expression of fie1 appears to be highly regulated. fie1 is only expressed in the endosperm (Figure 3C). fie1 is not expressed in unfertilized ovules, but after fertilization, expression increases gradually reaching its maximum at ~9DAP (Figure 3A and 3B). In contrast, fie2 is constitutively expressed and although fie2 is expressed in both the embryo and endosperm the levels of transcription are higher in the embryo when compared to levels in the endosperm. fie2 is also expressed at all times during kernel development, unlike fie1. The spatial and temporal expression differences between fie1 and fie2 suggest that the biological function of these two genes is not redundant.

In Arabidopsis only one FIE gene has been identified and it works to suppress endosperm development before fertilization. The Arabidopsis FIE gene is expressed in both embryo and endosperm. In maize, two genes were identified and because of their high homology to FIE are believed to be putative repressors of endosperm development. However, no mutants of the fie genes in maize have been identified and as such their biological role is not known. Since fie1 is not expressed in the ovule and is only expressed during early endosperm development it cannot act to repress endosperm development before fertilization, leaving fie2 as the putative homologue of the Arabidopsis FIE.
The Arabidopsis FIE gene is expressed in both reproductive and vegetative tissues (Ohad et al, 1999, Luo et al., 2000). Loss of function alleles of FIE produces pleiotropic phenotypes, including initiation of endosperm development without fertilization, embryo abortion at early stages, premature flowering by seedling shoots and the formation of flower-like structures along the roots and hypocotyls. (Ohad et al, 1999, Kinoshita et al. 2001). These results suggest the FIE protein encoded by a single-copy gene in the Arabidopsis genome may form distinct complexes in different plant tissues and participate in repression of several different developmental programs. We hypothesize that in maize, the dual function performed by the Arabidopsis FIE, is carried out by the fie1 and fie2 genes. For instance, fie2 may be involved in repression of endosperm development before fertilization whereas fie1 may be involved in a more specialized function in endosperm development. The conservation of protein structure suggests that both FIE proteins are involved in chromatin mediated gene silencing and function as global negative regulators of transcription. But because of distinct spatial and temporal patterns of expression, they may interact with different partners to regulate different pathways.

**The fie1 gene is regulated by imprinting**

Analysis of the fie1 and fie2 transcripts in developing kernels revealed that fie1 is an imprinted gene. Only the maternally derived allele is expressed during early endosperm development, whereas, the paternal allele remains silenced at all times (Figure 4).

Although fie2 is not an imprinted gene, it still showed a delay of paternal expression in developing kernels. fie2 paternal expression was not detected early in endosperm development. We first detected fie2 paternal expression at ~5DAP. The paternal expression delay of the fie2 is similar to the delay observed in the Arabidopsis FIE gene. The expression pattern of fie2 parallels the expression pattern of Arabidopsis FIE supporting the hypothesis that fie2 and not fie1 is the functional homologue of the Arabidopsis FIE.
Methods

Gene specific probes

To discriminate between *fie1* and *fie2* during hybridization experiments, gene specific primers were designed to the 3'UTR of each gene. The *fie1* specific primers (5'-CTGCTTCCAGCTCAAAC-3' and 5'-TTATTCATCTCATCCACGGTG-3') were designed to amplify a 287bp fragment. *fie2* specific primers (5'-ATCCGAGCTCCAGAAACTGA-3' and 5'-ATGATTTAACGTTATCTGTTACCCA-3') were designed to amplify a 270bp fragment.

Cloning and sequencing of *fie* genomic fragments

The Mo17 BAC genomic library was screened with full-length *fie1* and *fie2* cDNAs. Five BAC clones for each gene were identified and confirmed by DNA gel blot hybridization with gene-specific probes. *HindIII* and *EcoRI* BAC fragments were subcloned into pBluescript II KS+ (Stratagene, Cedar Creek, TX) and hybridized with *fie*-specific probes, and positive clones were submitted for sequencing.

DNA sequence analysis

DNA assembly was performed using the Sequencher program (Sequencher, Gene Code Corp., Ann Arbor, MI). Sequence analysis was performed with GCG programs (Genetics Computer Group, Madison, WI).

RNA gel blot analysis

Total RNA was extracted from 1g of material using a hot phenol extraction procedure and selective precipitation with 4M LiCl to remove traces of DNA and small RNA species (Brugiere et al., 1999; Verwoerd et al., 1989). For each time point, kernels were collected from two ears harvested from two different plants (replications) from either the B73 or Mo17 inbreds. RNA was quantified using a spectrophotometer (Beckman Instruments, Fullerton,
CA) at 260 nm. Poly-A was prepared from total RNA (400 µg) using the Oligotex poly-A purification kit (Qiagen, Valencia, CA). Electrophoretic separation was performed on 1.5% agarose gels containing 5% (v/v) of a solution of 37% formaldehyde in 3-(N-morpholino)-propanesulfonic acid buffer [0.02 M 3-(N-morpholino)-propanesulfonic acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA]. Gels were blotted to nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany) using the TurboBlotter (Schleicher & Schuell, Keene, NH), with 20 x SSC (1 x SSC is 150 mM NaCl, 15 mM sodium citrate) as the transfer buffer. Blots were probed with $^{32}$P-labeled PCR fragments of fie1 or fie2 cut from the 3’ UTR of the appropriate EST clones. Probe sequences share no homology, which avoids cross-hybridizations.

**Distinguishing fie mRNAs in reciprocal crosses**

Reciprocal crosses between B73 and Mo17 inbred lines were made, and F1 kernels sampled at 2, 5, 10 and 15 days after pollination. Total RNA was isolated using Purescript RNA Isolation Kit (catalog number R-5000A) from Gentra Systems (Minneapolis, MN). cDNA synthesis was performed with Superscript First-Strand Synthesis System (catalog number 11904-018) from Invitrogen (Carlsbad, CA). PCR reactions were performed with Pwo DNA polymerase (catalog number 1644947) from Roche Molecular Biochemicals (Germany). Primers used to amplify fie1 cDNA were 5’-

AGGCGAGATCTATGTCTGGGAAGTGCAGTC-3’ and 5’-

CAACCAGCACGGAGTACGATCGATGTGAA-3’. The product was 300bp, and the B73 allele differed from the Mo17 allele by a 12bp deletion. Primers designed to amplify fie2 were based on the MITE insertion in the B73 allele. The forward primer 5’-

CGTGAAGGCAAAATCTACGTGTGG-3’, positioned within exon 11, is common to both genotypes. In B73, fie2 poly-A transcripts terminate in the middle of MITE insertion that became part of the 3’UTR. The B73-specific reverse primer is 5’-

CATTACGTTACAATATGTGAACCGCAAACG-3’ and was designed for the MITE sequence. The Mo17-specific reverse primer is 5’-CAGAACAAACAGATGACAACGGTGTCCCAAAG-3’, and was designed for the 3’ UTR of cDNA that has no homology with the MITE. PCR fragments
were separated on 1% agarose gel. This primer combination allows the monitoring of paternal alleles expression in reciprocal crosses. Primers for the biallelic expressed gene were 5'-GGGACGAAGATAAAACG-3' and 5'-GCCAAACACATTTTGATAT-3'. Tubulin primers were 5'-AGCCCCATGGCCACCATGCCCAGTGATACCT-3' and 5'-AACACAAAGATCCCTGCAGCCCAGTGC-3'.

References


MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell 12, 2367-2382.
Figure 1. Genomic structure of fie loci.
Genomic segments (12-kb) of the fie1 (chromosome 4, bin 4.05) (A) and fie2 (Chromosome 10, bin 10.03) (B) are shown. Genes were mapped previously (Springer et al., 2002). The predicted start and stop codons of the fie coding regions are indicated by an ATG and TGA, respectively. Positions of nucleotides are relative to the translation start codon ATG (+1). The putative transcription and translation start sites are shown as bent arrows, exons are shown as tall vertical boxes, UTRs as shorter boxes, and introns as thick dark lines. Regions that have homology to retrotransposons are stippled. The direct repeats positioned upstream of fie2 are marked by large arrows. (C) Shows sequence alignment of the A, B, and C repeats. Nucleotide identities are shaded.
Figure 2. Expression pattern of \textit{fie}1 and \textit{fie}2.

RT-PCR was performed in various vegetative and reproductive tissues in maize using gene specific primers for \textit{fie}1 and \textit{fie}2. \textit{tubulin} was used as control.
Figure 3. *fie* expression in developing kernels.

(A) RT-PCR was used to determine expression of the *fie1* and *fie2* genes in developing kernels ranging from 3DAP to 20DAP. Two biological samples were used for each time period, indicated by the horizontal lines. The stage of the kernel is labeled at the top. The gene assay is marked at the right. (B) mRNA was isolated from ovules and kernels at 3, 6, 9, 12 and 15 days after pollination (DAP). Three mg of poly-A RNA were loaded into each lane. Blots were probed with $^{32}$P labeled 300bp specific probes from the 3'UTR of *fie1* or *fie2* cDNA. Probes have no homology to each other and do not cross hybridize. The actin probe was used as a loading control. (C) RT-PCR of *fie1* and *fie2* in embryo and endosperm. Embryo and endosperm were isolated under a dissecting microscope from 16DAP kernels. RT-PCR was performed with primers specific for *fie1* or *fie2*. RT-PCR of *tubulin* was used as a positive control.
Figure 4. Pattern of paternal and maternal *fie1* mRNA accumulation in developing kernels. Graphs represent the size-dependent separation of RT-PCR amplification products by the WAVE denaturing HPLC system. Larger fragments are retained longer on the cartridge, resulting in an accurate quantitative separation of a complex mixture. Total RNA was isolated from 15DAP kernels from Mo17 x B73 reciprocal crosses. Unfertilized ovules and 11DAP kernels from self-pollinated plants were sampled from both inbred lines as controls. Total RNA was extracted from whole kernels and RT-PCR was performed with primers positioned around a 12bp deletion in the 3'UTR of the Mo17 allele. (A) Expression of the maternal Mo17 allele in the Mo17 x B73 cross. (B) Expression of the maternal B73 allele in the reciprocal cross. (C) Biallelic expression of a control gene detected in the same RNA samples. The ratio of maternal to paternal peaks is 2:1:1. RT-PCR primers were designed around an insertion/deletion in the EST sequence. MEST80-E04.T3 (courtesy of Mei Guo, Pioneer Hi-Bred International)
Figure 5. Pattern of paternal and maternal fie2 mRNA accumulation in developing kernels. (A) fie2 Mo17 and B73 alleles are polymorphic due to a MITE insertion in the 3’ UTR in B73. Positions of a common forward primer (F) in exon 11 and genotype-specific reverse primers (R) in the 3’UTR are indicated by arrows. (B) DNA sequence of the 185bp MITE insertion into the 3’UTR of the fie2 B73 allele. The target site duplications are boxed and arrowheads mark the 14bp terminal inverted repeats. (C) Allele-specific expression in developing kernels from reciprocal crosses. Reciprocal crosses between B73 and Mo17 were performed and kernels collected at 2, 5, 10, and 15DAP. Total RNAs were isolated and amplified by RT-PCR with the primers described in (A). Ovules and 11DAP selfed kernels were used as controls to test primer specificity. tubulin expression was used as a loading control.
A

Exon 11

TGA

Mo17

F

R2

B73

F

R1

Mile

B

C

Mo17 allele

B73 allele

tubulin

♀ Mo17 x B73♂  ♀ B73 x Mo17 ♂
IMPRINTING OF THE MAIZE ENDOSPERM GENE FIE1 IS CONTROLLED BY DNA METHYLATION

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Abstract

Maize fie1 is a highly regulated gene that is expressed only during early endosperm development. The expression of fie1 is controlled by imprinting. Only the maternally transmitted fie1 alleles are active, whereas the paternal allele remains silent. Methylation sensitive restriction enzymes and bisulfite sequencing were used to investigate the role of DNA methylation in fie1 imprinting in the endosperm. Methylation sensitive restriction enzymes detected cytosine methylation at CG and CNG sites in exons 1-8 of fie1 in all tissues tested and a significantly lower level of methylation in the downstream exons 10-12. Methylation was also detected at CG or CNG sites in the promoter region of fie1, as shown by bisulfite sequencing. High methylation levels of fie1 were detected in leaf and embryo tissues in which fie1 is not expressed but less methylation was detected in the endosperm, where maternal alleles are expressed. SNPs (single nucleotide polymorphism) between B73 and Mo17 parental lines were used to demonstrate that the maternally transmitted fie1 alleles are unmethylated in the endosperm, but the paternally transmitted allele is methylated. These findings indicate that DNA methylation plays a role in imprinting of fie1. We propose a model in which the methylated state is a default for fie1 in all tissues except the endosperm where transcriptional activation of the maternal fie1 complements is achieved through their demethylation.

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Introduction

Genomic imprinting is an epigenetic mechanism by which a gene is differentially expressed based on the sex of the parent from which it was transmitted. Non-equivalent expression of a single gene depending on the parent-of-origin has been described in various organisms including insects, mammals and plants (de la Casa-Esperon and Sapienza, 2003). Imprinting was first described in maize by Kermicle (Kermicle, 1970). Imprinting has been shown to be important for proper seed development in plants (Baroux et al., 2002; Grossniklaus et al., 2001; Kermicle and Alleman, 1990). Pronuclear transplantation experiments have also demonstrated non-equivalent expression of paternal genomes in mammals (McGrath and Solter, 1984; Surani and Barton, 1983). Imprinting in mammals plays a significant role during embryo development (Kaneko-Ishino et al., 2003), and abnormal imprinting has been shown to be the cause of several human genetic diseases (Walter and Paulsen, 2003).

Flowering plants undergo sexual reproduction by a process known as double fertilization. During double fertilization, two sperm cells from one pollen grain fertilize the egg cell (1N) and the central cell (2N) of the female gametophyte. This process results in the diploid embryo (2N) and the triploid endosperm (3N). The embryo and the endosperm are genetically identical but differ only by the balance of maternal and paternal genomes (Reiser and Fischer, 1993). In maize, both maternal (1m:0p) (maternal:paternal) and paternal (0m:1p) haploids are viable. These haploids may arise either spontaneously or due to a mutation, indeterminate gametophyte (igl) (Kermicle, 1969; Kindiger and Hamann, 1993). Recovery of mature haploid plants indicates that one genome, maternal or paternal is sufficient for a plant to develop (Alleman and Doctor, 2000).

In contrast, proper maintenance of the maternal to paternal ratio in the endosperm has proven to affect seed development. Interploidy crosses have shown that failure to maintain the appropriate maternal to paternal genome balance can have different effects on development of the embryo and the endosperm (Haig and Westoby, 1989; Lin, 1984). In Arabidopsis, crosses between a diploid mother and a tetraploid father produce a triploid embryo with a 1m:2p ratio and a tetraploid endosperm with a 2m:2p ratio (Scott et al., 1998).
In this case, seed development is abnormal with a bigger embryo and smaller endosperm. In the reciprocal cross in which a tetraploid mother is crossed with a diploid father, a triploid embryo $2m:1p$ and an endosperm with $4m:1p$ are obtained: but in this latter case the endosperm and embryo are smaller when compared to normal seeds (Scott et al., 1998). In maize maintaining the proper maternal to paternal ratio in the endosperm is more stringent. Lin (1984) produced maize lines with endosperm ploidy levels from diploid (2n) to nanoploid (9n) using an $igl$ mutant. The only combinations that produce normal endosperms are $2m:1p$ or $4m:2p$, in which the normal maternal to paternal ratio is maintained, any deviation causes seed abortion.

The observation that proper endosperm ploidy needs to be maintained for proper endosperm development is known as the ploidy barrier (Lin, 1984). The ploidy barrier is believed to be caused by differences in maternal and paternal genome contributions. The ploidy barrier may be interpreted as that the parental genomes are epigenetically different in the endosperm, although dosage effect *per se* may contribute to this effect as well (Birchler, 1993). In either genome, a set of different genes may be silenced in a sex-specific manner by genomic imprinting and both genomes are required for proper endosperm development.

Haig and Westoby (1989) proposed that imprinting has evolved as a conflict between males and females over the allocation of the maternal resource to offspring (Haig and Westoby, 1989; Haig and Westoby, 1991; Wilkins and Haig, 2003). The hypothesis predicts that there is a class of genes that are expressed only from the maternal genome and a class of genes expressed only from the paternal genome. Paternally expressed genes should promote growth and maternally expressed genes should slow it down. According to this theory, imprinted genes are likely to be the key regulators of endosperm growth and development.

A number of genes in which expression depends on the parent-of-origin are known in maize. The imprinted genes in maize include $r$, a transcription factor involved in the anthocyanin pigment pathway (Kermicle, 1970); *zein-protein regulator (dzrl)*, which regulates low accumulation of the 10-kDa zein (Chaudhuri and Messing, 1994), 19-kDa and 22-kDa zeins and *α-tubulin* (Lund et al., 1995a; Lund et al., 1995b). However, none of these genes are involved in seed morphogenesis. Moreover, these genes show imprinting only for
particular alleles of some maize lines, arguing that their imprinting is not a general feature of these genes. However the maize gene, *dap* (*defective aleurone pigmentation*) is maternally expressed but paternally silenced and shows defects in aleurone and sub-aleurone cells, suggesting that this imprinted gene does affect endosperm development (Gavazzi et al., 1997). So far, the *dap* gene is not cloned and no molecular analysis has been done.

Several imprinted genes that function in endosperm development were found in Arabidopsis. The *MEDEA* (*MEA*) gene of Arabidopsis (Grossniklaus et al., 1998), has been shown to be imprinted (Kinoshita et al., 1999; Vielle-Calzada et al., 1999). MEA is a Polycomb group protein which works in a complex with other Polycomb proteins, FIE (FERTILIZATION INDEPENDENT ENDOSPERM) preventing endosperm development before pollination (Luo et al., 2000; Spillane et al., 2000; Yadegari et al., 2000). MEA is a negative regulator of cell proliferation affecting embryo and endosperm growth (Grossniklaus et al., 1998; Reyes and Grossniklaus, 2003). *MEA* is expressed in the embryo, endosperm and vegetative tissues (Kinoshita et al., 1999; Spillane et al., 2000). However, *MEA* is regulated by imprinting only in the endosperm, where the paternal *MEA* allele is transcriptionally silenced during all stages of development, but both alleles are active in vegetative tissues (Kinoshita et al., 1999; Vielle-Calzada et al., 1999).

Kinoshita et. al., (Kinoshita et al., 1999), have convincingly shown that the maternal and paternal *MEA* alleles are expressed in the embryo at the torpedo and later stages of development but Vielle-Calzada et. al., (2000) have also demonstrated that the paternal *MEA* allele is not active during early seed development, around 54 hours after pollination, when the embryo is at the midglobular stage. These results indicate a delay of paternal *MEA* allele expression in the embryo. Nevertheless silencing of the paternal *MEA* allele in the endosperm and enlarged size of the *mea* mutant seeds agree with the parent-offspring theory for the evolution of imprinting (Haig and Westoby, 1989; Haig and Westoby, 1991; Kinoshita et al., 1999).

Imprinting of recently discovered genes in the maize endosperm, *Fie1* and *NRPI* (*NAM RELATED PROTEIN1*) has been demonstrated at the transcriptional level, showing expression of maternally transmitted alleles, but not paternal alleles during all stages of
endosperm development (Danilevskaya et al., 2003; Guo et al., 2003). This type of expression was found in several inbred lines providing evidence that parent-of-origin effect is gene specific and not allele specific. However, the role of these genes in endosperm development remains to be discovered.

In mammals, it is widely accepted that imprinting is controlled by differential DNA methylation of maternal versus paternal alleles, a process that takes place during gametogenesis (Brannan and Bartolomei, 1999; Kaneko-Ishino et al., 2003). DNA methylation consists of 5-methylcytosine instead of cytosine in the CpG sequence. The major function of cytosine methylation is a repression of transcription. Most of the CpG sites in higher eukaryotes are methylated with the exception of CpG islands, which are stretches of DNA enriched in CG di-nucleotides usually associated with the transcriptional active state of genes (Ponger et al., 2001). However, imprinted mammalian genes show differential DNA methylation in CpG islands (Reik and Walter, 2001). A number of experiments suggest that methylation may also play a role in gene imprinting in plants. For example, in Arabidopsis reciprocal crosses between hypomethylated Arabidopsis plants carrying a transgene for METHYLTRANSFERASE 1 antisense (MET1a/s), an enzyme involved in maintenance of the methylation state, and wild type diploid plants affect seed size (Adams et al., 2000). In crosses in which hypomethylated plants are used as females and wild type plants as males, larger seeds with overproliferating endosperm are produced. When the reciprocal crosses are carried out, small seeds with reduced endosperm are observed. The observed phenotypes mimic phenotypes seen in crosses between diploid and tetraploid plants. Large embryo and endosperm proliferation resembles a paternal excess phenotype. On the other hand, small embryo and reduced endosperm resembles a maternal excess phenotype of tetraploid by diploid crosses (Adams et al., 2000; Luo et al., 2000).

In Arabidopsis, autonomous endosperm development may be induced by the combination of maternal demethylation (MET1a/s) or loss of FIE function suggesting that demethylation lifts repression from some genes whose functions are required for endosperm development (Spielman et al., 2003; Vinkenoog et al., 2000). More recently, another imprinted gene in Arabidopsis (FWA) has been shown to be affected by the level of
methylation (Kinoshita et al., 2004). *FWA* is a homeodomain transcription factor and only the maternal allele is expressed in the endosperm whereas the paternal allele remains silent. It has been shown that paternal *FWA* repression, as well as silencing of both maternal and paternal alleles in non-expressing tissues, is related to the maintenance of methylation at the promoter region of the gene. Research indicates that MET1 is involved in the maintenance of the methylation state of *FWA* as part of the mechanism responsible for gene silencing.

To study the role of DNA methylation in parental imprinting, and tissue specific expression, we made use of the two *fie* genes in maize. The endosperm specific gene *fie1* shows permanent paternal allele silencing during endosperm development (Danilevskaya et al., 2003). The duplicate gene *fie2* has a broader pattern of expression. *fie2* is expressed in vegetative tissues as well as in reproductive tissues, such as embryo and endosperm, and is an example of a non-imprinted gene (Danilevskaya et al., 2003; Gutierrez-Marcos et al., 2003).

We looked at the level of DNA methylation across the entire *fie1* gene as a way to test for its role in imprinting. We used methylation sensitive enzymes *HpaII/MspI* followed by PCR amplification across the restriction sites as well as bisulfite sequencing to determine *fie1* methylation states in DNA isolated from leaf, embryo and endosperm tissues. The study showed that DNA methylation occurred in the promoter region of the *fie1* gene and extends through exons 1-8. The study also showed a correlation between expression of *fie1* and its methylation state. Methylation was only observed in tissues in which *fie1* was not active. In DNA isolated from leaves and embryos methylation of *fie1* was observed in both maternal and paternal alleles. In contrast, in DNA isolated from endosperm, methylation was observed in the paternal allele and not in the maternal alleles. The study also showed no DNA methylation in *fie2*, which is a good correlation with the expression pattern of this gene in all tissues.
Results

DNA methylation assay of the maize *fie* genes in leaves, embryos and endosperms

To determine the level of cytosine methylation within the *fie* genes, we used a quick and simple method based on DNA digestion with methylation sensitive restriction enzymes, followed by PCR amplification across the restriction sites. If the template region was digested by the restriction enzyme, no PCR product was produced. In contrast, if the cytosines at CCGG sites in the template region were methylated, the DNA was protected from digestion and PCR product was produced. Two commonly used enzymes *Hpa*I and *Msp*I were chosen for this analysis. Both enzymes recognize CCGG sites but show different sensitivities to cytosine methylation. *Hpa*I does not cut DNA if either of the cytosines (CCGG) is methylated, whereas *Msp*I does not cut if the external cytosine is methylated. If none of the cytosines, or only the internal cytosine, are methylated *Msp*I cuts the DNA and no PCR product is produced. PCR products obtained from genomic DNA treated with *Hpa*I indicated that the CCGG sites in the amplified region were fully or partially methylated. PCR products obtained from genomic DNA treated with *Msp*I indicated that only the external cytosine was methylated.

The restriction maps of *fie1* and *fie2* genomic sequences show the distribution of *Msp*I/*Hpa*I sites along the gene (Figure 1). Sites are scattered along *fie1* and grouped in one cluster on *fie2*. As we reported, the *fie1* gene has two GC rich segments defined as CpG islands (Danilevskaya et al., 2003). The first island is located within the 1st exon and contains two *Msp*I/*Hpa*I sites. The second island covers the 11th and 12th exons and the 3'UTR, containing three *Msp*I/*Hpa*I restriction sites. The *fie2* gene has one CpG island within the 1st exon with eight *Msp*I/*Hpa*I restriction sites. No *Msp*I/*Hpa*I sites are present in any other segments of the *fie2* gene.

Four pairs of reverse and forward primers were designed around clusters of CCGG sites of the *fie1* gene (Figure 1A) and one pair of primers was designed for the *fie2* gene. Reciprocal crosses between the public inbred lines B73 and Mo17 were made and DNA samples were isolated from embryo and endosperm tissues of 14 DAP (Days After Pollination) kernels. DNA samples were digested with *Hpa*I or *Msp*I restriction enzymes.
DNA extracted from leaves of B73 inbred was used as a vegetative tissue control. PCR amplification, using equal amounts of undigested or digested DNA were performed, using the gene specific primers for each of the MspI/HpaII regions in fie1 and fie2. The PCR products were then visualized on an agarose gel (Figure 1B).

Results showed a specific pattern of cytosine methylation across the fie1 gene (Figure 1B). PCR products were obtained in the region amplified with the primers F1 x R1 and F2 x R2 in all DNA samples (leaves, embryos and endosperm) from both reciprocal crosses B73/Mol7 and Mol7/B73. These results showed that the CCGG sites within the 1st and 8th exon were methylated at both cytosines. In contrast, the second CpG island located in the downstream segment of the gene, regions amplified with F3 x R3 and F4 x R4 primers, showed a lower level of methylation. A very weak PCR product was detected in the same regions on DNA samples isolated from embryo and leaf tissues, in both reciprocal crosses, when cut with HpaII or MspI. Barely detectable products were obtained from DNA isolated from endosperm tissues. These observed lower levels of PCR amplification indicated a lower level of DNA methylation in regions three and four of fie1 genomic DNA. Our results point to a possible gradient of cytosine methylation along the fie1 gene with methylated sites at the beginning and unmethylated sites at the end of the gene. Conversely none of the samples digested with HpaII or MspI allowed for amplification within the fie2 gene, with primers F5 x R5, in any of the tissues tested. The lack of PCR amplification indicated that some or all of the CCGG sites in this region of fie2 were not methylated. Our results were in agreement with the expression pattern of fie1 and fie2. Methylation of fie1 was detected in all tissues tested at various levels depending on the tissue tested. On the other hand, in fie2, which is expressed in all tissues, methylation was not detected in any of the samples. These findings indicated a correlation between the methylation and expression of the fie genes.

The fie1 promoter region is methylated

To further investigate the methylation state of fie1 and its possible role in gene regulation, we looked at methylation along the promoter region of fie1. Due to the absence of MspI/HpaII sites in the promoter region we were not able to use the methylation sensitive
restriction enzymes assay. Sequence analysis revealed eleven possible CG/CNG methylation sites in the promoter region of fiel (Figure 2A). Using bisulfite treatment, which acts on non-methylated cytosines and converts them to uracils, we analyzed the methylation state of the promoter region of fiel. Genomic DNA from reciprocal crosses was isolated from embryo, endosperm and leaf tissues. After bisulfite treatment, PCR was performed along two regions of the fiel promoter using gene specific primers. The two regions, primers F6 x R6 and F7 x R7, encompass six out of the eleven predicted methylation sites. We were not able to develop primers that amplified other areas in the promoter region of fiel. The PCR products were then subcloned into a sequencing vector and multiple clones were sequenced (Figure 2B). Sequence analysis showed that almost all Cs in the bisulfite treated DNA were converted to Ts (Figure 2B). Most importantly, the results also showed that methylation did occur at the six predicted methylation sites. The level of methylation observed was dependent on the tissue analyzed. In DNA isolated from leaf and embryo tissues, where fiel is not expressed, all of the six possible methylation sites were methylated in at least 3 out of 4 molecules sequenced. In contrast, in samples from endosperm tissue, where fiel does express, all of the six possible methylation sites showed a low methylation level, where only 1 of every 4 molecules sequenced was methylated. Similar results were observed in the reciprocal crosses, B73 / Mo17, (data not shown).

Together the results obtained, from methylation sensitive restriction enzymes and bisulfite sequencing, showed that fiel was methylated at the promoter and coding region from the 1st to 8th exons (Figure 3). No significant methylation was detected downstream from the 8th exon. fiel methylation correlates with gene expression. Methylation was observed only in tissues in which the gene was not expressed (embryo and leaves). In contrast, partial demethylation was detected in the endosperm were fiel does express.

**Maternally derived fiel alleles are unmethylated in the endosperm**

In order to determine cytosine methylation of maternally and paternally transmitted fiel alleles in endosperm DNA, we took advantage of two SNPs (single nucleotide polymorphism) present in the 1st exon of B73 and Mo17 inbred lines (Figure 4A and 4B).
PCR primers were designed to amplify the fie1 region containing the two SNPs and two CCGG sites. If both alleles were methylated at CCGG sites, the sequences of the PCR products would show traces of both SNPs. If one allele was methylated at the CCGG sites, the sequence of the PCR product should have SNPs only from one parent. To facilitate direct sequencing of PCR products, fie1 gene-specific primers were extended with T3 and T7 primers at their 5’ ends. DNA isolated from embryo and endosperm tissues of the B73 and Mo17 reciprocal crosses were digested to completion with HpaII or MspI enzymes, amplified by PCR, and fragments were sequenced with T3 and T7 primers. Chromatograms of the nucleotide traces of the sequencing reaction of PCR products from digested embryo DNAs showed the presence of SNPs from both parents, B73 and Mo17 (Figure 4A and 4B), strongly supporting that both parental alleles were methylated in the embryo. Conversely, the chromatograms of PCR products generated from digested endosperm DNA showed the presence of SNPs only from the paternally transmitted alleles and a complete absence of traces from the maternally transmitted alleles. These results indicated that only the paternal silent allele of fie1 was methylated in the endosperm. As a control, undigested DNA was used and the chromatograms showed a mixture of traces from both parents B73 and Mo17. Thus we have shown the fie1 paternal allele remains methylated in the endosperm, but the maternal allele is unmethylated.

Discussion

DNA methylation pattern of the fie genes correlate with their transcriptional activity.

The maize genome contains two homologous fie genes but differences in their expression pattern suggest distinct mechanisms of regulation, as well as different functions for each of the genes. fie1 is a highly regulated gene expressed only during early endosperm development. fie1 is imprinted with a maternally active but paternally silenced allele. In contrast fie2 is not an imprinted gene and it has a broad pattern of expression (Danilevskaya et al., 2003). To investigate the possible role of DNA methylation in gene regulation, analysis of the methylation state of fie1 and fie2 was done in genomic DNA isolated from embryo, endosperm and leaf tissues. Methylation sensitive restriction digestion followed by PCR was
used to determine if CCGG sites of fie1 and fie2 were methylated (Figure 1). Results showed that the fie1 gene was methylated across exons 1-8, whereas a significantly reduced level of methylation was seen in the downstream segments of the gene.

The fie2 gene did not produce any detectable PCR product indicating that it is not methylated. Because of the high number of CCGG sites, it is possible that some sites may be methylated and that the lack of PCR product is due to only digestion of one CCGG site. Due to the close proximity and repeated nature of this region we were not able to assay each individual CCGG site for methylation.

Bisulfite sequencing results (Figure 2) demonstrate that methylation also took place along the promoter region of the fie1 gene. Two regions in the fie1 promoter containing six possible methylation sites were analyzed and all six regions showed methylation in DNA isolated from embryo, leaf and endosperm tissues. The results from the methylation assays indicated that methylation of fie1 expands from the promoter through the 8th exon (Figure 3).

Bisulfite sequencing revealed that although methylation was observed in all six methylation sites, the percentage of molecules that were methylated was different in DNA isolated from embryo and endosperm tissues. PCR products obtained from embryo DNA showed a higher level of methylation in which three out of four molecules sequenced were methylated. In contrast, in DNA isolated from the endosperm only one out of four molecules was methylated. Together these results point to a correlation between methylation and expression. In the endosperm where fie1 is expressed, methylation was lower. On the other hand, in leaf and embryo tissues where fie1 does not express, methylation was higher. We propose, that methylation of fie1 is a part of the mechanism that regulates its expression. Demethylation permits expression of fie1 during early endosperm development, possibly by allowing access of transcription factors to the promoter region of the gene.

**Demethylation of maternally transmitted alleles causes transcriptional activation of the fie1 gene in the endosperm**

Based on the results obtained by the methylation assays, we conclude that a direct correlation exists between methylation of fie1 and gene expression. We expected that in the
endosperm only the paternal silent allele would be methylated, whereas the maternal active allele would be nonmethylated. We confirmed this hypothesis by using SNP between the parental lines B73 and Mo17. The results showed the paternal allele of fiel was methylated and therefore protected from the restriction enzymes. The absence of maternal traces (SNP) indicated that the maternal allele was not methylated and was digested by the restriction enzymes. The results confirmed that methylation of fiel directly correlates with its expression. In tissues where the gene is not expressed, fiel is highly methylated. Our results suggest that in the endosperm where fiel does express, the paternal allele remains silent due to methylation but in the maternal active allele methylation is released and expression is allowed to take place.

A model for the control of fiel imprinting in the endosperm

Based on our results, we propose a model in which methylation of fiel is part of a mechanism that inhibits fiel expression. This model is based on two major experimental observations. 1) The fiel gene is expressed only in the endosperm and only from the maternally transmitted alleles. 2) The fiel gene is methylated in all tissues except in the endosperm where the silent paternal allele is methylated but the active maternal allele is unmethylated. We propose that the methylated state of fiel, including the promoter region and extending through the 8th exon, is the default state of the gene. This explains the lack of expression in all other tissues. In the embryo sac before fertilization, the fiel allele undergoes demethylation in the central cell but remains methylated in the egg cell (Figure 5). After fertilization, the demethylated fiel allele becomes active in the developing endosperm, but remains silenced in the embryo. This specific spatio-temporal demethylation takes place only in the central cell before fertilization. This specific mechanism explains imprinting of fiel that allows for maternal activation but paternal silencing in the endosperm. It remains to be discovered if demethylation of fiel in the central cell may be due to a global demethylation effect or to a gene specific effect.

A similar mechanism has been described by Kinoshita et al., (Kinoshita et al., 2004). They have shown that FWA, a homeodomain containing transcription factor, which controls
development in Arabidopsis is an endosperm specific imprinted gene whose expression is in part controlled by methylation in the 5' region of the gene (Soppe et al., 2000). DEMETER (DME), a DNA glycosilase protein, works to activate FWA. Kinoshita et al., proposed that flowering plants use a one-way control for the imprinting regulation of FWA in which demethylation of the maternal allele allows for gene expression. In this model de novo methylation of the paternal component is not required.

MEA is another endosperm-imprinted gene in Arabidopsis. Like FWA, MEA expression is also affected by methylation. Expression of the maternal MEA allele in the central cell requires functional DEMETER (DME) (Choi et al., 2002). At this time we do not know what role DME homologs may play in maize and in the regulation of field imprinting. In Arabidopsis, DME is only expressed in the central cell before fertilization and a similar enzyme may play a role in demethylation in the central cell in maize. Our data supports the “one way control of imprinting” proposed by Kinoshita et. al., (2004). Because the endosperm is a terminal tissue that does not contribute genetically to the next generation, loss of demethylation does not need to be reestablished. This one way control is different from the mechanism in mammals in which the methylation is released and then reestablished.

Methods

Plant material

Reciprocal crosses between inbred lines B73 and Mo17 were performed in the field in the summer of 2002. Embryos and endosperm tissues were dissected from kernels at 14 DAP (days after pollination) and 20 DAP. Dissections were performed under a dissecting microscope to avoid tissue cross contamination. Pericarp tissue was removed from the kernels. Tissues were immediately frozen in liquid nitrogen and stored at –80 °C. Leaf tissues were obtained from leaf blades of mature B73 or Mo17 plants.
DNA isolation and PCR reactions.

Genomic DNA was isolated from 10 to 20 mg of frozen embryo and endosperm tissues using Puregene™ DNA isolation components (Gentra Systems Minneapolis, MN, catalogs numbers D-50K2 and D-50K3). 0.5 µg of genomic DNA was digested at 37°C overnight in a 50µl reaction with 25U of either HpaII or MspI (New England Biolabs, Beverly, MA). After an overnight incubation, 25 more units of the appropriated RE were added and the reaction was incubated for 2hr at 37°C.

PCR amplification was performed using Expand Long Template (Roche, Germany). 2µl of the restriction enzyme reaction was used for PCR amplification in a 50µl Vom. The PCR conditions used were 95°C for 5 min followed with 35 cycles of 95°C for 45sec, 60°C for 45sec, 72°C for 1min and a final extension of 72°C for 10min. The primers used were: for fie1 F1 (CGCCGCCACCATAAGAACCACCTTATC), R1 (ATGGCAACTGCGATGGCC), F2 (ATGAGATAAGGACTCATGCCTCGAAGCCA), R2 (CCCACCTACGTGCGATGAAAG), F3 (AGGCGAGATCTATGTCTGGGAAGTGCAGTC), R3 (ATCGGCGACTTGCATTCC), F4 (AATGCAAGTCCCGAATAAGGCAAGACCAG) and R4 (CAACCAGCAGGAGTGACGATGATGAA). For fie2 the primers used were F5 (CGCGACACTAGTTAAGGTCTACACCA) and R5 (CGCGTCATGAAGTGAACCCCGATAG). For the paternal allelic analysis the primers used were: F (AATTAACCTCATAAAGGCGGCAGCCACCATAGAACCAC) and R (GATATACGACTCTATAGGGCATGATGGCCAGGGATG). PCR product was sequenced using the T3 and T7 primers.

Biulfite sequencing

Genomic DNA isolated from embryo, endosperm and leaf tissues was treated with bisulfite using EZ DNA Methylation Kit™ (Zymo Research, Orange, CA Cat No D5001). In a 50µl reaction 1µg of genomic DNA was treated according to the manufacturer’s recommendations. After bisulfite treatment, PCR was performed using gene specific primers for the bisulfite converted DNA sequence. The primers used were F6
(TAGTGGAGGTTGGATTTATT), R6 (TCACCTACACCTAACCTAATCCTAC), F7 (ATGGTACCACTTAGGTCTTAATTC) and R7 (AAAAATTATTTCCCCCCTAATCCT).

PCR amplification was performed using Expand Long Template (Roche, Germany). 2ul of the restriction enzyme reaction was used for PCR amplification in a 50ul Vol. The PCR conditions used were 95°C for 5 min followed with 35 cycles of 95°C for 45 sec, 60°C for 45 sec, 72 °C for 1 min and a final extension of 72°C for 10 min. PCR products were subcloned into pCR®4-TOPO vector (Invitrogen, Carlsbad, CA, Cat. No. K4575). Clones were then sequenced using M13F and M13R primers.

References


MEA genes that encode interacting polycomb proteins cause parent-of-origin effects on seed development by distinct mechanisms. Plant Cell 12, 2367-2382.
Figure 1. Methylation sensitive restriction enzyme assay of \textit{fie1} and \textit{fie2}.

(A) Genomic map of \textit{fie1} and \textit{fie2} showing \textit{MspI}/\textit{HpaII} restriction sites. Exons are marked with thick black arrows. Gene specific primes, shown by arrowheads, were used to amplify four regions of \textit{fie1} and one region of \textit{fie2} containing \textit{MspI}/\textit{HpaII} sites. (B) Agarose gel showing PCR products of the 5 analyzed regions of the \textit{fie} genes. Genomic DNA was isolated from embryo and endosperm tissues from reciprocal crosses of inbred lines B73 / Mo17 (female shown first). DNA was then treated with \textit{MspI} or \textit{HpaII} restriction enzymes and PCR was performed for one of the \textit{MspI}/\textit{HpaII} containing regions in \textit{fie1} and \textit{fie2}. The presence of PCR products indicates methylation of that particular region.
Figure 2. Bisulfite sequencing of the promoter region of *fie1*.

(A) Schematic representation of the promoter region of *fie1*. Eleven possible CG/CNG methylated sites are shown as open circles. Gene specific primers for the bisulfite converted DNA are show as small arrows F6, F7, R6, and R7. (B) Sequence of the PCR products from bisulfite treated DNA of three different tissues, Mo17 leaves, Mo17/B73 embryo and Mo17/B73 endosperm. Predicted methylation sites are framed. Gray shaded bases represent positions in which cytosines were changed to uracils. Yellow shaded bases indicate protected cytosines. Methylated sites are marked with an asterisk. The genomic sequence without bisulfite treatment is labeled Unt (untreated). Sequences labeled 1-4 represent four independent clones obtained from bisulfited treated genomic DNA.
Figure 3. Methylated sites across the *fiel* gene.

Black arrows indicate exons. Thirteen possible methylation sites were assayed by methylation sensitive restriction enzymes or by bisulfite sequencing. Methylated sites are indicated with closed circles and nonmethylated sites with open circles.
Figure 4. DNA methylation analysis of parental alleles of *fie1* in the endosperm and embryo. Two SNPs were identified in exon 1 of *fie1* between the two inbred lines B73 and Mo17 near two *HpaII* sites. DNA from embryo and endosperm tissues was digested with *HpaII* and PCR was performed along the region containing two *HpaII* sites as well as the two SNPs using *fie1* gene specific primers, containing T3 or T7 extensions. The PCR products were sequenced directly using the T3 and T7 primers. Uncut DNA was used as a control. The black shaded boxes indicate the two SNPs. The gray shaded bar marks the location of the SNP in the chromatograms. (A) Chromatograms from B73 x Mo17 cross. (B) Chromatograms from Mo17 x B73 cross.
A

SNP No. 1
B73
TGGGCTGT
TGGGCTGT
Mo17
SNP No.2
AACA GACA

Hpall

B73 / Mo17
Embryo

Uncut

Hpall

B73 / Mo17
Endosperm

Uncut
B

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<th>SNP No.1</th>
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**Hpall**

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<th>Mo17 / B73 Endosperm</th>
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<td>TGGG CTGTT</td>
<td>TGGG CTGTT</td>
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<td>AACA GACA</td>
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Figure 5. Proposed model for control of *fie1* imprinting.

Spatial and temporal demethylation allows for expression of only the maternal allele of *fie1* in the endosperm. Because demethylation occurs in the central cell before fertilization the paternal allele will remain methylated in the endosperm and both maternal and paternal alleles will also remain methylated in the embryo.
meiosis

IP >
microspore

megaspore

mother cell

mother cell

male gametophyte

Fertilization

Paternal genes stay methylated in the sperm.

Maternal genes are demethylated in the central cell.

Non Methylated DNA

Methylated DNA

M-maternal

P-paternal

endosperm

embryo

kernel

Paternal genes stay methylated in the sperm.

Maternal genes are demethylated in the central cell.

Non Methylated DNA

Methylated DNA

M-maternal

P-paternal

endosperm

embryo

kernel
GENERAL CONCLUSION

Summary

In this project we characterized the mRNA expression of the maize *fie1* and *fie2* genes. We determined that although these two genes are 78% homologous in their coding region, their promoter regions contain no similarity. We demonstrated that the genes *fie1* and *fie2* have different patterns of mRNA expression. *fie1* mRNA was detected only in the endosperm, during early stages of development. In contrast, *fie2* mRNA was constitutively expressed in all tissues tested. More significantly we showed that *fie1* is an imprinted gene in the endosperm.

Analysis of the DNA methylation state of *fie1* revealed a correlation between methylation and repression of gene expression. We detected methylation along the promoter and exonic regions of *fie1* only in tissues in which the gene is repressed. Lower levels of DNA methylation were detected in the endosperm, where *fie1* is expressed. We proposed a mechanism for the regulation of *fie1* imprinting. In this model the *fie1* maternal alleles are demethylated in the central cell of the female gametophyte before fertilization but remain methylated in the egg cell and in the male gametophyte. After fertilization, demethylation allows for expression of the maternal *fie1* alleles in the endosperm. However, the paternal allele remains methylated in the endosperm and therefore silent and both paternal and maternal alleles also remain methylated and silent in the embryo.

Recommendations for future research

In order to investigate if methylation of the promoter region of *fie1* is sufficient for silencing of the gene, I propose the use of a transgenic approach. In this experiment expression of a transgene under the control of the *fie1* promoter can be analyzed with the methods described in this dissertation. Some questions that can be asked are: Is the transgene expressed properly in the endosperm? Is the transgene expressed when it is maternally or paternally inherited? Is the promoter region of the transgene methylated?

If the technology allows, more experiments also need to be done to determine the methylation state of the *fie1* gene in the central cell and the egg cell. It is important to
continue genetic experiments to identify mutants for the $fie1$ and $fie2$ genes. The identification of mutants is crucial to assign function to these genes.

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