Plant WEE1 kinase is cell cycle regulated and removed at mitosis via the 26S proteasome machinery

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Abstract

In yeasts and animals, premature entry into mitosis is prevented by the inhibitory phosphorylation of cyclin-dependent kinase (CDK) by WEE1 kinase, and, at mitosis, WEE1 protein is removed through the action of the 26S proteasome. Although in higher plants WEE1 function has been confirmed in the DNA replication checkpoint, Arabidopsis wee1 insertion mutants grow normally, and a role for the protein in the G₂/M transition during an unperturbed plant cell cycle is yet to be confirmed. Here data are presented showing that the inhibitory effect of WEE1 on CDK activity in tobacco BY-2 cell cultures is cell cycle regulated independently of the DNA replication checkpoint: it is high during S-phase but drops as cells traverse G₂ and enter mitosis. To investigate this mechanism further, a yeast two-hybrid screen was undertaken to identify proteins interacting with Arabidopsis WEE1. Three F-box proteins and a subunit of the proteasome complex were identified, and bimolecular fluorescence complementation confirmed an interaction between AtWEE1 and the F-box protein SKP1 INTERACTING PARTNER 1 (SKIP1). Furthermore, the AtWEE1–green fluorescent protein (GFP) signal in Arabidopsis primary roots treated with the proteasome inhibitor MG132 was significantly increased compared with mock-treated controls. Expression of AtWEE1–YFP C (C-terminal portion of yellow fluorescent protein) or AtWEE1 per se in tobacco BY-2 cells resulted in a premature increase in the mitotic index compared with controls, whereas co-expression of AtSKIP1–YFP negated this effect. These data support a role for WEE1 in a normal plant cell cycle and its removal at mitosis via the 26S proteasome.

Key words: Arabidopsis thaliana, bimolecular fluorescence complementation (BiFC), BY-2 cell line, CDKA/B, cell cycle, F-box, green fluorescent protein (GFP), mitosis, Nicotiana tabacum, 26S proteasome SKIP1, WEE1.

Introduction

The cell cycle is conserved in all eukaryotes, with G₁/S and G₂/M being regulated by cyclin-dependent kinases (CDKs). In plants, >160 CDK-related genes have been cloned from >20 higher plant species and are apportioned into classes A–G and CDK-like (Dudits et al., 2007). The CDKB family is unique to plants. In Arabidopsis, CDKA;1 activity peaks at G₁/S and G₂/M, whereas CDKB2:1 peaks at G₂/M (Joube et al., 2000). In tobacco BY-2 cells, CDKA activity is relatively constant from S-phase to mitosis, whereas B-type activity peaks in mid-G₂ (Porceddu et al., 2001; Sorrell et al., 2001).

In Schizosaccharomyces pombe, the CDK, Cdc2, is phosphorylated in G₂, negatively by SpWeel kinase and...
positively by SpCdc25 phosphatase (Nurse, 1990). A partial WEE1 homologue was cloned in maize and inhibits CDK activity in vitro (Sun et al., 1999) whilst a full-length Arabidopsis WEE1 is highly expressed in meristems (Sorrell et al., 2002). T-DNA insertion lines are hypersensitive to DNA-damaging agents, demonstrating that AtWEE1 participates in the DNA damage and replication checkpoints (De Schutter et al., 2007). However, the role of WEE1 in a normal cell cycle remains uncertain since T-DNA insertion lines grow and develop normally. This is in contrast to Weel−/− mice that die during embryogenesis (Tominaga et al., 2006). However, a systematic analysis of cell cycle gene expression during Arabidopsis development shows tight regulation of WEE1 expression during the cell cycle in plants, as indicated by patchy expression patterns in parts of the young and mature leaves, shoot apical meristem, and young roots (de Almeida Engler et al., 2009). Furthermore, there may be a role for WEE1 during endoreduplication given that WEE1 transcript levels were high during this process both in the endosperm of Zea mays (Sun et al., 1999) and in tomato fruit (Gonzalez et al., 2004, 2007). The latter authors concluded that tomato WEE1 negatively regulates CDK activity to control cell size acting through a regulation of cell expansion and/or endoreduplication. However, in these systems, neither WEE1 protein nor the ability of WEE1 from plant protein extracts to inhibit CDK activity was measured and linked to the cell cycle phase.

Timely degradation of regulatory proteins has a critical effect on cell cycle progression. The most widely studied method of proteolysis in eukaryotes is the ubiquitin–26S proteasome pathway. Its mechanism is widely conserved among eukaryotes, including plants (reviewed by Sullivan et al., 2003). One of the components of the ubiquitin cascade is E3 ligase which provides the specificity to the target protein. Several types of E3 ligase exist in eukaryotes. The SCF E3 ligase complex consists of four subunits: S PHASE KINASE-ASSOCIATED PROTEIN 1 (SKP1) and CULLIN provide the structural backbone, while RBX is a ring finger protein which binds the E2, or ubiquitin-conjugating, enzyme. Finally an F-box protein binds to SKP1, providing specificity to the target protein (Skowyra et al., 1997; Deshaies et al., 1999; Zheng et al., 2002). In Arabidopsis, 692 F-box genes have been identified through homology searches (Xu et al., 2009), although only a relatively small number of these proteins have been studied functionally.

In budding yeast and mammalian cells, two E3 ligases, SCF and APC, are important components of the degradatory pathways that remove unwanted cell cycle proteins such as WEE1 kinase and cyclins. Their timely removal is followed by normal G2/M and metaphase/anaphase progression (King et al., 1995; Feldman et al., 1997; Skowyra et al., 1997). In Xenopus laevis, the degradation of WEE1 via the 26S proteasome is required for the correct timing of mitosis, and was blocked by inhibition of DNA replication, clearly indicating a link between the completion of S-phase and the progression of mitosis (Michael and Newport, 1998). The Saccharomyces cerevisiae WEE1 homologue, SWE1, is targeted for degradation by a SUMO (small-ubiquitin modifier protein, similar to ubiquitin) protein, SMT3, via the E3 ligase SIZ1 (Simpson-Lavy and Brandeis, 2010). F-box proteins including MET30 are implicated in WEE1 degradation, in S. cerevisiae (Kaiser et al., 1998), as is TOME-1 in Xenopus (Ayad et al., 2003). The most comprehensive study of WEE1 kinase activity during the cell cycle was carried out for HeLa cells, where WEE1 kinase activity was detected during interphase but not in mitosis (McGowan and Russell, 1995). In these assays, native WEE1 was pulled-down using human WEE1 antibody and then used to inhibit CDK activity in histone H1 kinase assays (McGowan and Russell, 1995).

The aim of this work was to test the hypothesis that plant WEE1 action is under cell cycle control, and investigate the mechanism by which CDKs are released from WEE1 inhibition as cells enter mitosis. Data presented here show, for the first time in plant cells, cell cycle regulation of WEE1 at the protein level and a drop in WEE1 inhibition of CDK activity during G2 that remained low as cells entered mitosis. Data presented here indicate that WEE1 protein degradation in plants is 26S proteasome dependent and targeted via the protein’s physical interaction with the 26S proteasome F-box protein, SKIP1.

Materials and methods

Cell cycle measurements

Tobacco (Nicotiana tabacum) BY-2 cells were subcultured every 7 d and synchronized as described previously (Francis et al., 1995). At hourly intervals following removal of aphidicolin, the mitotic index was derived from scoring ≥300 Hoechst-stained cells per slide in random transects using fluorescence microscopy (Olympus BH2, UV, λ = 420 nm).

Cloning Nicta;WEE1

Degenerate primers were designed based on the maize ZmWEE1 (accession no. AAD52983) and AtWEE1 (accession number: CAD28679) (Supplementary Table S2 available at JXB online) and used to amplify a 339 bp fragment of NtWEE1 from N. tabacum var. Samsun genomic DNA. The PCR product was cloned in pGEM-T-Easy (Promega, Southampton, UK) and sequenced. One cycle of 3′ rapid amplification of cDNA ends (RACE) and two cycles of 5′ RACE (using the BD SMART™ RACE cDNA amplification Kit, Clontech) furnished the whole open reading frame (ORF) (EMBL database accession nos: AJ866274, AJ866275, AJ866276, and AJ866277). The entire ORF was amplified (primers are given in Supplementary Table S2) from BY-2 cDNA and cloned into pTA7002 by digestion with Xhol/SpeI, creating pTA7002 NtWEE1. The ORF was fully sequenced (EMBL database accession no. AM408785). Clustal W within DNASTar (Lasergene), BIOEDIT version 7.0.1 (Hall, 1999), and MEGA software version 3.1 (Tamura et al., 2007) were used to compare the tobacco ORF with other weel sequences. pTA7002 NtWEE1 was transformed into Agrobacterium tumefaciens LBA4404 and GV3101 and used to transform BY-2 cells and Arabidopsis var. Columbia, respectively, as described previously (An, 1985; Clough and Bent, 1998; Orchard et al., 2005).

Semi-quantitative RT–PCR

Total RNA was extracted from BY-2 cells using TRI reagent (Sigma Aldrich, Gillingham, UK) and residual genomic DNA removed by DNase treatment (Ambion, Austin, TX, USA). RNA (5 μg) was reacted with Superscript II reverse transcriptase (GIBCO, Paisley, UK) and percursor RT was used to generate cDNA for semi-quantitative RT–PCR.
Histone H4 primers (Supplementary Table S2) were used to verify cell cycle stage, and 18S rRNA primers for normalization (Orchard et al., 2005). For all semi-quantitative RT–PCRs, the cycle number was optimized such that the amount of product was proportional to the amount of input total RNA, verified using a dilution series of cDNAs in each PCR. Products of three replicate PCRs were quantified using ethidium bromide-stained agarose gels and GeneGenius software (Syngene, Cambridge, UK).

**Protein expression and purification**

The coding sequences of \( N\text{t}WEE1 \) and \( \text{At}14\text{a}3-3 \text{~o} \) were PCR amplified (primers are listed in Supplementary Table S2 at JXB online) using \( Pfu \) polymerase and cloned into the pET15B vector system using \( NdeI/BamHI \). The insertions were verified by sequencing and the plasmids were transformed into \( \text{Escherichia coli} \text{ DE3 Rosetta} \) pLysS cells. Recombinant protein was induced with isopropyl-\( \beta\)-thiogalactopyranoside (IPTG) and the purity of the recombinant proteins was analysed by SDS–PAGE.

**Immunoprecipitation and kinase assay**

The CDK substrate for the kinase assays was pulled down from \( N. \text{tabacum} \) BY-2 cells using a p13GAL4-1 agarose conjugate (Upstate) from 100–250 µg of protein extract. WEE1 protein was immunoprecipitated from 100 µg of protein extracts from \( N. \text{tabacum} \) BY-2 cells at different times following synchronization using WEE1 antibody raised as described in Lentz Grønlund et al. (2009). The histone H1 assay was essentially as described in Cockroft et al. (2000) using 5 µl of \( N\text{t}WEE1 \) antibody. Samples were subjected to SDS–PAGE. Products were quantified from autoradiographs using GeneGenius software (Syngene, Cambridge, UK). Quantitated data presented are the means of three independent western blots for protein levels and two gels for the kinase assays (±SE).

**Two-hybrid analysis**

The bait plasmid pBD-Gal4-cam \( AtWEE1 \) was constructed as described in Lentz Grønlund et al. (2009). An Arabidopsis seedling root primary cDNA library was constructed in the HybriZAP-2.1 lambda vector (Stratagene) (Sorrell et al., 2003). The primary library was amplified and converted by \( \text{in vivo} \) excision into a GAL4 transcriptional activation domain pAD-GAL4-2.1 library according to the manufacturer’s protocol (Stratagene). Both bait and cDNA library were transformed into \( S. \text{cerevisiae} \) strain YRG-2 [MATa ura3-52 his3-200 ade2-101 lys2-801 trpl-901 leu2-3, 112 gal4-542 gal80-538 LYS2::UASGAL4-TATACTC-lacZ] (Stratagene). Approximately \( 1–2 \times 10^6 \) transformants were plated onto His− synthetic dextrose minimal medium and screened as described in Sorrell et al. (2003) using both the HIS3 and LacZ reporter genes. Interacting proteins were identified by colony PCR and sequenced. Sequences were identified using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST).

**Bimolecular fluorescence complementation (BiFC)**

The \( AtWEE1 \) ORF was amplified and cloned into the BiFC vector containing the C-terminal portion of yellow fluorescent protein (YFP), pkanII-SPYCE(M) (Wuadt et al., 2008) as described in Lentz Grønlund et al. (2009). The AtSKIP1 ORF was PCR amplified (primers are listed in Supplementary Table S2 at JXB online) and cloned using the Ascl/XmaI sites into the BiFC vector fusing the SKIP1 ORF in-frame with the N-terminal portion of YFP, pSPYNE (Walter et al., 2004). The constructs were transformed into \( A. \text{tumefaciens} \text{ strain} \text{ EHA105} \) and used to co-transform transiently (as described in Lentz Grønlund et al., 2009) and transform stably BY-2 cells as described previously (Orchard et al., 2005). Cells were more monitored for fluorescence using fluorescence microscopy (Olympus BX12, UV, λ=420 nm).

**AtWEE1 and AtWEE1–GFP transgenic BY-2 and Arabidopsis lines**

\( AtWEE1 \) under the 35S promoter in the BIN HYG TX vector was assembled as described in Spadafora et al. (2012). A WEE1–green fluorescent protein (GFP) fusion protein construct was created by amplifying the \( AtWEE1 \) ORF (the primers listed in Supplementary Table S2 at JXB online) and cloned into the Gateway (Invitrogen) vector system to create an entry clone in pDom207. The insert was then transferred to pGFP-N-Bin (Invitrogen) to create an N-terminal fusion. The constructs were transformed into \( A. \text{tumefaciens} \text{ strain} \text{ LB4404} \) for transformation into BY-2 cells and \( A. \text{tumefaciens} \text{ GV3101} \) for transformation into Arabidopsis var. Columbia as described above. The presence of the transgene was checked by PCR (for primers, see Table S2; data not shown).

The 35S::GFP BY-2-transformed line was kindly donated by Dr Lukas Fischer (Nocarova and Fischer, 2009). Both cell lines were synchronized with aphidicolin (Orchard et al., 2005) and samples were stained with Hoechst. GFP and Hoechst were visualized using an Olympus BX61 microscope at λ=488 nm or 530 nm. Where GFP signal was absent following Hoechst staining (35S::GFP line), further images were taken using differential interference contrast (DIC) microscopy.

**Arabidopsis lines**

Arabidopsis lines expressing WEE1–GFP were selected based on GFP fluorescence visualized as above and crossed with a line expressing AtSUN1–mRFP (monomeric red fluorescent protein) (Graumann et al., 2010). Root tips of 5- to 7-day-old seedlings were imaged using an oil immersion ×40 lens on a Zeiss LSM510 confocal microscope. GFP was excited with a 488 nm argon laser and fluorescence was captured with a 505–530 nm bandpass filter; mRFP was excited with a 543 nm helium–neon laser and fluorescence was captured with a 560–615 nm bandpass filter. Images were captured with the Zeiss LSM software and exported in TIF format.
solution 25 mg ml⁻¹ in dimethylsulphoxide (DMSO) diluted to
MG132 treatment of Arabidopsis seedlings

The H2B-YFP
Arabidopsis
and 650 nm. Images were captured using Leica confocal software. Not shown. The phase durations (shown above Fig. 1B) are
4 h (Fig. 1B). Mitotic indices peaked at 9 h (and also at 23 h),
generating histone H4 profiles showed an S-phase duration of
merase
Tobacco BY-2 cells were synchronized with aphidicolin and,
WEE1 activity drops during G2 and remains low as
proteins (Sun
Solanum lycopersicum
Z. mays
and
N. tabacum
with recombinant
E. coli
tested by western blotting, showing good specificity of the
protein using His-beads resulted in a single band of the
NtWEE1 antibody; purification of the recombinant WEE1
sampled from BY-2 cells at 1 h (early S-phase), decreased significantly
(S/G2; Fig. 1B) and is also highly comparable with previously published cell cycle data
highly comparable with previously published cell cycle data
for BY-2 cells (see Nagata et al., 1992; Sorrell et al., 2001;
Orchard et al., 2005). Expression of NtWEE1 peaked at 4 h
(S/G2; Fig. 1B) and is also highly comparable with published data (Gonzalez et al., 2004).
WEE1 protein level was highest at 5 h (early G2, Fig. 1C),
1 h following the WEE1 mRNA peak (Fig. 1B). WEE1 levels
dropped significantly (P < 0.05) at 7 h (mid-G2) and remained
low at 9 h (mid-G2 to mitosis) as more cells entered mitosis,
before showing a slight rise at 11 h (early G3) (Fig. 1C).
WEE1 inhibition of CDK activity (Fig. 1D) was maximal when WEE1 was pulled-down from proteins extracted
from BY-2 cells at 1 h (early S-phase), decreased significantly
(P < 0.05) by 4 h (S/G2) and again (P <0.05) from 4 h to 6 h
(early G3), and then remained at this level throughout G2 and
M phase. The data are thus consistent in showing a drop in WEE1 kinase activity during G2 that remained low when cells
entered mitosis.

Results

Recombinant N. tabacum WEE1 inhibits CDK activity
in vitro

The WEE1 antibody recognized a single 56 kDa band in pro-
teins extracted in lag (day 1), exponential (day 3), and sta-
tionary phase (day 5) of a 7 day subculture of BY-2 cells,
indicating good antibody specificity as shown previously with
Arabidopsis (Lentz Grønlund, 2009) (Supplementary Fig. S1
at JXB online).

The CDK inhibitory activity of NtWEE1 was investigated using a WEE1 kinase inhibition assay based on that used with recombinant Z. mays and Solanum lycopersicum WEE1 proteins (Sun et al., 1999; Gonzalez et al., 2007). This assay tests whether recombinant NtWEE1 can inhibit CDK phosphorylation of histone H1 in vitro. Total CDK activity in this assay is interpreted as the inverse of WEE1 activity, as previously demonstrated for Homo sapiens Wee1 by McGowan and Russell (1995). Induction of NtWEE1 in E. coli was tested by western blotting, showing good specificity of the NtWEE1 antibody; purification of the recombinant WEE1 protein using His-beads resulted in a single band of the expected 56 kDa on a Coomassie-stained gel (Supplementary Fig. S2 at JXB online).

Addition of recombinant NtWEE1 resulted in a 5-fold decrease in CDK activity (Fig. 1A) compared with CDK alone. This shows that recombinant NtWEE1 protein produced in E. coli can negatively regulate CDK activity in vitro as do other plant WEE1 kinases. This is taken to indicate that the WEE1 is inhibiting CDK activity through its kinase activity (see Sun et al., 1999).

WEE1 activity drops during G2 and remains low as
cells enter mitosis

Tobacco BY-2 cells were synchronized with aphidicolin and,
following removal of this reversible inhibitor of DNA poly-
merase α (Nagata et al., 1992), semi-quantitative RT–PCR
generated histone H4 profiles showed an S-phase duration of
4 h (Fig. 1B). Mitotic indices peaked at 9 h (and also at 23 h),
giving a cell cycle duration of 14 h (later part of the curve
not shown). The phase durations (shown above Fig. 1B) are
In the resultant line, the pattern of WEE1–GFP signal during the cell cycle is similar to that seen in BY-2 cells (Fig. 3). In interphase, a clear nucleoplasmic AtWEE1–GFP signal is visible, surrounded by nuclear envelope labelling of AtSUN1–mRFP (Fig. 3). During metaphase AtWEE1–GFP signal is essentially absent but a clear AtSUN1–mRFP signal can be observed in mitotic spindle membranes (Fig. 3). AtWEE1–GFP signal reappears in late anaphase/early telophase cells, while AtSUN1–mRFP is present in the reforming nuclear envelope, and, finally, strong GFP and RFP signals were observed during cytokinesis. Hence, there is a remarkably precise cell cycle regulation of WEE1 with presumed
degradation or destabilization of WEE1 when chromosomes align at the metaphase plate for both BY-2 cells and *Arabidopsis* root cells.

**WEE1 protein is not detectable in lateral root primordial cells**

The stability of WEE1 protein was also examined in lateral roots of two independent 35S::AtWEE1–GFP-expressing *Arabidopsis* lines, #10 and #67. In line #10, a weak GFP signal was observed in the nuclei of the basal cells of the lateral root primordia (Fig. 4A), but fluorescence was not detected in the rest of the primordium. In line #67, a fluorescent signal could not be detected in any of the cells of the lateral root primordia (Fig. 4B). In contrast, in the 35S::H2B–YFP line, strong YFP expression was observed throughout the lateral root primordia (Fig. 4C).

**AtWEE1 is degraded via the 26S proteasome degradation pathway**

The proteasome inhibitor MG132 was used to determine whether the reduced fluorescent signal observed in the AtWEE1–GFP lines was caused by proteasome-mediated protein degradation. GFP signal clearly increased in AtWEE1–GFP seedlings treated with MG132 relative to the mock-treated seedlings (Fig. 5A) both in the root tips and further up the root. Quantification of the fluorescent signal showed that there was a significant ($P < 0.05$) 2-fold increase in GFP signal in the MG132-treated seedlings compared with the mock-treated seedlings (Fig. 5B). This demonstrates that the AtWEE1–GFP protein persists when the degradation route via the 26S proteasome is blocked.

**In a yeast two-hybrid screen AtWEE1 interacts with components of the proteasome machinery**

AtWEE1 was used as a bait in a yeast two-hybrid screen to search for interacting proteins that might play a regulatory role in its turnover. Approximately $1 \times 10^7$ transformants were screened from a library generated using *Arabidopsis* primary and secondary root tips (Sorrell *et al.*, 2003). Over 900 interactors were detected by an ability to grow on His− medium and, in a second screen for β-galactosidase activity, 82 of these were confirmed. Sequencing of plasmid insertions revealed 60 different AtWEE1 interaction partners, of which 11 were identified multiple times (Supplementary Table S1 at *JXB* online). Functionally the interacting proteins could be divided into seven groups (Table 1). Of direct relevance to this work, four proteins associated with ubiquitin-mediated degradation were detected (Supplementary Table S1); each one was only detected once. One of these is a regulatory subunit of

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**Fig. 2.** In the AtWEE1–GFP line, GFP signal is greatly reduced in metaphase and early anaphase. (A) 35S::AtWEE1–GFP (GFP, Hoechst, and merged GFP–Hoechst; Hoechst $\lambda=420$ nm GFP $\lambda=530$ nm). Yellow arrows indicate a representative cell; scale bar=50 μm for all images. (B) Nuclear fluorescence frequency (%) of cells sampled from the synchronized 35S::AtWEE1–GFP cell line. $n$ values are indicated on each bar. Contingency $\chi^2=1511$ df 4, $P < 0.001$. 

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the 26S proteasome, while the other three are F-box proteins including SKIP1 (SKP1 INTERACTING PARTNER 1).

A protein–protein interaction between AtWEE1 and AtSKIP1 was confirmed in vivo through BiFC

BiFC in BY-2 cells was used to verify the AtSKIP1–AtWEE1 interaction in plant cells, initially by transient transformation. Interacting proteins were mostly localized in the nucleus; however, the interaction was also detected at the cell wall, especially at the junctions between adjacent cells (Fig. 6A).

The AtWEE1–YFPc (in the SPYCE vector) and AtSKIP1–

YFPN (in the SPYNE vector) constructs were stably cotransformed into BY-2 cells to study the dynamics of the interaction during the cell cycle. The frequency with which an interaction between AtWEE1 and AtSKIP1 was observed in each cell cycle phase was similar to that of AtWEE1–GFP, with a drop in interactions observed between interphase and prophase, and interactions were not observed during metaphase. Interactions were again observed during anaphase and telophase (Fig. 6B).

Expression of AtSKIP1 and AtWEE1 in BY-2 cells restores mitotic timing to wild-type levels compared with cultures expressing AtWEE1 alone

Given an interaction between AtWEE1 and AtSKIP1, the extent to which these genes affected aphidicolin-induced synchronized cell cycles in BY-2 cells was examined. Expression of AtWEE1–YFPc resulted in a mitotic peak at 7 h compared with 9 h in wild-type BY-2 cells (Fig. 7A, B). The mitotic peak was also earlier (at 4 h) when AtWEE1 was expressed in BY-2 cells in the BIN HYG TX vector under an attenuated 35S promoter (Supplementary Fig. S4 at JXB online). However, when AtSKIP1–YFPN was co-expressed with AtWEE1–

YFPc, there was an interaction between the two proteins, and the mitotic peak was restored to wild-type timing of 9 h (Fig. 7C).

Discussion

In the work reported here, the inhibitory effect of the WEE1 protein pulled-down from BY-2 cells on CDK activity drops

Fig. 3. AtWEE1–GFP and AtSUN1–mRFP expression in different cell cycle phases in roots of transgenic Arabidopsis expressing both genes. Green colouring indicates AtWEE1–GFP expression, while purple colouring indicates AtSUN1–mRFP expression. Scale bar=10 µm. Yellow arrows indicate a representative cell.
as cells traverse G₂ and enter mitosis. Remarkably, a GFP signal essentially disappeared at metaphase in both BY-2 cells and Arabidopsis lines expressing WEE1–GFP. The GFP signal was then restored towards the end of mitosis at late anaphase/early telophase. Previous work in Arabidopsis and tobacco BY-2 cells established that the G₂/M transition is regulated by both CDKA and CDKB. While CDKA activity is generally constant during S-phase and G₂, CDKB peaks in mid- to late G₂ phase (Porceddu et al., 2001; Sorrell et al., 2001; Orchard et al., 2005). In other words, WEE1 activity data are the opposite of the typical CDKB activity profile reported for BY-2 cells (Sorrell et al., 2001; Orchard et al., 2005). Such CDK activity is required until metaphase when partner mitotic cyclins are degraded via a proteolytic pathway that deploys the anaphase-promoting complex (APC-Cdh1; Peters, 2002). BY-2 cells transformed with non-degradable mitotic B1 cyclin exhibited normal prophase and metaphase, but from there on was a mitotic catastrophe (Weingartner et al., 2004). In other words, a normal mitosis is finely tuned and depends on CDK kinase activity persisting until metaphase but then finishing abruptly.

Another notable feature of the profile reported here is that NtWEE1 kinase-mediated inhibition of CDK activity is highest in early S-phase. Recently, it was shown in Arabidopsis that following hydroxyurea treatment, roots exhibited rapid accumulation of WEE1 transcripts at the start of S-phase, leading to the suggestion that WEE1 kinase has a role during S-phase following the induction of the DNA replication checkpoint (Cools et al., 2011). Thus data here support a role for plant WEE1 in S-phase although, of course, it is still possible that

Fig. 4. Lateral root primordia of AtWEE1–GFP lines #10 and #67, and H2B–YFP seedlings. (A and B) Green colouring indicates AtWEE1–GFP expression in two independent lines, #10 and #67. (C) Green indicates H2B expression. Red colouring is propidium iodide counterstain for the cell walls. Representative images of at least five seedlings examined. P, lateral root primordium; BC, basal cells of lateral root primordium. Note that the middle image of (A) is a primordium seen from above, and demarcated by propidium iodide counterstaining.
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there may also be other negative regulators of CDK activity in the in vivo plant cell cycle (e.g. ICK1/2), as proposed by Boudolf et al. (2006).

Unlike WEE1 kinase in the human cell cycle (McGowan and Russell, 1995), NtWEE1 activity never drops to zero. While the use of aphidicolin is an established and proven method for cell cycle synchrony (Nagata et al., 1992), it is not possible to achieve 100% synchronization of mitosis. Hence the residual protein and kinase activity is most probably due to WEE1 protein in interphase cells. Furthermore, BY-2 and Arabidopsis lines expressing AtWEE1–GFP exhibited WEE1 protein localization in mitotic phases other than metaphase. This is another reason why WEE1 kinase activity did not drop to zero during mitosis in synchronized BY-2 cells.

The 35S promoter normally confers strong, constitutive expression in BY-2 and Arabidopsis root tip cells. So the dramatic reduction of GFP signal in metaphase cells in both BY-2 and Arabidopsis root cells expressing WEE1 is consistent with the removal of the WEE1 protein at this stage of mitosis. In the 35S::GFP control BY-2 cells, GFP signal persisted in 100% of cells, regardless of mitotic phase.

A previous study of the interactions between rice cyclins and CDKs in BY-2 cells showed that there was a tight association of CDKB2;1–GFP and CycB2;2–GFP with chromosomes of transgenic BY-2 cells during an aphidicolin-induced synchronous cell cycle (Lee et al., 2003). This conclusion was supported by the finding that treatment with Triton X-100 resulted in a complete loss of GFP fluorescence in cells expressing GFP alone whereas GFP fluorescence was unaffected by the Triton treatment in the lines transformed with the CDK–GFP or cyclin–GFP fusion proteins. Exactly the same phenomenon was seen here when the GFP-expressing line was stained with Hoechst; this staining reaction depends on a pretreatment with Triton X-100. Hence, in the 35S::AtWEE1–GFP line, the data suggest that the loss of GFP signal is due to WEE1 degradation at metaphase and its re-appearance due to re-synthesis at anaphase. Moreover the same pattern of alteration of GFP signal was seen in root meristems of 35S::AtWEE1–GFP crossed with AtSUN1–RFP.

Table 1. Functional groups of proteins that interacted with AtWEE1 in a yeast two-hybrid library screen

| Functional group                                                                 | No. of proteins |
|---------------------------------------------------------------------------------|-----------------|
| Transcription factors/DNA- or RNA-binding proteins, histone modifications      | 9               |
| Plant growth regulation and signal transduction                                | 3               |
| Stress responses/detoxification/pathogen responses                              | 13              |
| Cell division/cell size/cell wall and cell growth                              | 8               |
| Ribosomes/protein biosynthesis                                                  | 4               |
| Ubiquitin-mediated degradation                                                  | 4               |
| Other                                                                           | 19              |

![Fig. 5.](https://example.com/fig5.png) Fig. 5. MG132- and mock-treated 5-day-old AtWEE1–GFP line # 67 seedlings. (A) Confocal images of root tips and more basal regions; green indicates AtWEE1–GFP expression and red is propidium iodide counterstain for the cell walls. Representative images of three seedlings examined for each treatment. To allow for accurate and direct comparison between the two treatments, the confocal settings were not altered between the imaging conditions. (B) GFP signal intensity (±SE). *Significant difference between treatments (P < 0.05; n= 466 to ≥2121).
The pattern of 35S::AtWEE1–GFP signal was also examined in lateral root primordia in two independent Arabidopsis lines. AtWEE1–GFP levels were reduced in both lines, to the point of being undetectable in line #67, whereas 35S::H2B–YFP was strongly and constitutively expressed in the lateral root primordia (Boisnard-Lorig et al., 2001). This implies a high turnover of AtWEE1 in lateral root primordia.

**Fig. 6.** (A) Tobacco BY-2 cells co-transformed with (i) AtWEE1–YFPC in the vector pSPYCE and AtSKIP1–YFPN in the vector pSPYNE; (ii) AtBZIP63 in both pSPYCE and pSPYNE (positive control); and (iii) AtWEE1–YFPC and AtBZIP63–YFPN (negative control); under UV light (left), white light (right), and the two merged (centre). Blue colouring indicates a positive interaction between the two proteins (representative images). (B) Mean nuclear fluorescence frequency (%; ±SE, n=3) in each cell cycle phase in cells from the following transgenic BY-2 lines: AtWEE1–GFP line # 4 (GFP–WEE1) and AtWEE1–YFPC/AtSKIP1–YFPN, line # 6 (WEE1–C/SKIP1-N6).

**Fig. 7.** Mitotic index (±SE) of BY-2 cell lines: (A) The wild type. (B) AtWEE1–YFPC and (C) AtWEE1–YFPC/AtSKIP1–YFPN line #6 synchronized with aphidicolin compared with the wild type. The timing of the first mitotic peak in each line is shown with an arrow.
At WEE1–YFP C alone, restoration of the normal functions of NtWEE1. However, co-expression of tobacco cellular machinery resulting in an interference and the AtWEE1 probably due to an interaction between mitosis, resulting in a larger cell phenotype. This result is 2002) and (Spadafora et al., 2012) tends to delay the cell cycle. Since pericycle cells in fission yeast (Sorrell et al., 2009), Xenopus (Michael and Newport, 1998), and humans (Watanabe et al., 2004), however, the mechanism for the degradation of WEE1 protein in plants is yet to be described. When a 26S proteasome inhibitor, MG132 (Rock et al., 1994), was supplied to seedlings of an AtWEE1–GFP-expressing line, the AtWEE1–GFP signal was significantly enhanced (Fig. 5A, B). These results strongly indicate that AtWEE1 protein is degraded via the 26S proteasome degradation pathway; note that GFP is unaffected by MG132 treatment (Song and Wu, 2005).

The finding that AtWEE1 interacts with several proteasome-related proteins in a two-hybrid screen further supports this route for its degradation. BifC was used to confirm the interaction between AtWEE1 and the F-box protein AtSKIP1. AtSKIP1 interacts with the SKP1/ASK1 subunit of SCF ubiquitin ligase (Risseeuw et al., 2003). It is not closely related to any other proteins in Arabidopsis; the closest homologue is a member of the RNI-superfamily, the Arabidopsis putative F-box/leucine-rich repeat protein 19 (At4g30640), which shares 40% gene sequence identity with AtSKIP1.

This interaction could provide the specificity for the targeted removal of AtWEE1, although the finding that AtWEE1 interacts with several F-box proteins indicates that different F-box proteins may mediate the specificity of WEE1 removal in different tissues or under different cellular conditions. If AtSKIP1 promotes AtWEE1 degradation, then co-expression of the two proteins might tend to destabilize the AtWEE1. This hypothesis was tested using synchronized BY-2 cells. Expressing AtWEE1 in tobacco BY-2 cells under the 35S promoter led to a shortening of G3 and a premature rise in the mitotic peak, as was the case in the AtWEE1–YFP C line. This was an unexpected result since expression of AtWEE1 in fission yeast (Sorrell et al., 2002) and Arabidopsis (Spadafora et al., 2012) tends to delay mitosis, resulting in a larger cell phenotype. This result is probably due to an interaction between AtWEE1 and the tobacco cellular machinery resulting in an interference in the normal functions of NtWEE1. However, co-expression of AtSKIP1–YFPN with AtWEE1–YFP C clearly delayed the mitotic peak compared with AtWEE1–YFP C alone, restoring the timing back to the wild type. This suggests that the removal of AtWEE1 by SKIP1 prevented a premature rise in the mitotic index in synchronized cells. Thus the hypothesis that the interaction between AtWEE1 and AtSKIP1 is indeed functional was supported.

In conclusion, a drop in WEE1 activity was discovered when synchronized BY-2 cells traverse G2 and enter mitosis. This is linked to a fine-tuned regulation of WEE1 protein, which essentially disappeared from chromosomes at metaphase in both BY-2 cells and Arabidopsis roots. Given what is known about CDK activity at G2/M and during mitosis, this suggests an inverse relationship between WEE1 and CDKB activity in the normal BY-2 cell cycle. The removal of WEE1 protein from lateral root primordia cells also supports the idea that WEE1 is degraded, enabling cell division in the pericycle and in developing lateral root primordia. Furthermore, the data here demonstrate for the first time that WEE1 protein is degraded via the 26S proteasome in plants, and that this may be mediated by an interaction with specific F-box proteins including SKIP1.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Proteins that interacted with AtWEE1 in a yeast two-hybrid library screen.

Table S2. Primer sequences for PCR analysis and vector construction.

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