Temporal and Spatial Regulation of Cell Cycle Genes during Maize Sex Determination

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Maize (Zea mays L.) pistil cell death and stamen cell arrest are pivotal process on the sex determination, which diverges from bisexual state of floral meristem to unisexual state in staminate or pistillate floret. We investigated the temporal and spatial distribution of cell cycle gene expression during maize sex determination. The positive regulatory genes of cell cycle, cyclin A, cyclin B, cyclin dependent kinase (CDK) and Mad2 were highly expressed in the developing pistil and stamen but the expression was disappeared in the dying pistil and arresting stamens. In contrast, the negative regulatory genes of cell cycle, Wee1 and CDK inhibitor (CKI) were expressed in the arresting stamens in the wild-type ear and tasselseed2 mutant tassel; however, these genes were not detected in dying pistil although the cyclin B gene expression was disappeared. These results suggest that both the pistil cell death and stamen cell arrest process in maize sex determination are involved in cell cycle regulation, but the different expression patterns of negative regulatory cell cycle genes in the arresting stamens and aborting pistils suggest that the two processes may have distinctive modes of action.

Key words – Maize, sex determination, cell cycle gene, pistil cell death, stamen arrest

Introduction

Most flowering plants are hermaphrodite, which produces both male and female reproductive organs within the same flower. In plant species, about 30% of them have evolved sex determination processes such as dicliny, dioecy, and monoecy that generate a unisexual state in the plant[3,7,15]. Maize is a monoecious plant that the male (tassel) and female (ear) florets are located in different space. within one plant and evolves into increasing heterogeneity. The maize spikelet initial develops acropetally to two floral meristems, upper (primary) and lower (secondary) floret. Each floral meristem initiates a series of floral organs, a palea, two lodicules, three stamen initials and a central pistil. After floral organ initiation, unisexual stage is reached from bisexual stage by selective pistil cell death, pistil cell protection, and stamen cell arrest[2,8,14,27]. In the tassel spikelet, all pistils abort while stamens sexually mature. The tasselseed genes are involved in the pistil cell death process resulted in staminate floret. In the tasselseed1 (ts1) and 2 (ts2) mutants, the sexual reverse occurred from staminate floret to pistillate floret[8,14,27]. The TS2 gene encodes a member of the short-chain dehydrogenase/reductase (SDR) family of enzyme[9]. This gene is expressed in subepidermis of pistil cells at the time of their abortion in both tassel spikelets and the secondary ear spikelets[2]. In the ear spikelet, stamens fail to mature not because of cell death but because they are developmentally arrested. This process of stamen arrest requires the phytohormone gibberellin—biosynthetic or perception gibberellins that mutations (dwarf or d1, d2, d3, d5, an1, and D8 mutants) prevent stamen arrest. In the dwarf mutants, all six stamens in the ear spikelets sexually mature, resulting in bisexual primary and staminate secondary florets[10,20]. In these mutations, exogenous treatment with biologically active gibberellins can reverse the mutant sexual phenotypes[19].

Plant cell cycle control is tightly linked to differentiation and development to accomplish normal plant growth[6], and plant cell divisions occur at specialized zones known as meristems, leaves and flowers are formed at the shoot and floral meristems, respectively[13]. The cell cycle comprises of G1, S, G2, and M-phase, and many cell cycle genes have shown restrict oscillatory expression pattern, phase-specific expression. Generally, cyclin A play roles in G1/S transition, therefore, these genes are expressed highly through these phases. In contrast, cyclin B genes are expressed specifically at G2/M phase during the cell cycle. The phase specific cyclin-dependent kinases (CDKs) play a
central role in mediating cell cycle progression[21,25]. The CDK activity is regulated by association with cyclin subunit, reversible phosphorylation and association with other regulatory factors. Both CDK A and B make a complex with cyclin A and cyclin B, respectively. The CDK/cyclin complexes are affected by a combination of different gene products and factors in which the progression of the cell cycle is initiated as an integral part of the growth and developmental program and in response to the environment. The mitotic arrest deficiency 2 (Mad2) spindle checkpoint protein inhibits anaphase-promoting complex or cyclosome (APC/C), a multi-subunit E3 ubiquitin ligase associated with proteosome-mediated proteinolysis, through binding to its mitotic specific activator, Cdc20[23,28]. It has been reported that the maize Mad2 localization patterns in mitosis are basically conserved among eucaryote, and functions roles in the spindle checkpoint[29]. On the other hand, Wee1 and CDK inhibitor (CKI) were act as a negative regulator of CDK/cyclin complexes by phosphorylating to this CDK subunit[5,6]. In plant, maize Wee1 gene expression is associated with endoreplication in highly polyploidy endosperm cells[26] and Arabidopsis Wee1 in fission yeast causes cell arrest[24]. However, it is not known well the relationship of cell cycle regulation with maize sex determination process in which the distinctive or specific regulation of cell cycle genes between pistil cell death and stamen cell arrest processes.

In this report, we examine the temporal and spatial expression of cell cycle genes in the two processing of maize sex determination, pistil cell death and stamen cell arrest. The positive regulator of cell cycle genes were expressed highly in the developing pistils and stamens but these gene expressions were declined in aborting pistils and arresting stamens. However, the negative regulator of cell cycle genes, Wee1 and CKI, were solely expressed in the arresting stamens but not in the aborting pistil. Therefore, the two distinctive maize sex determination processes, pistil cell death and stamen arrest, may partially be caused by the specific cell cycle gene regulation.

Materials and Methods

Plant materials

The wild-type maize (Zea mays L.) inbred line W22 was used in this study. The tasselseed2-reference (ts2) was obtained from E. Irish and backcrossed into the inbred line W22[9].

RT-PCR analysis and cloning of cell cycle genes

Total RNA from dissected maize tissues was purified by the guanidine thiocyanate method[4]. First strand cDNA was synthesized using Superscript II (Gibco BRL) and an oligo-d (T) primer according to manufacturer’s instructions. The cyclin A, cyclin B, CDK A (cd2), Mad2, Wee1, and CKI cDNA clones were obtained by PCR amplification of first strand cDNA using primers as follows - cyclin A (P888: 5'TACATTTGGGCAACA GACCAAGG3' and P889: 5' CCGACTCTGAGACAGCCTAGCAA3'); cyclin B (P903: 5'CCTGGAACAGTCTGGAACAGCCTACC3' and P889: 5'CCG ACTCTGAGACAGCCTAGCAA3'); CDK A (P905: 5'GG CATGGAACAGCCT AACCACATG3' and P919: 5'TGGA CAGAGAACAGCGTTAATCT3'); Mad2 (P958: 5'GAAG TACGGCTTACAGTCCGCT3' and P959: 5'AGCCGAG CCTTAAGGCGCTGACT3'); Wee1 (P904: 5'TCAGCTA TATGCT CCGGAAATTG3' and P920: 5'GGGTCCCGAAA TGCAACTGATAAC3'); CKI (P975: 5'GGGTACATTTGGGAG TTGAGGACCA3' and P976: 5'CATG GGAGGAACTAC AGGGAGAC3'). Maize actin gene was used as a positive RT-PCR control (P213: 5'-CATGAGGCACGTAACCGT ATC3' and P214: 5'TCATACCTCCCCATGGAGGATCCAC 3'). PCR amplification was performed using the Expand Long Template PCR system (Roche) using the manufacturer’s instructions under the following conditions: 94°C for 2 min, then ten cycles at 94°C for 10 sec, 65°C for 30 sec, and 68°C for 2 min, followed by an additional 20 cycles of PCR at 94°C for 10 sec, 65°C for 30 sec, 68°C for 2 min with additions of 20 sec at 68°C between each cycle. Amplification products used as in situ probes were cloned into the pCRII (Invitrogen), and sequenced to confirm their identity. The plasmid containing the cyclin A, cyclin B, CDK A, Mad2, Wee1, and CKI cDNAs are designated pYU1138, pYU1139, pYU1143, pYU1158, pYU1146, and pYU1174, respectively.

Preparation of in situ riboprobes

Plasmid DNA was prepared using Qiagen 100 columns (Qiagen Inc.) following the manufacturer’s instructions. DNA templates for sense and antisense riboprobes were linearized at a restriction site flanking the cDNA inserts by digestion with an appropriate enzyme, which leaves a 5’ overhang. In vitro transcription reactions using T7 or SP6 RNA polymerase and 11-digoxigenin-dUTP (Roche), riborobe DNase treatment, and hydrolysis was according to the manufacturer’s instructions. The riboprobe was subject to mild alkaline hydrolysis for varying times in 0.1 N NaOH to yield products in the 100-200 nucleotide range.
Cytological techniques and in situ hybridization

The formaldehyde tissue fixation, paraffin embedding and microtomy were essentially as described [16] except that Hemo-De (Fisher Scientific, Inc.) was substituted for Histoclear (National Diagnostics, USA), and the final concentration of riboprobe was adjusted to 10 ng/ml/kb and hybridization was performed at 55°C. After hybridization, slides were washed in 0.2X SSC (30 mM NaCl, 3 mM sodium citrate, pH 7.0) for 1.5 hrs (3 times for 30 min at 55°C, 60°C, 65°C, respectively), followed by a wash in 1X NTE (0.5 M NaCl 10 mM Tris, pH 7.5) for 10 min at 37°C. Sections were treated with 20 µg/ml RNase A in 1X NTE for 30 min at 37°C, washed in 1X NTE for 10 min at 37°C, followed by a incubation in 0.2X SSC for 1hr at 55°C, and treatment in 0.5% blocking reagent (Roche) dissolved in 1X TBS (150 mM NaCl, 100 mM Tris pH 7.5) for 1 hr at room temperature. Blocking reagent was decanted and slides were incubated with an antibody solution containing a 1:1000 dilution of anti-digoxigenin-alkaline phosphatase-conjugated antibody (Roche) in 0.3% Triton 100, 1% BSA in 1X TBS for 5 hrs at room temperature. After incubation with antibody, slides were washed four times in 1X TBS for 10 min each, followed by a 10 min incubation in substrate buffer, containing 100 mM Tris pH 9.5, 100 mM NaCl and 50 mM MgCl₂. Approximately 300 µl of substrate buffer containing 1 mM 4-nitroblue tetrazolium chloride and 0.4 mM 5-bromo-4-chloro-3-indolyl-phosphate (Roche) plus 1 mM levamisole (Sigma Co.) was pipetted onto each slide, a coverslip applied, and incubated for 3 days at room temperature in a dark, humid chamber. Slides were rinsed in 10 mM Tris pH 7.5, 1 mM EDTA, counterstained for 5 min at room temperature and mounted in 70% glycerol and 30% PBS.

Microscopy and image processing

All sections were examined using an Axioplan 2 microscope with digital image capturing on an HR and processing using AxioVision v4.4 (Carl Zeiss Inc.). Composites figures were generated and labeled using Adobe Photoshop 7.0 (Adobe systems Inc.).

Results and Discussion

Isolation of cell cycle genes from various maize tissues

To investigate the distribution of cell cycle genes during maize sex determination processes, we were trying to perform in situ hybridization using maize flowers, tassel and ear florets. To this end, we firstly examined RT-PCR analysis to verify which genes are expressed in dissected maize tissues and isolate cell cycle genes. Both tassel and ear samples were divided into two stages according to its size, early (<15 mm floret length) and late (15 - 20 mm floret length) stages. The early stage can be defined bisexual state in which the floral organ initiation growth and mature to early gynoecium or androecium development. In the late stage, the unisexual state was accomplished by pistil cell death or stamen arrest. Furthermore, other tissues such as root, stem, leaf, embryo and endosperm were collected. As shown in Fig. 1, the positive-regulated cell cycle genes such as cyclin A, cyclin B and CDK A, and a mitotic spindle checkpoint protein Mad2, were highly expressed in floral organs compared to other vegetative tissues. In the early and late stages of ear and tassel tissues, no big differences of these gene expression levels were observed. In case of negative-regulated cell cycle genes, Wee1 gene was expressed highly in the late stage of floral tissues rather than the early stage. This result suggests that the Wee1 protein activates on the sex determining stages in which the pistil cell death and stamen arrest are occurred. On the other hand, the CKI gene, another cell cycle inhibitor, was also expressed in the tassel and ear.

Fig. 1. RT-PCR analyses of cell cycle genes with various maize tissues. Total RNA was isolated from dissected maize tissues, and the first cDNA strand was synthesized using reverse transcriptase. RT-PCR was performed by its cell cycle gene specific primers. The actin gene was used as a positive control. M, size marker; 1, early ear; 2, late ear; 3, early tassel; 4, late tassel; 5, root; 6, shoot; 7, embryo & endosperm; 8, leaf.
through all stages but the expression levels were shown to reverse compared to Wsll gene expression pattern. Taken together, these results indicate that both the positive- and negative-regulated cell cycle genes used in this study are good indicators for cell cycle analysis on the sex determination process. Thus, all tested cell cycle genes were cloned and sequenced for in situ probes.

Temporal and spatial distribution of positive-regulated cell cycle genes in maize sex determination

To examine the temporal and spatial regulation of cell cycle genes in the dying pistil and arresting stamens, we conducted in situ hybridization using maize wild-type and tasselseed2 mutant florets using digoxigenin-labelled antisense RNA probe. A spotty expression pattern was observed in all probes (Fig. 2). As shown in wild-type tassel (Fig. 2A-D), the positive-regulated cell cycle genes, cyclin A, cyclin B, CDK A and Mad2 are highly expressed in the developing stamens but these expression was not detected in aborting pistil. In the wild-type ear (Fig. 2E, G and H), the developing pistils have shown to have these gene expression levels but these gene expression in the arresting stamens was not observed. The maize tasselseed2 mutant tassel reverses sexual state resulted in pistillate[8,14,27]. As shown in the growing pistil of tasselseed2 mutant tassel (Fig. 2F), the positive-regulated cell cycle gene (cyclin B) was expressed highly, but the arresting stamens were not expressed. The other positive cell cycle genes are working on the same way in the tasselseed2 mutant (data not shown). Based on these results, we suggest that the spatial regulation of cell cycle genes is involved in the sex determination processes, pistil cell death and stamen cell arrest. To verify the temporal regulation of these genes, the cyclin B gene were chosen and performed in situ hybridization from very early to late stages of tassel florets. The cyclin B gene acts as a positive regulator in the G2/M phase of the cell cycle and its RNA is short-lived and present only in dividing cells in the G2/M transition[11]. As shown in Fig. 3A and B, the cyclin B gene was highly expressed in both pistil and stamen primodia at the very young bisexual

![Image of in situ expression of positive regulated cell cycle genes with wild-type and tasselseed2 mutant tassel florets.](image1)

Fig. 2. In situ expression of positive regulated cell cycle genes with wild-type and tasselseed2 mutant tassel florets. The wild-type tassel (A-D), wild-type ear (E, G and H), and the tasselseed2 tassel (F) florets were used in this experiment. The antisense riboprobes, cyclin A (A and E), cyclin B (B and F), CDK A (C and G), and Mad2 (D and H) were hybridized with longitudinal sections. P, pistil; S, stamen.

![Image of development time.](image2)

Fig. 3. Cyclin B gene expression in maize wild-type tassel according to developmental time. In situ hybridization was performed using cyclin B gene according to development time, from bisexual to unisexual state in the wild-type tassel. P, pistil S, stamen; scale bar = 100 μm.
stage. As tassel florets matured, the cyclin B transcripts became disappeared in the dying pistil and stopped the expression while stamine tassel mature (Fig. 3C-E). However, the growing stamens still have shown to high cyclin B gene expression (Fig. 3C-E). The same result occurred in the ear floret, the cyclin B gene expression was stopped in the arresting stamens (data not shown). These results implicate that the temporal regulation of cell cycle genes are important in the floral organ development. Taken together, these results suggest that cell cycle arrest or cell division cessation involved in the pistil cell death and stamen arrest process.

Arresting stamen specific expression of negative-regulated cell cycle genes

To examine whether the negative-regulated cell cycle genes are also expressed in the aborting pistil and arresting stamens, two negative regulator of mitosis genes such as Wee1 and Cki were selected (Fig. 1). The Wee1 protein is a Thr/Tyr protein kinase that inhibits Cdc2 (CDK) activity and the progression of cells from G2/M transition in the cell cycle[18,22]. The maize Wee1 gene expression is associated with endoreduplication in highly polyploid endosperm cells[26]. Also the Cki has been shown to be participated in the control of both the cell cycle and the endoreduplication cycle[1,5]. As shown in Fig. 4A and B, the arresting stamens in wild-type ear displayed specific expression of Wee1 and Cki genes, respectively. The result coincided in the tasselseed2 mutant tassel in which the arresting stamens have accumulated Wee1 and Cki transcription messages (data not shown). Intriguingly, these two genes were not expressed in the aborting pistil in the wild-type tassel (Fig. 4C). These results refer a cell cycle block, possibly mediated by Wee1 and/or Cki, negative regulators of cell cycle, in the process of arresting stamen cells but not in the pistil abortion process. The different expression patterns of those genes between arresting stamen and aborting pistil tissues may refer that the pistil cell death and stamen cell arrest mechanisms are quite distinctive even though the disappearances of cyclin B gene expression was occurred in the both tissues(Fig. 2 and 3). In regarding to stamen arrest, it seems that the negative regulator of cell cycle genes function predominately. All together, we propose the extend mechanism of the maize sex determination that the temporal and spatial regulation of cell cycle genes play pivotal roles in pistil cell death and stamen arrest. Particularly, the stamen arrest event in the wild-type ear and tasselseed2 mutant tassel may cause by cell cycle arrest with expressing negative regulated cell cycle genes such as Wee1 and/or Cki.

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초록: 옥수수 성 결정에 있어서 세포주기 유전자들의 시간적, 공간적 조절

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옥수수 (Zea mays L.) 뿌음 억압 세포사멸과 수술 세포 성장정지 등을 통하여 양성상태에서 단성 상태로 성장정지를 완성한다. 본 논문에서는 옥수수 성 결정 중간 세포주기 유전자들의 시간적, 공간적 발현조절을 조사하였다. 세포주기의 양성조절 인자 즉 cyclin A, cyclin B, cyclin dependent kinase A (CDK A), Mad2 유전자들은 성장하는 억압과 수술에서 늦게 발현되는 반면 죽어가는 억압과 성장이 정지되는 수술에서는 이들의 발현이 사라진다. 이와 달리, p53과 CDK inhibitor (CKI) 같은 세포주기 억제조절유전자들은 악생염 억압과 tasselseed2 돌연변이 수술의 성장이 정지하고 있는 수술에서 발현이 증가되었지만, 홍무률에도, 이들 유전자들은 죽어가는 억압세포에서는 발현되지 않았다. 이들 결과들을 통하여 옥수수 성 결정 과정 중에서 억압 세포사멸과 수술세포 성장정지를 세포주기조절과 밀접한 관계가 있으며, 특히 성장이 정지하는 수술과 죽어가는 억압에서의 응성 세포주기 조절 유전자들의 다른 발현양상은 이들의 성 결정 메커니즘을 구별 될 것이라고 사료된다.