Characterization of maize (Zea mays L.) Wee1 and its activity in developing endosperm

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ABSTRACT We report the characterization of a maize Wee1 homologue and its expression in developing endosperm. Using a 0.8-kb cDNA from an expressed sequence tag project, we isolated a 1.6-kb CDNA (ZmWee1), which encodes a protein of 403 aa with a calculated molecular size of 45.6 kDa. The deduced amino acid sequence shows 50% identity to the protein kinase domain of human Wee1. Overexpression of ZmWee1 in Schizosaccharomyces pombe inhibited cell division and caused the cells to enlarge significantly. Recombinant ZmWee1 obtained from Escherichia coli is able to inhibit the activity of p135-adsorbed cyclin-dependent kinase from maize. ZmWee1 is encoded by a single gene at a locus on the long arm of chromosome 4. RNA gel blots showed the ZmWee1 transcript is about 2.4 kb in length and that its abundance reaches a maximum 15 days after pollination in endosperm tissue. High levels of expression of ZmWee1 at this stage of endosperm development imply that ZmWee1 plays a role in endoreduplication. Our results show that control of cyclin-dependent kinase activity by Wee1 is conserved among eukaryotes, from fungi to animals and plants.

The cell cycle in eukaryotes is controlled by a family of conserved cyclin-dependent protein kinases (CDKs) (reviewed in refs. 1–6). The catalytic subunit of these enzymes, such as p34CDC2 for CDK1 and p33CDC2 for CDK2, contains a conserved protein kinase domain whose activity depends on association with a cyclin regulatory subunit (reviewed in ref. 6). CDK activity fluctuates in a regulated manner during the cell cycle, triggering essential events. This oscillation is safeguarded by sophisticated regulatory networks that involve transcriptional and posttranscriptional mechanisms (reviewed in refs. 6 and 7). By integrating intracellular and extracellular signals that modulate CDK activities, this control network modulates cellular proliferation with developmental and environmental cues.

In multicellular eukaryotes, phosphorylation and dephosphorylation of threonine 14 and tyrosine 15 of the catalytic subunit of CDKs regulate their activity and determine the timing of G1 and mitosis (8). Phosphorylation of tyrosine 15 inactivates CDK1, whereas dephosphorylation by CDC25 activates the enzyme, triggering the G1-to-M-phase transition (9). Threonine 14 and tyrosine 15 are buried beneath a loop structure, and cyclin binding induces a conformational change that makes these residues accessible for phosphorylation (10). Extracellular stimuli, such as high osmolarity (11), calcium (12), and UV irradiation (13), affect cell division by regulating these phosphorylation events.

Phosphorylation of CDKs at tyrosine 15 is mediated by the related kinases Wee1 (14–16), Mik1 (15, 17), and Myt1 (18, 19). The first of these to be identified was Wee1 from Schizosaccharomyces pombe, which causes a delay in mitosis by phosphorylating the M-phase promoting factor on tyrosine 15. Myt1, a membrane-associated Wee1 homologue from Xenopus (18) and humans (19), preferentially phosphorylates threonine 14 and, to a lesser extent, tyrosine 15. Wee1/Mik1-type kinases were identified in a number of eukaryotes, such as Saccharomyces cerevisiae (20), humans (21–25), Drosophila (26), Xenopus (27), and mouse (28). The molecular size of Wee1/Mik1 kinases ranges from the 107-kDa Swe1 in S. cerevisiae (20) to the 68-kDa Wee1 from Xenopus (27). Most of the sequence variation in Wee1 occurs in the amino-terminal domain, whereas the carboxy-terminal region, where the protein kinase domain resides, is more conserved (27, 29).

Wee1 activity is regulated in multiple ways. In synchronized S. pombe cells, Wee1 mRNA does not fluctuate during the cell cycle, but the abundance of Wee1 protein undergoes moderate oscillation (29), suggesting the involvement of either translational or proteolytic regulation. In S. cerevisiae, Swe1 is regulated by proteolysis via the Met30-containing F-box ubiquitination pathway (30), and in Xenopus, proteolysis of Wee1 via the ubiquitin-dependent pathway may be required for mitosis (31). Nim1, a protein kinase in S. pombe, phosphorylates Wee1 and inhibits its kinase activity in vitro (32, 33). Overexpression of Nim1 rescues S. pombe mutants lacking CDC25, whereas the down-regulation of Nim1 results in enlarged cells (14), a phenotype analogous to Wee1 overexpression. Multiple phosphorylation targets for CDK are present in the NH2-terminal domain of Wee1 from S. pombe (29). The cyclin B/CDK1 complex is able to phosphorylate human Wee1 in vitro (25), although the biological significance of this is unknown. The activity of Wee1 also may be regulated through compartmentalization. In HeLa cells, Wee1 is localized exclusively in the nucleus during interphase, but it becomes redistributed to the cytoplasm when cells enter mitosis (34).

Though most of the early research on Wee1 focused on its role at the G2/M transition (8), evidence is accumulating that Wee1 also functions in S phase. Human Wee1 can phosphorylate mitotic CDK1/cyclin B, CDK2/cyclin E, and CDK2/cyclin A, the latter two of which are required for entry into and progression of S phase (25, 35). Consistent with this, the phosphorylation status of CDK2 changes during the cell cycle. In synchronized HeLa cells, tyrosine 15 phosphorylation of CDK2 reaches a maximum during S phase (36), when it is complexed primarily with cyclin A. Expression of a mutant CDK2 that cannot be phosphorylated by Wee1 is lethal in human cells (37). Microinjection of an antibody against CDC25A, which is expressed predominantly in late G1,
blocked the G1/S-phase transition (38). Together, these observations imply that phosphorylation and dephosphorylation of tyrosine 15 regulate entry into S phase.

Tissues undergoing endoreduplication provide useful systems to explore the role of Wee1 in S phase. During endoreduplication, cells engage in multiple rounds of DNA synthesis without chromosome condensation, segregation, and cytokinesis (reviewed in refs. 39–41). This process involves a simplified cell cycle with only S phase and gaps. In the Rcho-1 rat choriocarcinoma cell line, the onset of endoreduplication coincides with a decrease in cyclin B transcript abundance and the activity of cyclin B-containing CDKs, whereas the progression of endoreduplication is associated with oscillation of E- and A-dependent kinase activities (42). Most endosperm cells in maize kernels switch from mitotic to endoreduplication cell cycles between 10 and 12 days after pollination (DAP) (41, 43). This change is accompanied by a reduction in the activity of mitotic CDKs, the induction of S-phase CDKs, and the phosphorylation of retinoblastoma homologues (44). In this study, we cloned and characterized Wee1 from maize, ZmWee1, and showed its expression is up-regulated during endosperm development, suggesting that ZmWee1 influences CDK activity during endoreduplication.

MATERIALS AND METHODS

Plant Materials. Maize (Zea mays L.) inbred line W64A+ was grown at the University of Arizona research farm in Tucson during 1995 and 1996. Immature ears and developing kernels were harvested, frozen in liquid nitrogen, and stored at −80°C. For RNA isolation, the embryo and scutellum were removed by hand dissection. Protein extractions were performed in NETN (20 mM Tris-HCl, pH 8.5/100 mM NaCl/1 mM EDTA/1 mM DTT/0.5% Triton X-100) supplemented with 5 mM NaF, 1 mM sodium orthovanadate, and 0.1 mM PMSF.

Isolation of a ZmWee1 cDNA Clone from Maize Endosperm. A cDNA homologous to Wee1 was identified in the maize expressed sequence tag (EST) database at Pioneer. A cDNA homologous to Wee1 was identified in the dosperm.

Overexpression of ZmWee1 in S. pombe. The coding sequence of ZmWee1 was amplified by using primers 5'-GTCCTATGCGACGATC-3' and 5'-TAGGGATCCCTCTGGGAGGGTC-3' by PCR under the conditions described above. The 1.2-kb fragment was ligated into the Ndel and BamH1 sites of pREP1 (54) to create pREP1ZmWee1. Transformation of S. pombe (PR109, -leu1, -ura4) was based on a protocol for transforming S. cerevisiae (55) with the following modifications. Ten milliliters of the yeast suspension culture was centrifuged at 5 K in a bench-top centrifuge. The pellet was washed once with 5 ml of sterilized water and once with 2.5 ml of LiSorb (10 mM Tris-HCl, pH 8/100 mM LiOAc/1 mM EDTA/1 M sorbitol) and resuspended in 0.6 ml of LiSorb. Fifty microliters of the suspension was mixed with 50 µl of plasmid DNA solution (LiSorb containing 10 µg of pREP1ZmWee1 and 200 µg of sheared salmon sperm DNA) before adding 900 µl of LiAc/TE (10 mM Tris-HCl, pH 8/100 mM LiOAc/1 mM EDTA). The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 7 min. An aliquot of 250 µl was plated onto an Edinburgh minimal medium plate (56) and incubated at 26°C for 4 days. The morphology of the transformants was analyzed with a Zeiss light microscope.

RNA Gel Blotting. RNA was isolated from 4-day-old seedlings and endosperm at 9, 11, 13, 15, and 17 DAP, as described by Jones et al. (57) with the following modifications. Ten grams of tissue was frozen in liquid nitrogen, ground into powder with a mortar and pestle, and resuspended in 10 ml of 50 mM Tris-HCl, pH 7.5/10 mM EDTA/1% SDS/1.5 M NaCl. The sample was extracted once with 1 vol phenol/chloroform/isoamyl alcohol (24:24:1) and once with 1 vol chloroform/isoamyl alcohol (24:1). RNA was precipitated with 2 vol ethanol and resuspended in diethylyrprocarbonate (DEPC)-treated water; LiCl was added to a final concentration of 2.8 M. The RNA was incubated on ice for 30 min and concentrated by centrifugation at 10,000 × g. The pellet was washed with 3 M LiCl and resuspended in DEPC-treated water. Poly(A)+ RNA was isolated by oligo(dT) celluose chromatography according to the manufacturer’s instructions (Promega). Five micrograms of poly(A)+ RNA from maize endosperm or 30 µg of total RNA from young seedlings was loaded on agarose gels, electrophoresed in the presence of 20% formaldehyde, transferred to a nylon membrane, and probed with 32P-labeled ZmWee1 (58).

RESULTS

Molecular Cloning of ZmWee1. An 807-bp EST was identified in the Pioneer EST database, and it showed 36% identity to the amino acid sequence of Drosophila Wee1. Using this clone as a probe, we screened a maize endosperm cDNA library constructed with poly(A) RNA from 9-DAP en-
dosperm (46). The analysis of 400,000 plaques led to the identification of two identical clones designated ZmWee1. The nucleotide sequence of ZmWee1 is identical to Pioneer’s EST sequence with an 807-bp overlap. As shown in Fig. 1, ZmWee1 is 1,601 bp in length with a large ORF of 1,211 bp. It encodes a protein of 403 aa residues with a calculated molecular size of 45.6 kDa. Because a translation-initiation sequence was not detected in the deduced nucleotide sequence and the ZmWee1 transcript is approximately 2.4 kb (see below), we believe ZmWee1 encodes only a portion of the maize Wee1 sequence.

ZmWee1 encodes the entire protein kinase domain of the enzyme including the well conserved ATP-binding site. As shown in Fig. 2, the amino terminus of Wee1 from different eukaryotic organisms is not highly conserved. In contrast, residues 179–403 of ZmWee1 show extensive similarity to the protein kinase domain of Wee1 from Drosophila, humans, S. pombe, and S. cerevisiae. Using the amino acid sequences of the protein kinase domain, we analyzed the relationship of Zm-Wee1 to the Wee1, Mik1, and Myt1 sequences from the fungi S. pombe, S. cerevisiae, and Emericella nidulans and from a wide range of animal species. Fig. 2B is a molecular phylogenetic estimation of the most parsimonious tree. This analysis shows three major clades: the fungal clade, the animal clade, and the plant clade. ZmWee1 consistently grouped with the animal Wee sequences, rather than with fungal Wee and Mik or animal Myt, regardless of the method or model for protein evolution (data not shown). However, the tree order, which indicates the relatedness of the three clades, varied between methods, displayed low bootstrap reproducibility, and some-
times remained unresolved (data not shown). When we performed pairwise comparisons of the conserved protein kinase domain, ZmWee1 showed greatest identity with human Wee1 (50%) and somewhat weaker identity with Wee1 from *S. cerevisiae* (40.5%) and *S. pombe* (43.1%). Therefore, ZmWee1 is more closely related to animal than yeast Wee1. Because the 49-kDa carboxyl-terminal sequence of human Wee1 is sufficient for p34cdc2 phosphorylation (22), we hypothesized that ZmWee1 would encode a catalytically active protein kinase.

**Overexpression of ZmWee1 in *S. pombe***. Wee1 was identified originally as an *S. pombe* mutant with a small cell, or “wee,” phenotype (59). Overexpression of Wee1 causes *S. pombe* cells to grow without division, increasing cell size in a gene dosage-dependent manner (14). We cloned ZmWee1 into pREP1, an *S. pombe* expression vector containing the thiamine-suppressible promoter, NMT1 (54). *S. pombe* cells transformed with pREP1 grew normally (Fig. 3A), whereas overexpression of ZmWee1 significantly inhibited cell division and caused the cells to increase in size (Fig. 3B).

**ZmWee1 Produced in *E. coli* Inhibits M-Phase Promoting Factor Activity.** To analyze the CDK inhibitory activity of maize Wee1, we cloned ZmWee1 into an expression vector, pGEX 2T, to create pGEXZmWee1. The resulting GST ZmWee1 fusion protein was purified by affinity chromatography on glutathione agarose. The purified recombinant protein then was tested for CDK inhibitory activity. We prepared maize CDK by incubating p13suc1 agarose with extract of immature ears, which exhibit high mitotic activity. Fig. 4A shows that neither GST nor the crude immature ear extract inhibited the CDK adsorbed by p13suc1, but GST-ZmWee1, with or without preincubation, inhibited CDK activity. To analyze the proportion of CDK that is susceptible to the inhibition of GST-ZmWee1, we incubated GST-ZmWee1 with crude extracts from immature ear. Fig. 4B shows that GST-ZmWee1 inhibits the histone H1-kinase activity in the extract. This result shows that all the detectable EGTA-insensitive histone H1 kinase activity in the immature ear is susceptible to inhibition by GST-ZmWee1.

**ZmWee1 Is a Single Locus on Maize Chromosome 4.** A database search with ZmWee1 identified a maize restriction fragment length polymorphism marker, UMC169, as Zm-Wee1. This genomic DNA fragment, generated as part of a genome-mapping project at the University of Missouri, contains three exon regions found in ZmWee1. Mapping experiments placed this locus at the bottom of the long arm of chromosome 4 at position 187.4, bin 4.11.

**ZmWee1 Transcripts Accumulate in Endosperm During the Period of Endoreduplication.** To investigate the expression of ZmWee1 in maize, we performed RNA gel blot analyses with transcrits from different tissues, including endosperm at several developmental stages. ZmWee1 RNA accumulated in the endosperm between 9 and 17 DAP, reaching a maximum level at 15 DAP. This coincides with the period when W64A endosperm nuclei are undergoing the maximum rate of endoreduplication (ref. 43; Y.S. and B.P.D., unpublished data). In young seedlings, the ZmWee1 transcript was easily detectable in 30 µg of total RNA and corresponded to a transcript of approximately 2.4 kb (Fig. 5B). The abundance of ZmWee1 RNA is highest in young seedlings, as compared with leaf, root, and endosperm tissues (data not shown).

**DISCUSSION**

We have isolated and characterized a Wee1 homologue from maize, ZmWee1, whose activity resembles related CDKs from other eukaryotes. Although Wee1 had not been identified in higher plants, circumstantial evidence predicted its existence,
and several studies suggested it plays an important role in regulating the plant cell cycle. Colasanti et al. (60) demonstrated that p34<sup>cdc2</sup> homologues from higher plants contain conserved threonine 14 and tyrosine 15 residues, the targets for Myt1, Mik1, and Wee1 phosphorylation. Tyrosine 15 phosphorylation could be an important control point in plant cell cycle regulation, because overexpression of yeast CDC25 kinase domain and that it shows considerable identity to Wee1 (62). Wheat seedlings subjected to water adsorbed protein kinase. Interestingly, the higher level of cytokinin to these cells increased the activity of p13 suc1-associated cyclin, and accumulation of CDK phosphatase 5 (45). It appears that both the catalytic and regulatory subunits of the M-phase CDK are present at the onset of endoreduplication in maize endosperm, and the reduction of CDK activity is due to the presence of a noncatalytic inhibitor (43). Along with this activity, ZmWee1 could provide a partially redundant function through the inhibitory phosphorylation of the CDK.

The progression of endoreduplication correlates with the cycling of cyclin E-associated CDK in endoreduplicating rat trophoblast cells (42). It was proposed that the fluctuation in activity of the S-phase-related CDK between DNA synthesis and the gap phase is required for the progression of an endoreduplication cell cycle (42). The down-regulation of S-phase CDK activity could be accomplished by Wee1, in addition to transcriptional regulation of cyclin transcripts, proteolytic degradation of cyclin, and accumulation of CDK inhibitors (7). Additional experiments, such as the genetic manipulation of ZmWee1 expression in maize endosperm and the isolation of transposon-tagged ZmWee1 mutants, are underway to test this hypothesis.

Because the size of the ZmWee1 transcript (2.4 kb) is significantly larger than the cDNA clone we isolated (1.6 kb), and no conserved translation initiation site was identified in the deduced amino acid sequence, we believe that ZmWee1 is truncated at the 5′ terminus. Repeated attempts to obtain additional 5′ sequence information were unsuccessful, and this could be a consequence of the high-GC content of this region. Nevertheless, the 45.5-kDa GST-ZmWee1 inhibits CDK activity in vitro (Fig. 4). These data are in agreement with the observation that the carboxyl-terminal domain of human Wee1 is sufficient for tyrosine kinase activity (21, 22). This region is highly conserved among higher eukaryotes (Fig. 2), and it contains the protein kinase domain. Because the NH<sub>2</sub>-terminal domain of Wee1 from <i>S. pombe</i> contains sequences necessary for negative regulation of Wee1 in vivo (29), we speculate that sequences with a regulatory function reside at the NH<sub>2</sub> terminus of ZmWee1. Experiments are ongoing to obtain the complete 5′ sequence of ZmWee1 and investigate its upstream regulators.

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During the transition from a mitotic cell cycle to endoreduplication in rat trophoblast cells, cyclin B-dependent CDK activity is reduced significantly, but cyclin B protein remains constant (42). At the onset of endoreduplication in maize endosperm, p13<sup>wee1</sup>-adsorbed kinase activity decreases, but the level of p34<sup>CDC2</sup> protein is more or less constant (43). It appears that both the catalytic and regulatory subunits of the M-phase CDK are present at the onset of endoreduplication in maize endosperm, and the reduction of CDK activity is due to the presence of a noncatalytic inhibitor (43). Along with this activity, ZmWee1 could provide a partially redundant function through the inhibitory phosphorylation of the CDK.

**Figure 5.** ZmWee1 RNA transcripts are increased in maize endosperm during the period of endoreduplication. RNA gel blot hybridization was performed with poly(A)<sup>+</sup> RNA from maize endosperm isolated between 9 and 17 DAP (A) or with total RNA from seedlings (B). The abundance of ZmWee1 transcripts peaked at 15 DAP in developing endosperm, coincident with the peak of endoreduplication activity.

<ref>Fig. 5</ref>

ZmWee1

ZmeEF1A

9 11 13 15 17 (DAP)

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