Effect of plant *WEE1* on the cell cycle and development in *Arabidopsis thaliana* and *Nicotiana tabacum*

A thesis submitted for the degree of Doctor of Philosophy

by

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List of Abbreviations

ATP	adenosine triphosphate
AtWEE1	*Arabidopsis thaliana WEE1*
bp	base pair
°C	degrees centigrade
cdc	cell division cycle
cDNA	complementary deoxyribonucleic acid
CDK	cyclin dependent kinase
Cyc	cyclin
C-terminus	carboxy-terminus
2,4-D	2,4-dinitrophenoxyacetic acid
DEX or dex	dexamethasone
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxy nucleotide triphosphate
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylenebis (oxyethylenenitrilo) tetra-acetic acid
EtBr	ethidium bromide
g	gravity force
G1	gap 1
G2	gap 2
Hyg	hygromycin
KH_2PO_4	potassium dihydrogen orthophosphate
M-phase	mitosis
M	molar
MI	mitotic index

MPF	Maturation promoting factor
M & S	Murashige and Skoog medium
mRNA	messenger ribonucleic acid
N-terminus	Amino-terminus
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
Rb	retinoblastoma protein
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
S	DNA synthesis phase
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SE	standard error
S. pombe	*Schizosaccharomyces pombe*
T	threonine
TBY-2	tobacco bright yellow var. 2 cell line
Thr	threonine
Tris	2-amino-2-hydroxymethyl-1-3-aminomethane
Tris-HCl	2-amino -2-hydroxymethyl-1-3- aminomethane hydrochloride
Tyr	tyrosine
UHP	ultra-high purity water
WT	wild type
Xgal	5-bromo-4-chloro-3-indoyl-β-D- galactopyranoside
Y	tyrosine

Abstract

In eukaryotes the regulatory cell cycle gene, *WEE1*, encodes a protein kinase. In late G2, it inactivates cyclin-dependent kinase (CDKs) in the CDK-cyclinA/B complexes, by phosphorylating the CDK on tyrosine 15. This can result in a delay in mitosis. Expression of *Arabidopsis thaliana* homologue of *WEE1* (*AtWEE1*) in fission yeast resulted in an elongated cell length phenotype in the same way as over expression of fission yeast *wee1*. I have tested whether *AtWEE1* could also induce this effect in tobacco cells and *Arabidopsis* plant roots.

The tobacco BY-2 cells have been transformed with *AtWEE1*, both under constitutive and inducible promoters. Phenotypic characteristics observed compared with the control are premature entry into mitosis and a reduced cell size through a shortening of the G2 phase with a compensatory increase in the duration of G1 phase. Hence, the phenotype and cell cycle response is the exact opposite of the known effect of expression of this gene in fission yeast. *NtWEE1* expression data revealed that the endogenous *WEE1* expression is delayed in transgenic lines, this results in a non-inhibition of CDKA and CDKB1 which are already active in early S-phase.

AtWEE1 was also employed to transform *Arabidopsis thaliana* plants, both under constitutive and inducible promoters. The effect of *AtWEE1* over expression was investigated on primary root growth and lateral root development. In particular, *AtWEE1* over expression lead to less primary root growth and a reduction in the frequency of lateral root primordia initiated when compared with wild type. *Arabidopsis* transgenic plants initiated fewer primordia both per unit time and per cm of primary root.

Chapter 1: General introduction

1.1. The eukaryotic cell cycle and its regulation

One of the key events in the life history of a cell is its division into two identical daughter cells, accomplished during a phase in its life known as mitosis (M phase). In proliferative cells interphase is a preparation for mitosis. The two periods, mitosis and interphase, constitute the well-organized sequence of events known as the cell cycle (Fig. 1.1).

The mitotic cell cycle consists of alternating rounds of DNA replication (which occurs during the S phase) and chromatid separation (which occurs during the M phase) interrupted by gaps known as G1 (the interval before S phase) and G2 (the interval after S phase) (Howard and Pelc, 1953; Taylor, 1958). G1 and G2 may be alternatively considered as periods of general cell growth: G1 represents a phase in which there is a great increase in the rate at which new components (except DNA) are made and G2 represents a period during which a small amount of further growth takes place. Events that occur in each phase are regulated to ensure that the DNA is replicated only once in each cell cycle and that each daughter cell arrives at G2/M with a complete complement of the genome (Eckardt, 2001).

Basic features of cell cycle control are remarkably conserved in all eukaryotes and principal control points at the G1/S and G2/M have been identified in yeast, animals, and plants (Van't Hof, 1966, 1974; Pines, 1995; Huntley and Murray, 1999). Progression through the successive phases of the cell cycle (S, G2, M and G1) in species as diverse as

yeast and humans is driven by a common class of heterodimeric protein kinases. These heterodimers consist of a cyclin-dependent kinase (CDK) and a non-catalytic cyclin (Evans *et al.*, 1983; Nigg, 1995).

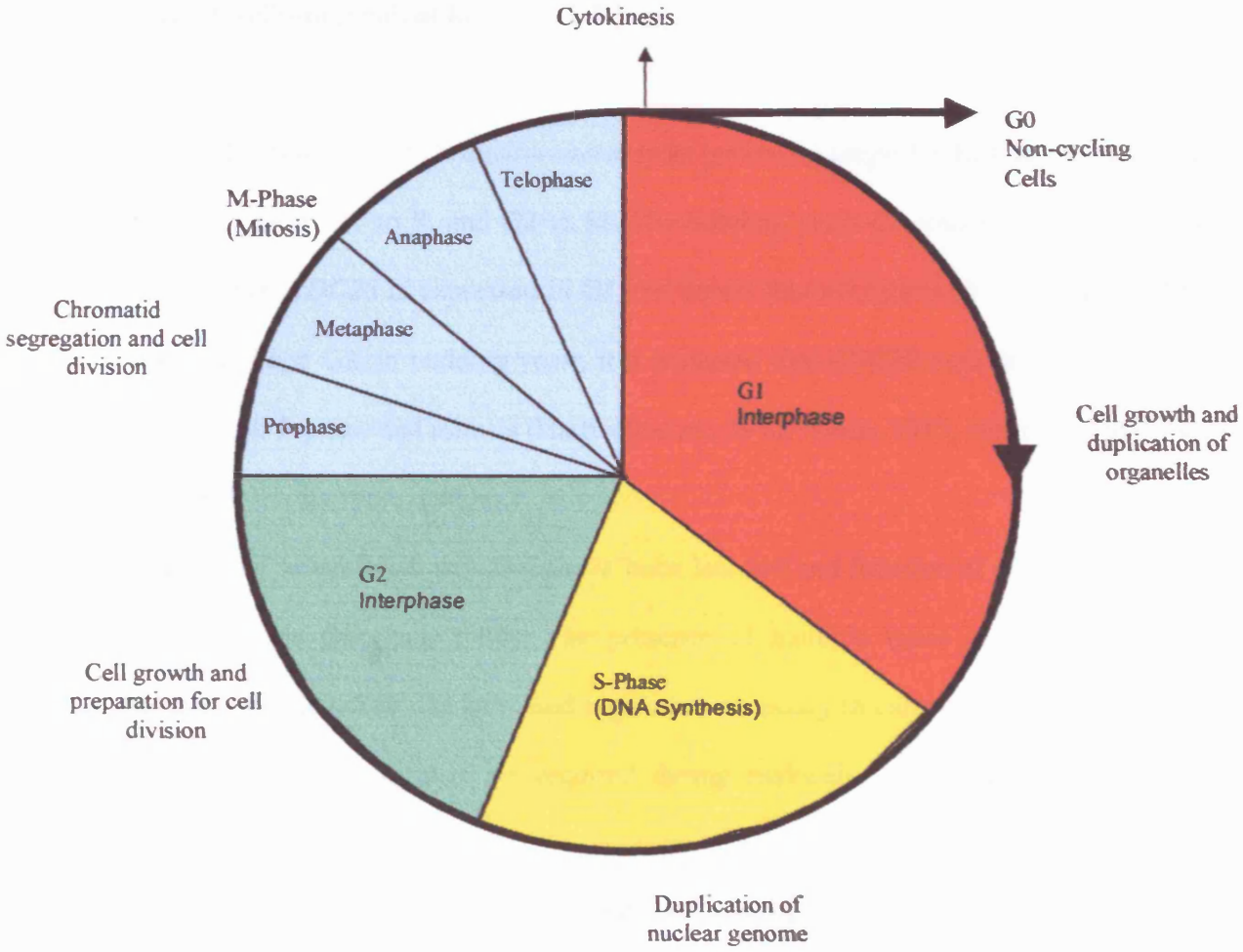


Fig. 1.1: The cell cycle and its component phases.

Studies of the cell cycle present complicated and confusing nomenclature. This is largely because fission yeast geneticists invented a unique nomenclature whereby the gene is

italic lower case while the protein is normal font but denoted by a capital letter beginning the description. The rest of the cell cycle community uses the conventional upper case italic for the gene and upper case normal font for the protein (i.e. Table 1.1).

1.1.1. Cyclin-dependent kinase (CDK)

In fission yeast (*Schizosaccharomyces pombe*) a single CDK, Cdc2 regulates the transition from G1 to S, and G2 to M. The budding yeast (*Saccharomyices cerevisiae*) homologue, *CDC28* is expressed in G1 and drives the cell into S-phase. Because of the poorly defined G2, in budding yeast, it is accepted that *CDC28* expression commits the cell to both S-phase and mitosis (Hartwell *et al.*, 1974; Nurse, 1975; Nasmyth, 1993) (see section 1.3.1. for more details).

In animals, several distinct CDKs have been isolated and function at different stages in the cell cycle (Morgan, 1997). The presence of multiple types of CDKs in higher eukaryotes may reflect the increased regulation necessary to carry out the more complex mitogenic instructions that are required during multicellar development (see section 1.3.2. for more details).

CDKs are not conserved between animals and plants and have variant sequences in their cyclin-binding domain. In animals the nomenclature CDK1 to CDK7 has been adopted (Pines, 1995), whereas in plants the lack of direct equivalents (except between CDK1 and CDKA) has led to the adoption of an alphabetical suffix (CDKA to CDKF) (Joubes *et al.*, 2000), which replaces an earlier confused nomenclature based on *cdc2* (Table 1.1).

At present, 46 CDKs have been identified in 23 different plant species that can be grouped into seven types and can be divided into two main different classes on the basis of their sequences (Segers *et al.*, 1997; Joubes *et al.*, 2000; Stals *et al.*, 2000). All of them possess a specific motif required for ATP and cyclin binding, as first described for the human CyclinA/CDK2 (Jeffrey *et al.*, 1995), as well as conserved sites of regulatory phosphorylation (Segers *et al.*, 1998).

The best characterized plant CDKs belong to the type A class. This class comprises kinases most closely related to the prototypical CDKs, yeast cdc2/CDC28 and animal CDK1 and CDK2, which share the conserved PSTAIRE (single letter code for amino acid) motif in the cyclin binding domain (Serges *et al.*, 1998; Huntley and Murray, 1999; Mironov *et al.*, 1999). These CDKs are also known as CDKA type (Table 1.1). In addition to this large group of CDKs, several non-PSTAIRE CDKs have been identified in plants, which are characterized by the variant sequences PPTALRE or PPTTLRE, and are known as CDKB types (Table 1.1) (Serges *et al.*, 1998; Huntley and Murray, 1999; Mironov *et al.*, 1999).

Table 1.1: Classification of CDKs in Plants based on their cyclin binding motif

Types of CDKs	Cyclin binding motif	New gene name	Old gene name	Species	Characteristics
CDKA	PSTAIRE	Allce;*CDKA;1*	Cdc2	*Allium cepa*	Involved in G1/S and G2/M.
		Antma;*CDKA;1*	Amcdc2a	*Antirrhinum majus*	
		Antma;*CDKA;2*	Amcdc2b	*Antirrhinum majus*	
		Arath;*CDKA;1*	Atcdc2a	*Arabidopsis thaliana*	
		Betvu;*CDKA;1*	cdc2	*Beta vulgaris*	
		Brana;*CDKA;1*	cdc2	*Brassica napus*	
		Cheru;*CDKA;1*	cdc2	*Chenopodium rubrum*	
		Glyma;*CDKA;1*	cdc2-S6	*Glycine max*	
		Glyma;*CDKA;2*	cdc2-S5	*Glycine max*	
		Lyces;*CDKA;1*	Lecdc2A-1	*Lycopersicon esculentum*	
		Lyces;*CDKA;2*	Lecdc2A-2	*Lycopersicon esculentum*	
		Medsa;*CDKA;1*	cdc2MsA	*Medicago sativa*	
		Medsa;*CDKA;2*	cdc2MsB	*Medicago sativa*	
		Nicta;*CDKA;1*	Ntcdc2-1	*Nicotiana tabacum*	
		Nicta;*CDKA;2*	Ntcdc2-2	*Nicotiana tabacum*	
		Nicta;*CDKA;3*	cdc2Nt1	*Nicotiana tabacum*	
		Orysa;*CDKA;1*	cdc2Os-1	*Oryza sativa*	
		Orysa;*CDKA;2*	cdc2Os-2	*Oryza sativa*	
		Peter;*CDKA;1*	cdc2	*Petroselinum crispum*	
		Pethy;*CDKA;1*	cdc2	*Petunia hybrida*	
		Picab;*CDKA;1*	cdc2Pa	*Picea abies*	
		Pinco;*CDKA;1*	cdc2Pnc	*Pinus contorta*	
		Pissa;*CDKA;2*	cdc2	*Pisum sativum*	
		Soltu;*CDKA;2*	cdc2	*Solanum tuberosum*	
		Sesro;*CDKA;1*	Srcdc21	*Sesbania rostrata*	
		Triae;*CDKA;1*	cdc2TaA	*Triticum aestivum*	
		Triae;*CDKA;2*	cdc2TaB	*Triticum aestivum*	
		Vigac;*CDKA;1*	cdc2	*Vigna acontifolia*	
		Vigum;*CDKA;1*	cdc2	*Vigna unguiculata*	
		Zeama;*CDKA;1*	cdc2A	*Zea mays*	
		Zeama;*CDKA;2*	cdc2B	*Zea mays*	

CDKB	PPTALRE;	Atma;*CDKB1;1*	Amcdc2c	*Antirrhinum majus*	Involved in
	PPTTLRE	Atma;*CDKB2;1*	Amcdc2d	*Antirrhinum majus*	G2/M.
		Arath;*CDKB1;1*	Atcdc2b	*Arabidopsis thaliana*	
		Dunte;*CDKB;1*	cdc2	*Dunaliella tertiolecta*	
		Medsa;*CDKB1;1*	cdc2MsD	*Medicago sativa*	
		Arath;*CDKB2;1*	Atcdc2d	*Arabidopsis thaliana*	
		Medsa;*CDKB2;1*	cdc2MsF	*Medicago sativa*	
		Meser;*CDKB2;1*	cdc2	*Mesembryanthemum crystallinum*	
		Orysa;*CDKB;1*	cdc2Os-3	*Oryza sativa*	
CDKC	PITAIRE	Arath;*CDKC;1*	cdc2At	*Arabidopsis thaliana*	Function is not
		Arath;*CDKC;2*	cdc2At	*Arabidopsis thaliana*	understood.
		Medsa;*CDKC;1*	cdc2MsC	*Medicago sativa*	
		Pissa;*CDKC;1*	Ps2cdc2	*Pisum sativum*	
CDKD	N(I/F)TALRE	Arath;*CDKD;1*	CAK2At	*Arabidopsis thaliana*	Involved in G1.
		Orysa;*CDKD;1*	R2	*Oryza sativa*	
CDKE	SPTAIRE	Medsa;*CDKE;1*	cdc2MsE	*Medicago sativa*	Function is not
		Arth;*CDKE;1*		*Arabidopsis thaliana*	understood.
CDKF		Arth;*CDKF;1*		*Arabidopsis thaliana*	Function is not understood.
CDKG	PLTSLRE	Arth;*CDKG;1*		*Arabidopsis thaliana*	Function is not understood.

The CDKB proteins fall into two subgroups on the basis of sequence relationships (Huntley and Murray, 1999; Umeda *et al.*, 1999; Joubes *et al.*, 2000) (Table 1.1). One group contains *Arabidopsis* cdc2b, *Antirrhinum majus* cdc2c and *Medicago sativa* cdc2MsD, which all contain the sequence PPTALRE and for which the name CDKB1 subgroup has been proposed (Hirayama *et al.*, 1991; Imajuku *et al.*, 1992; Hirt *et al.*, 1993; Forbert *et al.*, 1996; Segers *et al.*, 1996; Magyar *et al.*, 1997; Huntley and Murray, 1999). The other subgroup named CDKB2 contains *Antirrhinum majus* cdc2d, *Medicago sativa* cdc2MsF, *Oryza sativa* cdc2Os3 and *Arabidopsis* cdc2dAt (Hirt *et al.*, 1993; Kidou *et al.*, 1994; Forbert *et al.*, 1996; Magyar *et al.*, 1997; Umeda *et al.*, 1999; Huntley

and Murray, 1999). The genes from these two groups differ slightly in the timing of their expression during the cell cycle. CDKB1 transcripts accumulate during S, G2 and M phases, whereas CDKB2 expression is specific to G2 and M phase (Fobert *et al.*, 1996; Segers *et al.*, 1996; Magyar *et al.*, 1997; Umeda *et al.*, 1999). The uniqueness of this CDK has led to the view that CDKB is the kinase that drives plant cells into mitosis. Part of my thesis sheds new light on the timing of Nicta; CDKB1 enzyme activity.

A small group of four plant CDKs was characterized by the presence of the PITAIRE motif (Table 1.1). However, Mironov *et al.* (1999) argued against the involvement of Arath; CDKC in cell cycle control since an *in situ* hybridization signal for an *Arabidopsis* member of the CDKC family could not be obtained in actively dividing cells.

The CDKD proteins (Table 1.1) have a conserved N(I/F)TALRE motif closely related to the equivalent motif of CDK7 kinases from animals. CDKD proteins are considered to be bifunctional, involved in phosphorylation-dependent activation of other CDKs during the cell cycle, and in phosphorylation-dependent regulation of the activity of RNA polymerase II (Harper and Elledge, 1998). In synchronized suspension cells (Sauter, 1997), and during adventitious root growth (Lorbiecke and Sauter, 1999), a preferential expression of the rice *CDKD;1* gene was recorded in G1 and S phases.

Finally, the alfalfa Medsa;*CDKE;1* and Arath;*CDKE;1*, appear unrelated to any other plant sequence as they have a SPTAIRE motif; this CDK was named CDKE (Table 1.1). The involvement of CDKE genes in the plant cell cycle has yet to be proven because they display at the mRNA level a weak constitutive signal during a synchronized cell cycle (Magyar *et al.*, 1997). *Arabidopsis* CDKE might play a role in the specification of stamen and carpel identities and for the proper termination of stem cells in the floral meristem. It

might have a function similar to the differentiation function played by CDK8 in mammals (Wang and Chen, 2004).

In Arabidopsis, *Arath;CDKF;1* encodes a CAKAK (Cyclin dependent kinase activating kinase) (Shimotohno *et al.*, 2004).

CDKG is a relatively new-comer and has a PLTSLRE motif and shows homology to the human protein kinase p58/GTA, a member of the CDC2 kinase subfamily (Menges *et al.* 2005).

CDK activity is dependent on binding with a partener cyclin (see section 1.1.2 for more details) which determines the substrate specificity and the subcellular localization of the CDK complex (Pines, 1995). However, it also depend on a CDK-Activating Kinases (CAK) (see section 1.1.3 for more details) and on phosphorylation of T14 and Y15 residues of the CDK protein (Kugmagai and Dunphy, 1991) (see section 1.4 for more details). Negative regulation by cyclin-dependent kinase inhibitors (CKIs) deactivates the CDK complex (see section 1.1.4 for more details).

1.1.2. Cyclins

Cyclins are a diverse group of proteins with low overall homology that share a large, rather poorly conserved region responsible for their interaction with the CDK; this region is referred to as the cyclin core (Evans *et al.*, 1983). The cyclin core covers about 250 amino acid residues and is organized in two folds of five helices. The first fold is the cyclin box and comprises about 100 amino acid residues (Noble *et al.*, 1997), representing the region of highest conservation, although it contains only five absolutely

invariant positions. The crystal structure reveals the cyclin box as the face of interaction with the cognate CDK (Jeffrey *et al.*, 1995).

In budding yeast seven different cyclins (CLN and CLB proteins) were identified to play important roles at specific phases of the cell cycle (Nasmyth, 1993; Stuart and Wittenberg, 1995) (Table 1.2) (see section 1.2.1 for more details).

Table 1.2: Classifications of Cyclins in Budding Yeast

Cyclins	Characteristics
Cln3	Expressed from early G1 phase
Cln1	Expressed from G1 to beginning of S
Cln2	Expressed from G1 to beginning of S
Cln5	Expressed from S to G2 phase
Cln6	Expressed from S to G2 phase
Clb1	Expressed in M phase
Clb2	Expressed in M phase

In fission yeast six different cyclins (Cig 1 – 6) operate in a similar way (Martin-Castellanos *et al.*, 1996).

A classification based on sequence organization indicates that five types of cyclins exist in plants: A, B, C, D and H types. The first four classes of cyclins (A, B, C and D) are divided into subgroups (Table 1.3) (Renaudin *et al.*, 1996; Vandepoele *et al.*, 2002).

Table 1.3: Classifications of Cyclins in Plants

Cyclins	Species	Characteristics
CycA1	*Z. mays;* *N.tabacum;* *O. sativa.*	Expressed from S to M phase.
CycA2	*A. thaliana;* *Z. mays;* *N. tabacum;* *M. sativa.*	Expressed from S to M phase.
CycA3	*C. roseus;* *A. thaliana;* *N. tabacum;* *A. majus.*	Expressed from S to early G2 phase.
CycB1	*A. thaliana.*	Expressed from G2 to M phase.
CycB2	*M. sativa.*	Expressed from G2 to M phase.
CycB3	*A. thaliana*	Expressed from G2 to M phase.
CycD1	*A. thaliana;* *A. majus;* *H. tuberosus.*	Unknown.
CycD2	*A. thaliana;* *N. tabacum.*	Expressed from G1 to S phase.
CycD3	Widely identified.	Expressed from G1 to S phase.
CycD4	*A. thaliana.*	Unknown.
CycH	*A.thaliana* *O. sativa*	Unknown.

In plants, the major classes of cyclins have homology to animal groups, including the plant cyclins A, B and D (indicated in the Table 1.3 as CycA, CycB and CycD), and numerous examples continue to be isolated and analyzed (Hsieh and Wolniak, 1998; Day and Reddy, 1998; Nakagami *et al.*, 1999; Sorrell *et al.*, 1999).

In mammals, a single type of cyclin A is sufficient to promote CDK activity in S and G2 phase. The CycA-CDK2 complex plays a role in DNA replication and in transcriptional

regulation during S phase. In contrast, multiple A-type cyclins exist in plants. They can be grouped into three subgroups: CycA1, CycA2 and CycA3 (Table 1.3), according to sequence (Renaudin *et al.*, 1996; Chaubet-Gigot, 2000). The functional significance of their complex expression patterns remains unclear (Burssens *et al.*, 2000; Roudier *et al.*, 2000). In *Arabidopsis* 10 sequences (*CYCA1;1*, *CYCA1;2*, *CYCA2;1*, *CYCA2;2*, *CYCA2;3*, *CYCA2;4*, *CYCA3;1*, *CYCA3;2*, *CYCA3;3*, *CYCA3;4*) encoding A-type cyclins have been identified (Vandepole *et al.*, 2002). In most plants screened, members of all three subclasses of A-type cyclins have been found (Dewitte and Murray, 2003).

B-type cyclins (Table 1.3) are expressed specifically in late G2 and early M phase of the cell cycle (Ito, 2000). B type cyclins are distinguished from A-type cyclins, not only by their sequence differences, but also by their later expression pattern during the cell cycle. All identified B-type cyclins were subdivided into two subclasses, CycB1 and CycB2 (Renaudin *et al.*, 1996). However, recently a further B-type cyclin gene was discovered in the *Arabidopsis* genome; it encodes for a B-type cyclin-like protein without the typical B-type destruction box, and has been assigned to a third class, CycB3 (Vandepole *et al.*, 2002).

D-type cyclins (Table 1.3) were defined on the basis of a low sequence homology to animal D-type cyclins and the presence of the conserved LxCxE amino acid motif (single letter amino acid code, where x represents any residue), which is responsible for their interaction with retinoblastoma proteins (Rb) (Soni *et al.*, 1995). The LxCxE amino acid motif is conserved in both animals and plants and is located near the N-terminus of the protein (Ach *et al.*, 1997; Huntley *et al.*, 1998). D-type cyclins have been identified in a variety of plant species (Meijer and Murray, 2000). The recently completed *Arabidopsis*

genome sequence reveals the presence of 10 D-type cyclins sequences as well as several more distantly related cyclin-like genes (Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana,* 2000; Vandepole *et al.*, 2002).

On the basis of phylogenetic analysis, four groups of D-type cyclins exist in plants: CycD1, CycD2, CycD3 and CycD4 (Renaudin *et al.*, 1996; Meijer and Murray, 2000; Vandepole *et al.*, 2002, Boucheron *et al.,* 2005). Multiple pathways control D-type cyclin activity both transcriptionally and post-transcriptionally. *CycD3* transcription is regulated by cytokinins, but brassinosteroids also target *CycD3* expression (see section 1.2.3 Table 1.4 for more details) (Riou-Khamilichi *et al.*, 1999; Hu *et al.*, 2000). Moreover, sucrose has been shown to differentially regulate *Arabidopsis* D-type cyclin (CycD2 and CycD3) expression (Riou-Khamilichi *et al.,* 2000). The complex regulation of D-type cyclins further strengthens the notion that these molecules play a role throughout the plant cell cycle and are not only restricted to controlling the G1/S transition (Riou-Khamilichi *et al.,* 2000).

Genes encoding H-type cyclins (Table 1.3) have been identified in *Arabidopsis* and rice, but it is still unknown whether these proteins have a regulatory role in the plant cell cycle (Yamaguchi *et al.*, 2000; Vandepole *et al.*, 2002).

CycA and *CycB* cyclin genes are expressed in a cell cycle dependent manner, peaking transcriptionally at or near to the G2/M transition (A type cyclins are expressed somewhat earlier than B-type), and differences in the timing of expression exist even between genes encoding different sub-types of CycA cyclins (Reicheld *et al.*, 1996). *CycD* cyclin genes like their animal counterparts, show cell cycle independent expression, their transcription being induced by the presence of mitogens. The genes

encoding CycD cyclins are induced at specific times during cell cycle re-entry but generally remain expressed at a constant level in actively dividing cells (Soni *et al.*, 1995; Fuerst *et al.*, 1996; Sorrell *et al.*, 1999). In animals, E-type cyclins are strongly regulated transcriptionally at the G1/S boundary, but a direct equivalent has yet to be identified in plant cells (Dewitte and Murray, 2003).

The levels of cyclins are generally determined by specific protein-turnover mechanisms as well as highly regulated transcription (Ito *et al.*, 1998). The destruction of CycA and CycB cyclins at specific points during M phase depends on a destruction box motif which mediates their ubiquitin-dependent proteolysis (Glotzer *et al.*, 1991; Renaudin *et al.*, 1998). In contrast, most CycD cyclins contain PEST sequences, regions rich in proline, glutamate, serine and threonine, which are thought to be a signal for the rapid proteolysis of many proteins (Rechsteiner, 1990; Soni *et al.*, 1995; Sorrell *et al.*, 1999).

1.1.3. CDK-Activating Kinases

The basic mechanism and logic of cell cycle control, with some exceptions, is highly conserved in all eukaryotes, and so are the key genes that mediate cell cycle progression (Nasmyth, 1996; Novak *et al.*, 1998; Dewitte and Murray, 2003). Cyclin dependent kinases play a central role in mediating cell cycle progression (see section 1.1.2 for more details). CDK activity is regulated by association with cyclin subunits, reversible phosphorylation and association with other regulatory proteins. CDK activity is controlled by phosphorylation at three conserved sites, and many of the enzymes that act on these sites have been identified in many eukaryote organisms including plant

species (Lew and Kornbluth, 1996; Dewitte and Murray, 2003; Sorrell *et al.*, 2002, 2003). Cyclin dependent kinase activity not only requires binding of the CDK to a cyclin but also, phosphorylation of the CDK at a conserved threonine residue within the "T-loop" (residue Thr-160 or Thr-161). The T-loop can mask the catalytic site to prevent substrate binding, or swing open to permit substrate phosphorylation (Solomon, 1993). CDK-activating kinase (CAK), the enzyme responsible for the phosphorylation of the Thr-160 or 161 residues of CDKs (Fig. 1.2), has been identified in several organisms.

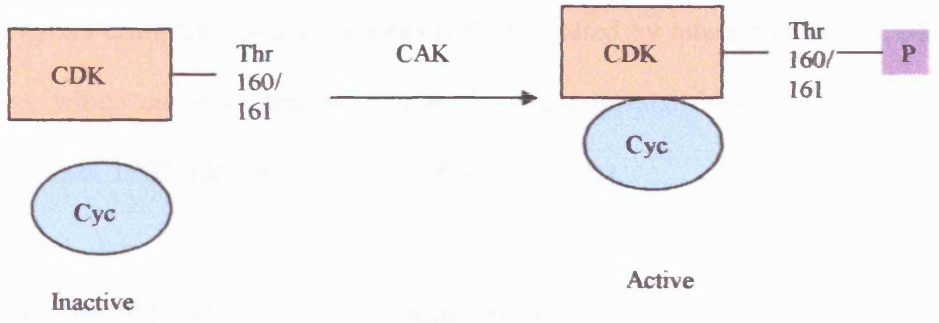


Fig. 1.2: CAK activates CDK by phosphorylation of threonine 160 or 161.

The most plausible candidate for the higher eukaryotic CAK is the CDK7/cyclinH/Mat1 complex, which was purified from frog, starfish, and mammalian cells (Nigg, 1996). An *Arabidopsis* cDNA, designated *CAK1Arath*, was isolated as a suppressor of a CAK mutation in budding yeast and fission yeast (Umeda *et al.*, 1998). The amino acid sequence of ArathCAK1 is related to animal CAKs, but similarities are restricted to the conserved kinase domain. ArathCAK1 can phosphorylate human CDK2 at the Thr-160

residue in the T loop (Umeda *et al.*, 1998). The rice R2, closely related to CDK7 of animals (Hata *et al.*, 1991), is structurally similar to the CAKs of metazoans and fission yeast, but is distinct from ArathCAK1 (Yamaguchi *et al.*, 1998).

In Arabidopsis, CAK comprises CyclinD and CDKD although CDKF might function equally well here. Note that in alfalfa, CDK;C1-cyclin T phosphorylates RNA polymerase II at its C-terminal (Fulop *et al.*, 2005).

In higher eukaryotes, the activated cyclin-bound CDK can be inhibited by phosphorylation of two conserved residues within the catalytic cleft at residues Y15 and T14 (Lew and Kornbluth, 1996). However, in fission yeast only Y15 is phosphoregulated (Russell and Nurse, 1987) (see section 1.4 for more details). The catalytic activity of CDK/cyclin complex can also be negatively regulated by interaction with CDK inhibitor (CKIs), which cause extensive structural changes through binding (Martin-Castellanos and Moreno, 1997) (see section 1.1.5 for more details).

1.1.4. Cyclin-dependent kinase inhibitors (CKIs)

CKIs are low molecular mass proteins that bind and negatively regulate the catalytic activity of the CDK/Cyclin complex by causing extensive structural changes through their binding (Martin-Castellanos and Moreno, 1997). In mammals, several inhibitors have been isolated, grouped into the Ink4 or the Cip/Kip1 family, according to functional and sequence similarity (Sherr and Roberts, 1995; Burssens *et al.*, 1998). In fission yeast and budding yeast cells, CDK inhibitors prevent premature initiation of DNA replication by inhibiting the CDK/Cyclin complex in early G1 phase (Sanchez-Diaz

et al., 1998). In *S. cerevisiae* degradation of the CDK inhibitor is required for initiation of S phase (Schwob *et al.*, 1994). Plant CDK/cyclin complex inhibitory proteins are known as ICK (inhibitor of Cdc2 kinase) or KRP (Kip-related proteins) and bind both CDK and cyclin subunits (Wang *et al.*, 1997, Zhou *et al.*, 2003, Verkest *et al.*, 2005). KRPs can inhibit both CYCD2/CDKA and CYCD2/CDKB (Nakai *et al.*, 2006).

Using the yeast two hybrid system, the first plant *CDK* inhibitor gene was identified in *A. thaliana* (Wang *et al.*, 1997, Weinl *et al.*, 2005), where the *CKI* genes constitute a small gene family (Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana,* 2000). The plant CKI protein restricts CDK activity *in vitro* and *in vivo* (Wang *et al.*, 1998, 2000).

1.2. Comparison between the CDK/cyclin complexes during the different phases of the cell cycle in yeast, animals and plants

1.2.1. Budding yeast cell cycle

In early interphase (G1) of the budding yeast cell cycle, cells rapidly increase in volume. This cell growth is accompanied by a gradual increase of G1-phase Cln3 cyclin (a "non-cycling" cyclin) that promotes the accumulation of cyclins Cln1 and Cln2 at the transcriptional level (Nasmyth, 1993; Stuart and Wittenberg, 1995) (Fig. 1.3 a). These associate with CDC28 kinase, the budding yeast homologue to Cdc2, to regulate the transition through the G1 checkpoint known as "START" (Nasmyth, 1993) (Fig. 1. 3 b).

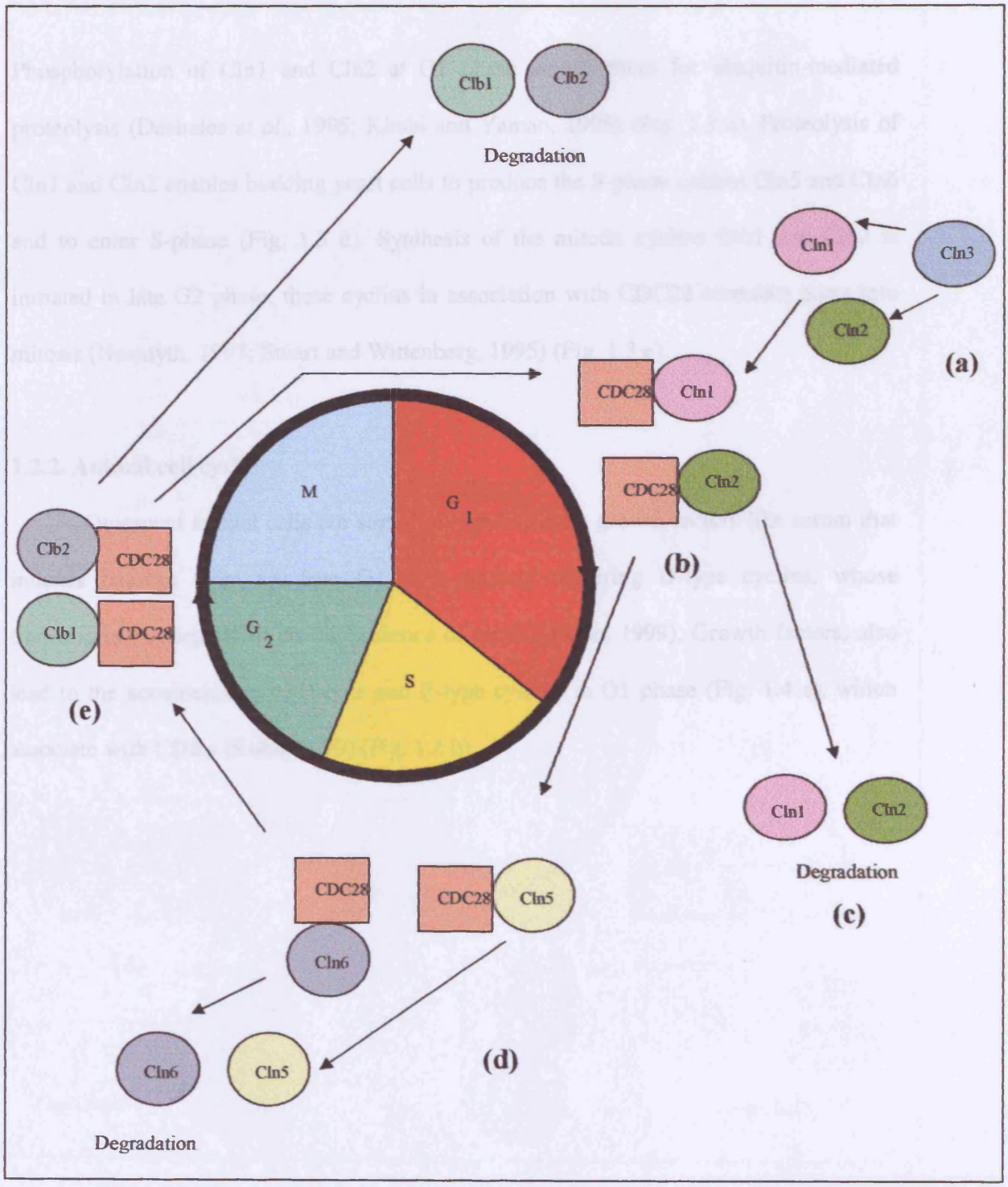


Fig.1.3: Schematic model of budding yeast cell cycle which shows interactions between CDC28 and different cyclins during G1, S, G2 and M phase.

Phosphorylation of Cln1 and Cln2 at G1 phase targets them for ubiquitin-mediated proteolysis (Deshaies *et al.*, 1995; Kirshi and Yamao, 1998) (Fig. 1.3 c). Proteolysis of Cln1 and Cln2 enables budding yeast cells to produce the S-phase cyclins Cln5 and Cln6 and to enter S-phase (Fig. 1.3 d). Synthesis of the mitotic cyclins Clb1 and Clb2 is initiated in late G2 phase, these cyclins in association with CDC28 stimulate entry into mitosis (Nasmyth, 1993; Stuart and Wittenberg, 1995) (Fig. 1.3 e).

1.2.2. Animal cell cycle

Quiescent animal cells are stimulated to divide by growth factors like serum that induces passage from G0 into G1 in a process requiring D-type cyclins, whose transcription is dependent on the presence of serum (Kohn, 1999). Growth factors, also lead to the accumulation of D-type and E-type cyclins in G1 phase (Fig. 1.4 a), which associate with CDKs (Kohn, 1999) (Fig. 1.4 b).

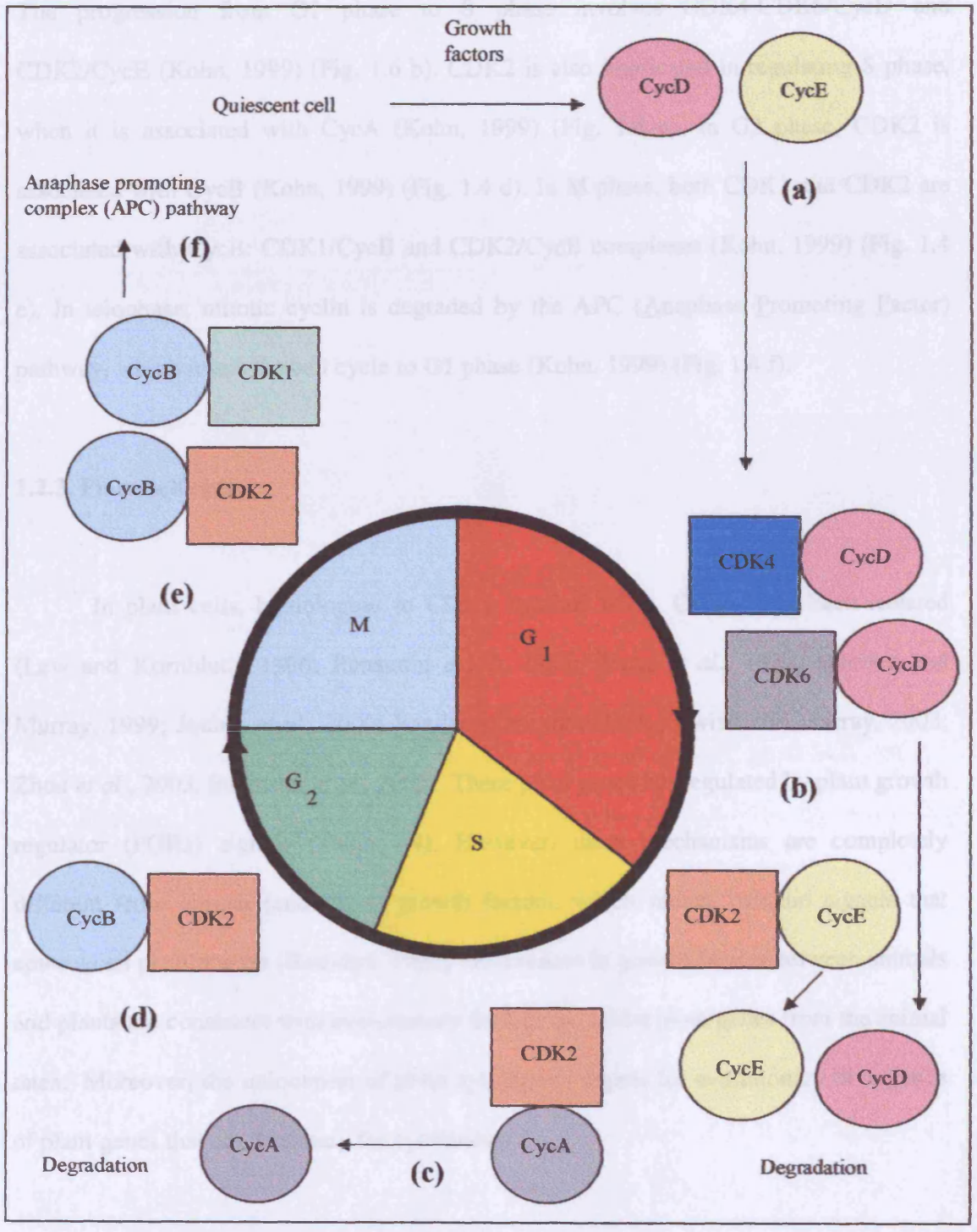


Fig. 1.4: Schematic model of animal cell cycle which shows interactions between different CDK and cyclins during G1, S, G2 and M phase.

The progression from G1 phase to S phase involves CDK4-CDK6/CycD and CDK2/CycE (Kohn, 1999) (Fig. 1.6 b). CDK2 is also implicated in regulating S phase, when it is associated with CycA (Kohn, 1999) (Fig. 1.4 c). In G2 phase, CDK2 is associated with CycB (Kohn, 1999) (Fig. 1.4 d). In M phase, both CDK1 and CDK2 are associated with CycB: CDK1/CycB and CDK2/CycB complexes (Kohn, 1999) (Fig. 1.4 e). In telophase, mitotic cyclin is degraded by the APC (Anaphase Promoting Factor) pathway, which resets the cell cycle to G1 phase (Kohn, 1999) (Fig. 1.4 f).

1.2.3. Plant cell cycle

In plant cells, homologues to CDKs, cyclins, ICKs, CAKs have been isolated (Lew and Kornbluth, 1996; Renaudin *et al.*, 1996; Wang *et al.*, 1997; Huntley and Murray, 1999; Joubes *et al.*, 2000; Vandepoele *et al.*, 2002; Dewitte and Murray, 2003; Zhou *et al.*, 2003, Beemster *et al.*, 2005). These plant genes are regulated by plant growth regulator (PGRs) signals (Table 1.4). However, these mechanisms are completely different from animal (and yeast) growth factors, which induce calcium signals that control cell proliferation (Berridge, 1995). Differences in growth factors between animals and plants are consistent with evolutionary divergence of the plant genes from the animal ones. Moreover, the uniqueness of plant cytokinesis argues for evolutionary divergence of plant genes that are necessary for cytokinesis.

Table 1.4: Effects of exogenous PGRs and sucrose on the plant cell cycle

PGRs	Characteristics	Published examples
Cytokinins (i.e. kinetin, zeatin, benzyladenine)	Promote plant cell division, can cause non-cycling cells to divide. Can act at the G1/S transition by stimulating CycD3 and at the G2/M transition by stimulating activation of b-type CDK.	Riou-Kamlichi *et al.*, 1999. Zhang *et al.*, 2005. Menges *et al.*, 2006.
Brassinolides	Activate cell proliferation.	Miyazawa *et al.*, 2003.
Gibberellins (GAs)	Induce cell cycle activation at the G2/M transition by increasing the level of CDK kinase.	Sauter *et al.*, 1997.
Auxin	Required to initiate cell division in the pericycle by acting on D-type cyclin.	De Veylder *et al.*, 1999.
Abscisic acid (ABA)	Prevents DNA replication by keeping the cells in the G1 phase inducing ICK1 activity.	Wang *et al.*, 1998; Swiatek *et al.*, 2002.
Ethylene	Delays the entry of cells into mitosis and induces cell death at the G2/M transition.	Herbert *et al.*, 2001.
Sucrose	Stimulates G0 to G1 progression by inducing CycD2. Enables cells arrested in G1 to enter S phase or cells arrested in G2 to enter M phase.	Soni *et al.*, 1995; Sorrell *et al.*, 1999.

Cytokinins, brassinolides and sucrose are molecules that regulate plant cell division. In G1 phase CycD, the partner of CDKA, is rapidly induced in response to the accumulation of the above regulators (Fig. 1.5 a). During S-phase, CycA begins to accumulate as a partner for CDKA (Fig. 1.5 b). In G2 phase and in the transition from G2 to M phase, CycA is replaced by CycB as partner of both CDKA and CDKB (Fig. 1.5 c).

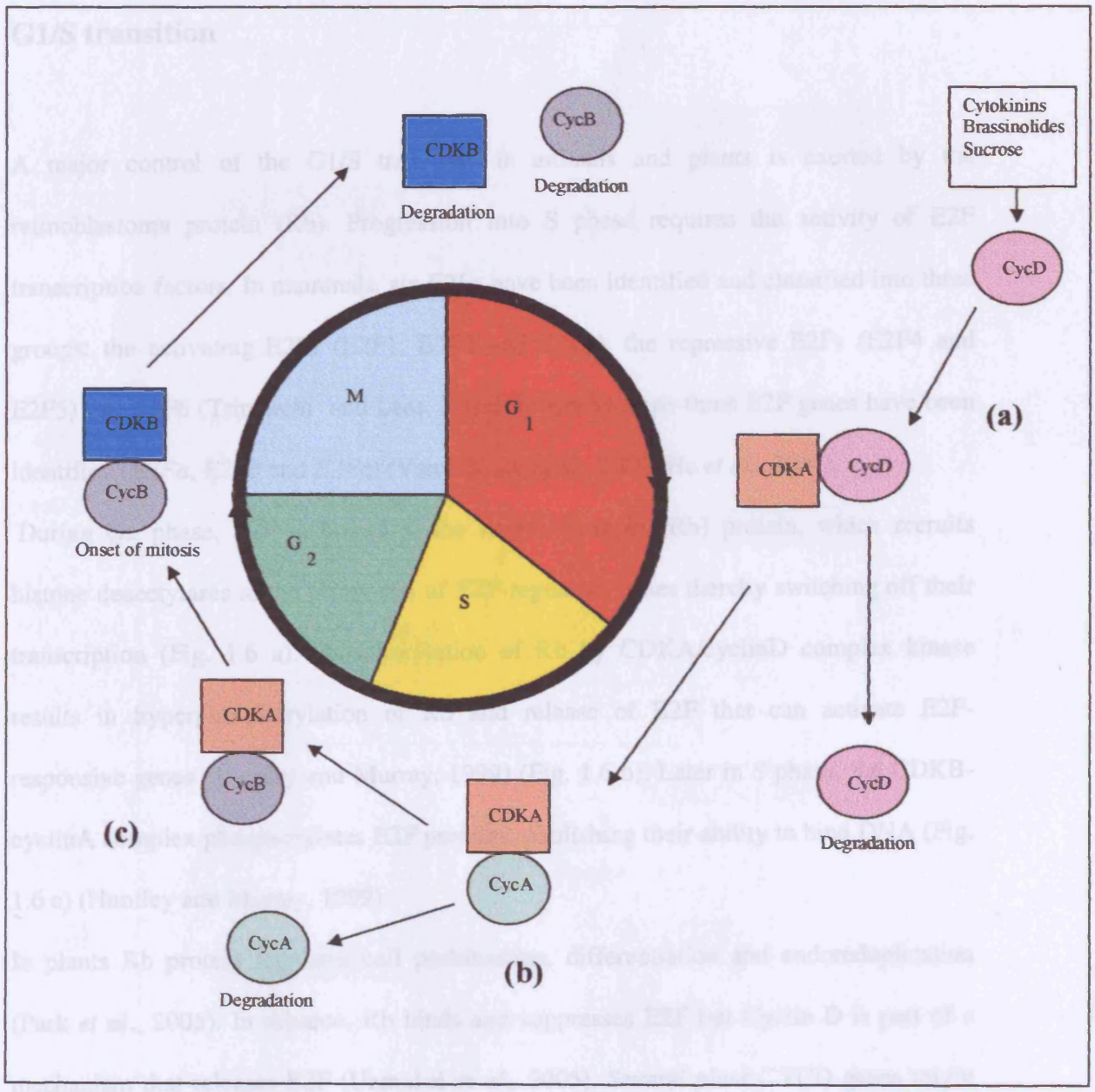


Fig. 1.5: Schematic model of plant cell cycle. Various cell cycle regulatory proteins are degraded through the ubiquitin/proteasome pathway at specific cell cycle stages in plant cells.

1.3. Retinoblastoma protein and the E2F transcription factor regulate G1/S transition

A major control of the G1/S transition in animals and plants is exerted by the retinoblastoma protein (Rb). Progression into S phase requires the activity of E2F transcription factors. In mammals, six E2Fs have been identified and classified into three groups: the activating E2Fs (E2F1, E2F2 and E2F3), the repressive E2Fs (E2F4 and E2F5) and E2F6 (Trimarchi and Lees, 2002). In *Arabidopsis* three E2F genes have been identified (*E2Fa*, *E2Fb* and *E2Fc*) (Vandepoele *et al.*, 2002; He *et al.*, 2004).

During G1 phase, E2F is bound to the Retinoblastoma (Rb) protein, which recruits histone deacetylases to the promoters of E2F-regulated genes thereby switching off their transcription (Fig. 1.6 a). Phosphorylation of Rb by CDKA/cyclinD complex kinase results in hyperphosphorylation of Rb and release of E2F that can activate E2F-responsive genes (Huntley and Murray, 1999) (Fig. 1.6 b). Later in S phase, the CDKB-cyclinA complex phosphorylates E2F proteins, abolishing their ability to bind DNA (Fig. 1.6 c) (Huntley and Murray, 1999).

In plants Rb protein regulates cell proliferation, differentiation and endoreduplication (Park *et al.*, 2005). In tobacco, Rb binds and suppresses E2F but Cyclin D is part of a mechanism that releases E2F (Uemukai *et al.*, 2005). Several plant CYCD genes might bind with Arath; CDKA and replace animal cyclin E in hyperphosphorytion of Rbs. (Menges *et al.*, 2005).

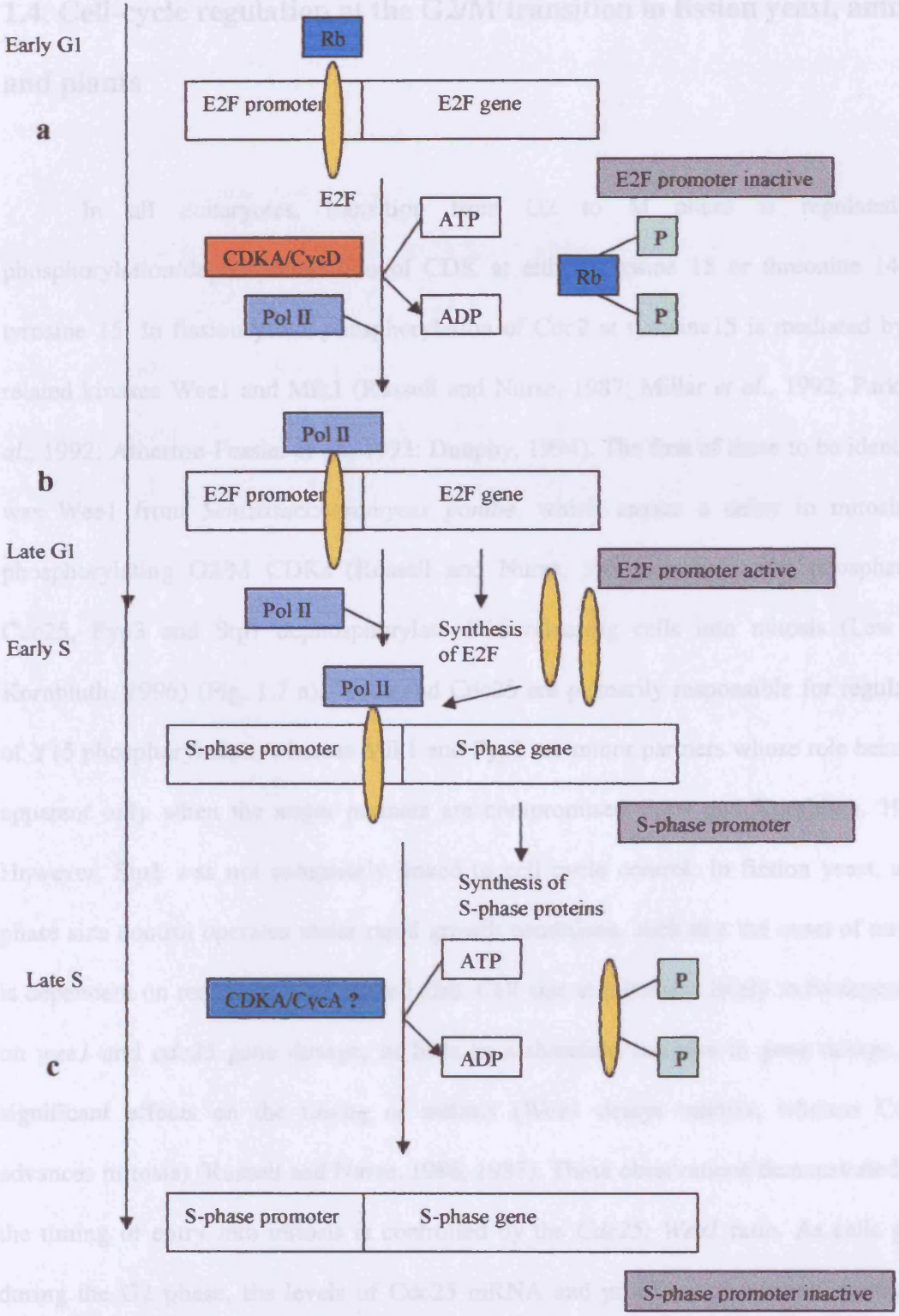


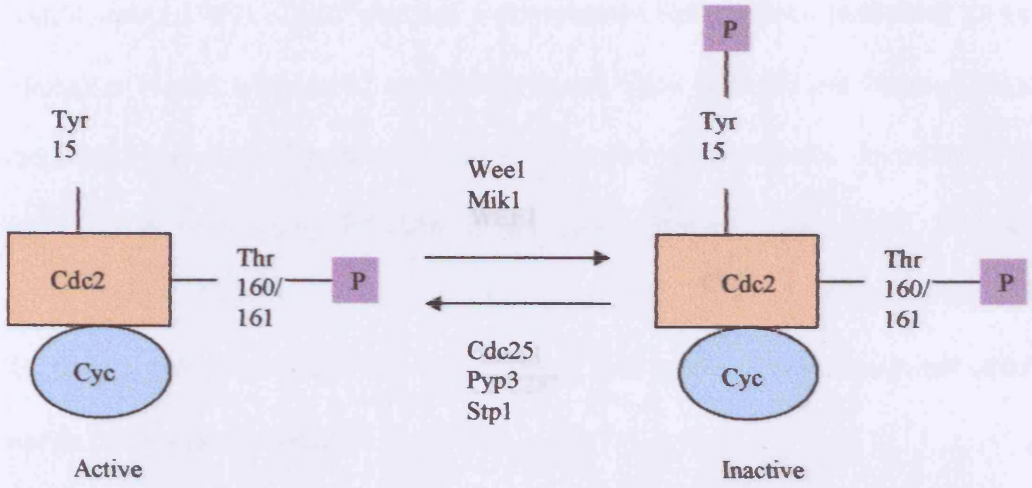
Fig. 1.6: Schematic model for the control of G1/S transition in plants, involving Retinoblastoma protein (Rb) and E2F transcription factor (adapted from Huntley and Murray, 1999).

1.4. Cell-cycle regulation at the G2/M transition in fission yeast, animals and plants

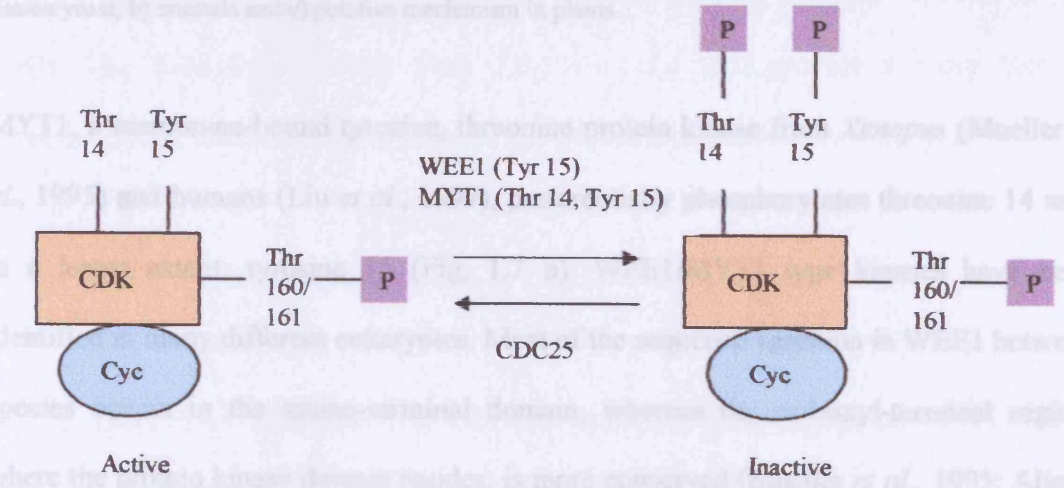
In all eukaryotes, transition from G2 to M phase is regulated by phosphorylation/dephosphorylation of CDK at either tyrosine 15 or threonine 14 and tyrosine 15. In fission yeast, phosphorylation of Cdc2 at tyrosine15 is mediated by the related kinases Wee1 and Mik1 (Russell and Nurse, 1987; Millar *et al.*, 1992; Parker *et al.*, 1992; Atherton-Fessler *et al.*, 1993; Dunphy, 1994). The first of these to be identified was Wee1 from *Schizosaccharomyces pombe,* which causes a delay in mitosis by phosphorylating G2/M CDKs (Russell and Nurse, 1987), whereas the phosphatases Cdc25, Pyp3 and Stp1 dephosphorylate Y15 releasing cells into mitosis (Lew and Kornbluth, 1996) (Fig. 1.7 a). Wee1 and Cdc25 are primarily responsible for regulation of Y15 phosphorylation, whereas Mik1 and Pyp3 are minor partners whose role becomes apparent only when the major partners are compromised (Lew and Kornbluth, 1996). However, Stp1 was not completely linked to cell cycle control. In fission yeast, a G2 phase size control operates under rapid growth conditions, such that the onset of mitosis is dependent on reaching a critical cell size. Cell size at mitosis is likely to be dependent on *wee1* and *cdc25* gene dosage; as little as a threefold increase in gene dosage, has significant effects on the timing of mitosis (Wee1 delays mitosis, whereas Cdc25 advances mitosis) (Russell and Nurse, 1986, 1987). These observations demonstrated that the timing of entry into mitosis is controlled by the *Cdc25: Wee1* ratio. As cells grow during the G2 phase, the levels of Cdc25 mRNA and protein increase until mitosis is triggered, after which there is a sharp drop in both mRNA and protein abundance

(Moreno *et al.*, 1990). It has been proposed that this accumulation pattern results in a gradually increasing *cdc25: wee1* ratio during the G2 phase, and that the ratio becomes sufficient to trigger mitosis when cells reach a critical size (Lew and Kornbluth, 1996).

In multicellular eukaryotes, phosphorylation and dephosphorylation of both threonine 14 (animals) and tyrosine 15 (animals and plants) of the catalytic subunit of CDKs regulate their activity and determine the timing of G2 phase and mitosis (Dunphy, 1994). Phosphorylation on tyrosine 15 and threonine 14 inactivates CDK, whereas dephosphorylation by CDC25 activates the enzyme, triggering the G2 to M phase transition (Kugmagai and Dunphy, 1991). Threonine 14 and tyrosine 15 are buried beneath the T loop structure, and cyclin binding induces a conformational change that makes these residues accessible for phosphorylation (De Bondt *et al.*, 1993) (Figure 1.7 b, c).


Tyr
15
Cdc2
Cyc
Thr
160/
161
P
Active
Wee1
Mik1
Cdc25
Pyp3
Stp1
P
Tyr
15
Cdc2
Cyc
Thr
160/
161
P
Inactive

a


Thr
14
Tyr
15
CDK
Cyc
Thr
160/
161
P
Active
WEE1 (Tyr 15)
MYT1 (Thr 14, Tyr 15)
CDC25
P
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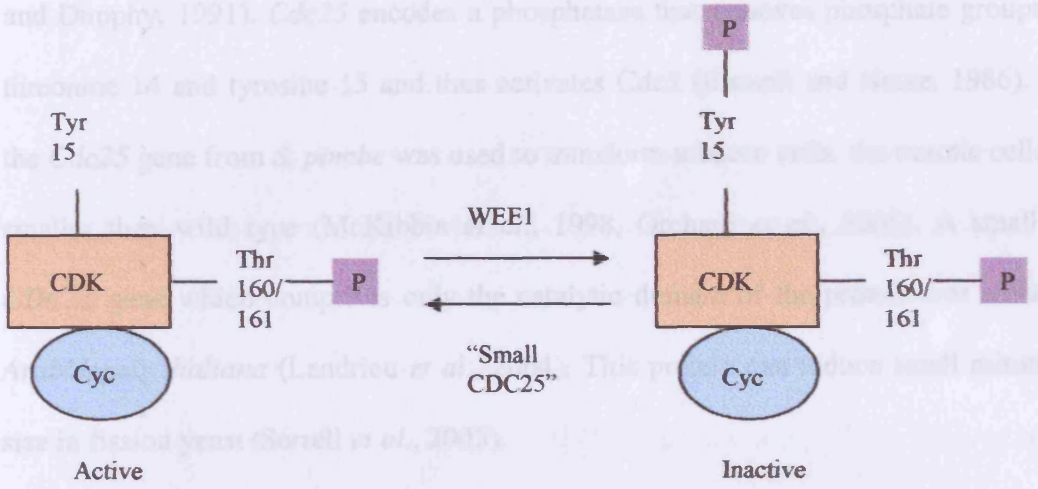
Thr
14
Tyr
15
CDK
Cyc
Thr
160/
161
P
Inactive

b



c

Fig. 1.7: CDK regulation by phosphorylation/dephosphorylation of threonine 14 and tyrosine 15 in **a)** fission yeast, **b)** animals and **c)** putative mechanism in plants.

MYT1, a membrane-bound tyrosine, threonine protein kinase from *Xenopus* (Mueller *et al.*, 1995) and humans (Liu *et al.*, 1997), preferentially phosphorylates threonine 14 and, to a lesser extent, tyrosine 15 (Fig. 1.7 b). WEE1/MYT1 type kinases have been identified in many different eukaryotes. Most of the sequence variation in WEE1 between species occurs in the amino-terminal domain, whereas the carboxyl-terminal region, where the protein kinase domain resides, is more conserved (Mueller *et al.*, 1995; Aligue *et al.*, 1997). In plants, CDK regulation by phosphorylation/dephosphorylation of tyrosine 15 is likely mediated by WEE1 and CDC25 respectively (Sun *et al*, 1999; Sorrell *et al.*, 2002, 2005) (Fig. 1.7 c).

In *S. pombe,* the *Cdc25* gene is a mitotic inducer controlling the G2-M transition (Russell and Nurse, 1986). In humans three homologues of CDC25 have been detected:

HsCDC25A, B and C. HsCDC25C is functionally homologous to SpCdc25 (Kumagai and Dunphy, 1991). *Cdc25* encodes a phosphatase that removes phosphate groups from threonine 14 and tyrosine 15 and thus activates Cdc2 (Russell and Nurse, 1986). When the *Cdc25* gene from *S. pombe* was used to transform tobacco cells, the mitotic cells were smaller than wild type (McKibbin *et al.*, 1998, Orchard *et al.*, 2005). A small plant *CDC25* gene which comprises only the catalytic domain of the protein was isolated in *Arabidopsis thaliana* (Landrieu *et al.*, 2004). This protein can induce small mitotic cell size in fission yeast (Sorrell *et al.*, 2005).

1.5. The role of *WEE1* during the G2/M transition

The focus of my thesis was to gain an understanding of *WEE1* function in plant cells. The following sections from 1.5.1 to 1.5.4 will provide a more thorough introduction to the role of WEE1 kinase and its regulation during the cell cycle.

1.5.1. WEE1 plays an important role in checkpoint control

To maintain a specific size, cells must coordinate their growth and division. Yeast cells are thought to use cell size checkpoints to coordinate these two processes (Nurse, 1975; Fantes and Nurse, 1977; Hartewell and Unger, 1977; Johnston *et al.*, 1977; Rupes, 2002). Cell size checkpoints prevent passage through key cell cycle transitions until cells have reached a critical size. In fission yeast, the critical size requirement is exerted primarily at the G2/M transition, whereas in budding yeast it is exerted primarily at the

G1/S transition (Rupes, 2002). Although the existence of cell size checkpoints was proposed over 20 years ago, the underlying molecular mechanisms have remained elusive. Moreover, it is not understood how a cell monitors its size/growth. Genetic analysis of cell cycle checkpoints is difficult because mutations that accelerate or delay cell cycle progression may have indirect effects on cell size (Kellogg, 2003). Despite these difficulties, early work in fission yeast suggested that the Wee1 kinase plays an important role in a checkpoint that coordinates cell growth and cell division at the G2/M transition (Nurse, 1975; Fantes and Nurse, 1978; Thuriaux *et al.*, 1978). Thus cloning of a *WEE1* in *Arabidopsis* (Sorrell *et al.*, 2002) became an extremely interesting tool as a putative cell size controller in plants.

1.5.2. The WEE1 protein kinase is regulated at multiple levels

Early work in fission yeast suggested that Wee1 is part of a cell-size checkpoint that prevents entry into mitosis until cells have reached a critical size (Russell and Nurse, 1987). Recent experiments on *Swe1*, the budding yeast homologue of *wee1*, have provided new support for this idea (Jorgensen *et al.*, 2002; Harvey and Kellog, 2003). During the cell cycle, WEE1 protein kinases can be negatively regulated by phosphorylation; in addition to phosphorylation WEE1 is also regulated by interaction with 14-3-3 proteins (Honda *et al.*, 1997; Lee *et al.*, 2001).

In budding yeast intricate signaling networks are required for regulation of *Swe1* (Harvey and Kellogg, 2003). WEE1 kinase from *Xenopus,* fission yeast and budding yeast undergo extensive hyperphosphorylation during mitosis. Moreover, the

hyperphosphorylated form of *Xenopus* WEE1, isolated from mitotic extracts, has reduced kinase activity (Tang *et al.*, 1993; Muller *et al.*, 1995; Sreenivasan and Kellogg 1999; Harvey and Kellogg, 2003). Hyperphosphorylation of WEE1 in *Xenopus* extracts is dependent upon mitotic CDK1 activity, that can phosphorylate WEE1 *in vitro* (Tang *et al.*, 1993; Muller *et al.*, 1995). Experiments with fission yeast and budding yeast have identified several kinases required for regulation of WEE1 kinase *in vivo*. In fission yeast, Wee1 is phosphorylated and inactivated by the Cdr/Nim1 protein kinase complex (Coleman *et al.*, 1993; Parker *et al.*, 1993). Wee1 can also be phosphorylated by the Chk1 and Cds1 checkpoint kinases (O'Connel *et al.*, 1997; Boddy *et al.*, 1998). In budding yeast, a complex signaling network is required for regulation of Swe1 and for coordination of cell growth and cell division at G2/M. This network includes the kinases, Gin4, Hsl1, Cla4 and Elm1. In addition, a number of proteins required for regulation of these kinases have been identified, including Nap1, Hsl7 and a family of proteins called the septins (Fig. 1.8) (Kellogg and Murray, 1995; Ma *et al.*, 1996; Altaman and Kellogg, 1997; Carroll *et al.*, 1998; Tjandra *et al.*, 1998; Barral *et al.*, 1999; Edgington *et al.*, 1999; Shulewitz *et al.*, 1999; Sreenivans and Kellogg, 1999; Longitane *et al.*, 2000).

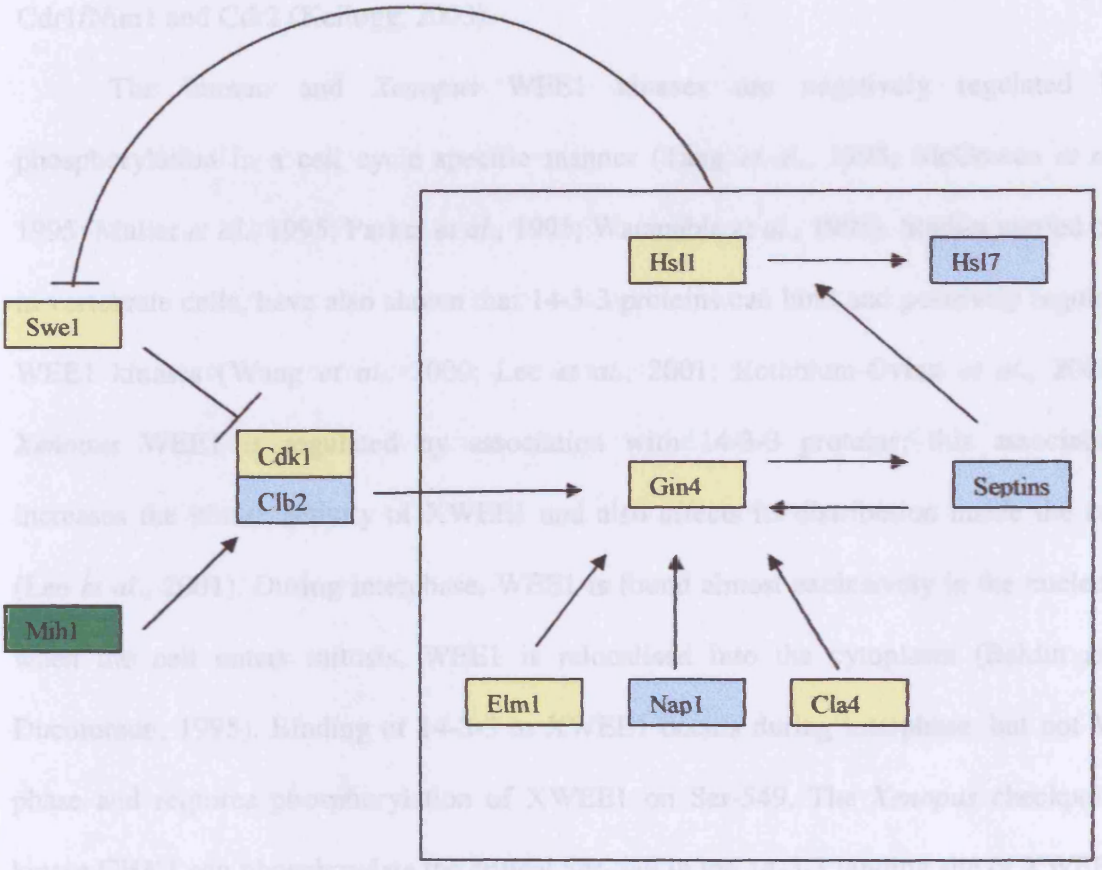


Fig. 1.8: Signaling network required for regulation of Swe1 in budding yeast. Kinases are shown in yellow, proteins required for regulation of kinases are shown in blue and phosphatase is shown in green (adapted from Kellog, 2003).

However, it is unclear how the kinase signaling network regulates Swe1 because none of these proteins has been found to phosphorylate Swe1 directly. In addition the physiological signals that regulate the network are poorly understood, although there is some evidence that components of the network respond to nutritional cues (Fig. 1.8) (Garrett, 1997; Cullen and Sprague, 2000; La Valle and Wittemberg, 2001). Many of the proteins that function in the network are highly conserved, suggesting a similar network

in all eukaryotes. For example, budding yeast Gin4 and Hsl1, are related to fission yeast Cdr1/Nim1 and Cdr2 (Kellogg, 2003).

The human and *Xenopus* WEE1 kinases are negatively regulated by phosphorylation in a cell cycle specific manner (Tang *et al.*, 1993; McGowan *et al.*, 1995; Muller *et al.*, 1995; Parker *et al.*, 1995; Watanable *et al.*, 1995). Studies carried out in vertebrate cells, have also shown that 14-3-3 proteins can bind and positively regulate WEE1 kinases (Wang *et al.*, 2000; Lee *et al.*, 2001; Rothblum-Oviatt *et al.*, 2001). *Xenopus* WEE1 is regulated by association with 14-3-3 proteins; this association increases the kinase activity of XWEE1 and also affects its distribution inside the cell (Lee *et al.*, 2001). During interphase, WEE1 is found almost exclusively in the nucleus; when the cell enters mitosis, WEE1 is relocalised into the cytoplasm (Baldin and Ducommun, 1995). Binding of 14-3-3 to XWEE1 occurs during interphase, but not M-phase and requires phosphorylation of XWEE1 on Ser-549. The *Xenopus* checkpoint kinase CHK1 can phosphorylate the critical Ser-549 in the 14-3-3 binding site of XWEE1 (Lee *et al.*, 2001). Similarly in humans, phosphorylation of WEE1 on SER-642 is essential for the binding of 14-3-3 proteins to WEE1 (Wang *et al.*, 2000). Like *Xenopus* WEE1, interactions of 14-3-3 proteins with human WEE1 are reduced during mitosis, but not during interphase when interaction between 14-3-3 proteins and WEE1 increases the enzymatic activity of WEE1; this indicates that 14-3-3 proteins function as positive regulators of the human WEE1 protein kinase (Rothblum-Oviatt *et al.*, 2001)

1.5.3. WEE1 protein levels fluctuated during the cell cycle

In addition to phosphorylation and interaction with 14-3-3 proteins, human WEE1 is also regulated at the level of protein synthesis and stability (Watanabe *et al.*, 1995). WEE1 protein levels rise during the S and G2 phases of the cell cycle because of increased synthesis, and WEE1 protein levels fall during M phase because of decreased synthesis combined with proteolysis (Rothblum-Oviatt *et al.*, 2001). Experiments using budding yeast have shown that the Swe1 protein is stable during G2/M and is not degraded until exit from mitosis (Sreenivasan and Kellogg, 1999; Harvey and Kellogg, 2003). It was also concluded that Swe1 is targeted for destruction by the SCF ubiquitin ligase complex (Sia *et al.*, 1998; McMillan *et al.*, 1999, 2002).

1.6. Tobacco BY-2 cells

Dispersed plant cell suspension cultures allow the study of cell division free of developmental constraints, by providing a homogeneous population of near-identical cells (Gould, 1984). Synchronization of such cultures can provide material representative of specific cell cycle phases, and this may be achieved either by removal and subsequent re-supply of a compound required for growth, such as phosphate, nitrate, hormones or sucrose (King *et al.*, 1973; Amino *et al.*, 1983; Kodama *et al.*, 1991; Nishida *et al.*, 1992; Riou-Khamlichi *et al.*, 1999; Riou-Khamlichi *et al.*, 2000), or by applying reversible blocks at different stages of the cell cycle using specific inhibitors (Gould, 1984; Nagata *et al.*, 1992; Magyar *et al.*, 1993; Perennes *et al.*, 1993; Fukuda *et al.*, 1994; Glab *et al.*,

1994; Lucretti and Dolezel, 1995; Ito *et al.*, 1997; Planchais *et al.*, 1997, 2000; Binarova *et al.*, 1998; Roudier *et al.*, 2000). However, in such plant cell suspensions, a synchronous population rarely reaches a mitotic index >10%. The notable exception is the tobacco BY-2 cell line, which is widely used in cell cycle studies (Nagata *et al.*, 1992; Samuels *et al.*, 1998).

The TBY-2 cell line was established from callus induced from seedlings of *Nicotina tabacum* L. cv. Bright Yellow 2 in the Central Research Institute of the Japan Tobacco and Salt Public Corporation (now the Tobacco Science Research Laboratory, Japan Tobacco, Inc.) (Kato *et al.*, 1972). It is propagated in the medium of Linsmaier and Skoog (1964) supplemented with sucrose and 2, 4-dichlorophenoxyacetic acid (2, 4-D). According to Kato *et al.* (1972) the TBY-2 cell line was the most proliferative among the lines examined created from 40 species of *Nicotina* and three species of *Populus*, which suggests that this cultivar of tobacco had unique characteristics. Also, unlike unstable cultures that exhibit mixoploid cells, the TBY-2 line is remarkably stable.

TBY-2 cells exhibit rapid growth in one week of batch culture at 27°C (Fig. 1.9) and can be easily synchronised using the reversible DNA polymerase α inhibitor, aphidicolin (Sala *et al.*, 1980). Following the synchrony with aphidicolin, TBY-2 cells are the only plant cell line that can attain mitotic indices of 40 to 50% (% frequency of cells in division) (Sorrell *et al.*, 2001).

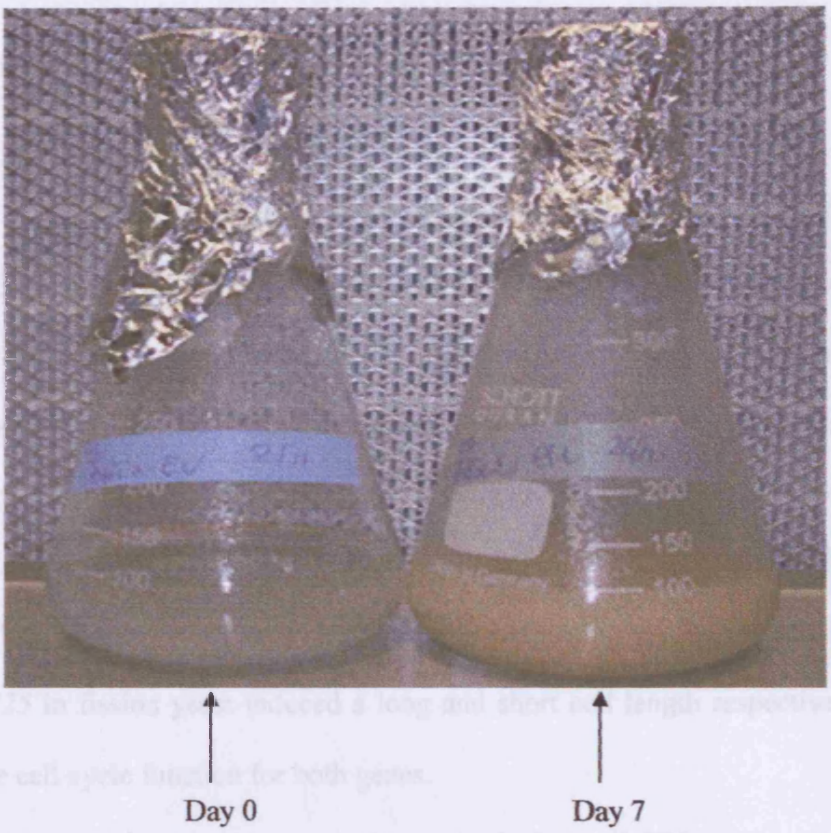


Fig. 1.9: TBY-2 batch cultures at day 0 and day 7

A wide variety of cell cycle processes, including phase specific gene expression (Reichheld *et al.*, 1995; Combettes *et al.*, 1999; Sorrell *et al.*, 1999), CDK activity (Porceddu *et al.*, 2001; Sorrell *et al.*, 2001) and microtubule rearrangements (Hasezawa and Nagata, 1991) have been studied using this model cell line.

1.7. Aims

In reviewing the plant cell cycle, much is now known about plant CDKs and plant cyclins. However, until fairly recently very little was known about the phosphoregulation of CDK complexes at G2/M transition. The first report of a partial plant *WEE1* cDNA from *Zea mays* (*ZmWEE1*) appeared seven years ago (Sun *et al.*, 1999), and a full length clone of *Arabidopsis thaliana WEE1* (*AtWEE1*) was first presented by Sorrell *et al.* (2002). Moreover, the first report about a putative plant *CDC25* has only been published in the last 12 months (Sorrell *et al.*, 2005). Interestingly, expression of *AtWEE1* and *AtCDC25* in fission yeast induced a long and short cell length respectively. These data indicate cell cycle function for both genes.

My work began with a central hypothesis that *AtWEE1* regulates plant cell size. I transformed the TBY-2 cell line and *Arabidopsis* plants with *AtWEE1* which then enabled me to test this hypothesis by analyzing the effect of this *transgene* on the:

- TBY-2 cell cycle
- Mitotic cell area

As well as exploiting the benefit of TBY-2 cells for cell cycle work, this strategy also prevented gene silencing that might have occurred by over-expressing *AtWEE1* in *Arabidopsis* cells.

- I also cloned a portion of *Nicotoiana tabacum WEE1* (*NtWEE1*) thereby enabling an examination of the effects of *AtWEE1* expression on *NtWEE1* expression
- Tobacco CDKA and CDKB1 kinase activity were investigated in TBY-2 cells expressing *AtWEE1*

- Primary root growth and primordial formation were investigated in *Arabidopsis* plants over-expressing *AtWEE1*.

Chapter 2: Materials and Methods

2.1. BIN HYG TX and pTA7002 plasmids

2.1.1. Construction of plasmids

The *Arabidopsis thaliana WEE1* gene *(AtWEE1)* was used to make constructs for transformation into tobacco BY-2 cells. Two different constructs (BIN HYG TX-*AtWEE1* and pTA7002-*AtWEE1*) were made and used independently to transform tobacco cells, alongside the empty vectors (BIN HYG TX and pTA7002). BIN HYG TX is a constitutive system (Fig. 2.1), originally designed for use in a tetracycline inducible system (Gatz *et al.*, 1991, 1992; Böhner *et al.*, 1999), but in this work only the constitutive component was used. BIN HYG TX is composed of a promoter derived from the cauliflower mosaic virus 35S gene promoter (35S TX promoter), which is attenuated by approximately 20X. The *AtWEE1* open reading frame (*AtWEE1* ORF), is inserted between the *Sma*I and *Sal*I restriction sites, and its transcription is terminated by a terminator (ocs) (Table 2.1). The selectable marker for plant transformation is hygromycin (hpt) which has its own promoter (nos) and terminator (Ag7).

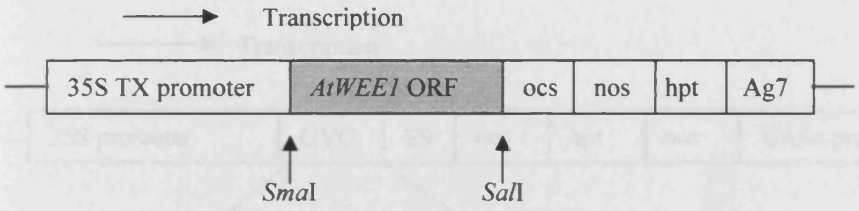


Fig. 2.1: Constitutive promoter BIN HYG TX-*WEE1*

Plasmid pTA7002 is based on an inducible system developed by Prof. N.H. Chua (Aoyama and Chua 1997). (Fig. 2.2). This system has been developed using the regulatory mechanism of vertebrate steroid hormone receptors. It is composed of a transcription factor (GVG), consisting of the DNA-binding domain of the yeast transcription factor (GAL4), the transactivating domain of the herpes viral protein (VP16) and the receptor domain of the rat glucocorticoid receptor (GR). GR is not only a receptor molecule but also a transcription factor which, in the presence of a glucocorticoid like dexametasone (DEX), activates transcription from promoters containing glucocorticoid response elements. The 35S promoter drives the *GVG* gene, which is terminated by E9. In the second transcription unit six copies of the GAL4 upstream activating sequence (GAL4 UAS) are fused to the promoter (UASc promoter) to control transcription of the target gene (*AtWEE1*) inserted between the *Xho*I and *Spe*I restriction sites. Transcription of *AtWEE1* is terminated by 3A. These two transcription units are separated by a selectable marker for plant transformation (hygromycin (hpt)) which has its own promoter (nos) and terminator (nos) (Table 2.1).

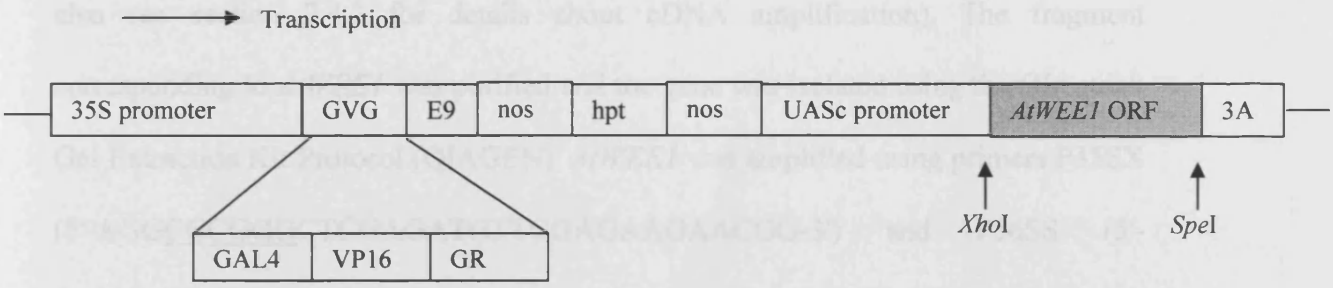


Fig. 2.2: Inducible promoter pTA7002-*WEE1*

Both plasmids BIN HYG TX and pTA7002 also contain a kanamycin resistance gene active in bacteria (Table 2.1).

Table 2.1: Details of the transgene vector and antibiotic selection

Vector	**Transgene**	**Antibiotic selection: *Agrobacterium tumefaciens* and *Escherichia coli***	**Antibiotic selection: Tobacco TBY-2**	**Notes on Vector**
BIN HYG TX-*AtWEE1*	*AtWEE1*	Kanamycin	Hygromycin	It has an attenuated form of the standard CaMV35S promoter
BIN HYG TX	none	Kanamycin	Hygromycin	It has an attenuated form of the standard CaMV35S promoter
pTA7002-*AtWEE1*	*AtWEE1*	Kanamycin	Hygromycin	CaMV35S
pTA7002	none	Kanamycin	Hygromycin	CaMV35S

To construct BIN HYG TX-*AtWEE1*, the *AtWEE1* gene was amplified from *Arabidopsis thaliana* cDNA by PCR (see section 2.4.1, 2.4.2 and 2.4.3 for details

about RNA extraction, DNase treatment of cDNA and cDNA synthesis respectively; also see section 2.4.3 for details about cDNA amplification). The fragment corresponding to *AtWEE1* was purified and the gene was isolated using the QIAquick Gel Extraction Kit Protocol (QIAGEN). *AtWEE1* was amplified using primers P35SX (5'-AGGCCCGGGCTCGAGATGTTCGAGAAGAACGG-3') and P36SS (5'-GCACACTAGTCGACTCAACCTCGAATCCTAT-3'), that included *Sma*I and *Sal*I sites respectively. PCR reactions were made as follows: 1 µl of cDNA was amplified with 0.625 U of Qiagen Taq polymerase, Qiagen buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP and 100 ng of each of the primers. Thermocycle conditions were: 15 min 95°C hot start followed by 35 cycles of 95°C (1 min), 60°C (1 min) and 72°C (1 min). The PCR machine used was an MJ Research PTC-100™.

The *AtWEE1* (2 µg) PCR product was then digested with *Sma*I and *Sal*I restriction enzymes (Promega) using Buffer D (10%) (Promega) in a total volume of 50 µl for 2 hours at 37°C. Digests were purified using the QIAquick Purification Kit Protocol (QIAGEN).

BIN HYG TX vector was extracted from *E. coli* cells (see paragraph 2.2.1 for details about vector extraction) and 2 µg were restriction digested in two separate reactions using either *Sma*I or *Sal*I restriction enzymes (Promega) to ensure that both enzymes were able to cut the vector. The reactions were performed in a total volume of 50 µl using MCx10 (10%) (Promega) and incubated at 37°C for 2 hours. Digests were restricted with the second enzyme *Sma*I or *Sal*I respectively and then purified using the QIAquick Purification Kit Protocol (QIAGEN).

The restricted vector (100 ng) was then ligated (using T4 DNA ligase enzyme (Promega)) to the PCR-amplified and restricted *AtWEE1* (40 ng). The reaction was

performed in a total volume of 10 µl using ligase buffer (10%) and incubated at 4°C over-night.

To construct pTA7002-*AtWEE1*, the vector was extracted from *E. coli* cells (see paragraph 2.2.1 for details about vector extraction), cut with *Xho*I and *Spe*I restriction enzymes and ligated to the PCR-amplified ORF of *AtWEE1* (see above for details). The same primers were used to amplify the *AtWEE1* ORF, P35SX (5'-AGGCCCGGGCTCGAGATGTTCGAGAAGAACGG-3') and P36SS (5'-GCACACTAGTCGACTCAACCTCGAATCCTAT-3'), which included *Xho*I and *Spe*I restriction sites respectively.

2.2. Transformation into bacterial cells and tobacco cells

2.2.1. *Escherichia coli* DH5α transformation

Plasmids BIN HYG TX-*AtWEE1*, BIN HYG TX-empty vector, pTA7002-*AtWEE1* and pTA7002-empty vector were used to transform *Escherichia coli* DH5α competent cells.

E. coli DH5α cells were made competent by growing them into 5 ml of 2xYT-medium (per litre: 16 g bacto-tryptone; 10 g bacto-yeast extract; 5 g NaCl; pH=7) over-night. Four ml of the over-night culture were inoculated in 1 l of 2xYT and grown to OD_{600}=0.5, cells were then centrifuged at 6000 g for 15 min at 1°C (Beckman Coulter J-E centrifuge, rotor JA-14) and resuspend in 10 ml of cold 0.1 M $CaCl_2$ plus 2.5 ml of 80% glycerol. Cells were then aliquoted into microfuge tubes, incubated on ice for 30 min and stored at -80°C.

One hundred µl of *E. coli* DH5α competent cells were added to 2 µl of the ligation mixture in a 14 ml sterile tube and left on ice for 20 min, then heat shocked (42°C for

45 seconds) and returned to ice for a further 2 min. Subsequently, the cells were added to 900 µl of LB medium (per litre: 10 g bacto-tryptone; 5 g bacto-yeast extract; 10 g NaCl; pH=7) and incubated at 37°C gently shaking for 1 hour, 200 µl of cells were spread onto Petri dishes containing LB-medium (0.8%) agar plus kanamycin (final concentration 50 µg/ml) and incubated at 37°C over-night.

To extract plasmids from clones, the cells were inoculated in 10 ml LB with kanamycin (final concentration 50 µg/ml), and incubated over-night at 37°C with shaking at 200 rpm. Bacterial cells were harvested by centrifugation at 6000 g for 15 min at 4°C (Beckman Coulter J-E centrifuge, rotor JA-14). DNA was extracted using the Qiagen midiprep kit (QIAGEN). This kit is designed for preparation of up 100 µg of high- or low-copy plasmid DNA. The kit provides lysis buffers to disrupt the bacterial cell wall and membrane and ribonuclease (RNase A) to remove the RNA molecules. Cell debris and proteins are separated from the plasmid DNA by centrifugation. The supernatant containing the plasmid DNA is applied to the QIAGEN-tip 100 column. This column contains a resin able to bind the plasmid DNA, but not other elements that contaminate the DNA, which are washed through. Elution DNA buffer is then applied to the column to release the plasmid DNA from the resin. The DNA is then precipitated by adding isopropanol to the eluted DNA and centrifuged. The pellet is washed with 70% ethanol and centrifuged. Ethanol is then removed and the pellet air-dried for 30 min, and redissolved in 50 µl Buffer EB (10 mM Tris-Cl, pH=8.5).

2.2.2. *Agrobacterium tumefaciens* LBA4404 transformation

One μg of plasmid DNA was added to 100 μl of competent *Agrobacterium tumefaciens* LBA4404 cells (kindly provided by Dr. David Sorrell) and frozen in liquid nitrogen for 10 sec before being thawed for 5 min at 37°C and added to 1 ml of 2xYT-medium. The cells were incubated for 4 h at 30°C with shaking, centrifuged at 1000 rpm for 1 min in a microcentrifuge (Eppendorf MiniSpin) and resuspended in 100 μl of 2xYT. One hundred μl of cells were plated on a Petri dish containing 2xYT-media and kanamycin (final concentration 50 μg/ml). Transformed *Agrobacterium tumefaciens* LBA4404 cells grew in 3-4 days at 30°C.

2.2.3. Transformation of tobacco BY-2 cells with *AtWEE1*

Stable transformation of TBY-2 cells was achieved using a modified version of the method described by An (1985). Isolated colonies of *Agrobacterium tumefaciens* LBA4404 containing either BIN HYG TX-*AtWEE1*, BIN HYG TX-empty vector, pTA7002-*AtWEE1* or pTA7002-empty vector were picked from fresh 2xYT-kanamycin (50 μg/ml) (Table 2.1) plates and cultured over-night in 7 ml 2xYT, without antibiotic, in 50 ml conical flasks at 30°C with shaking. Four ml aliquots of 6 day old stationary phase TBY-2 cells containing 20 μM of freshly added Acetosyringon (Sigma-Aldrich) were co-cultivated with 100 μl of *Agrobacterium* culture in 90 mm Petri dishes sealed with Nescofilm, for two days at 27°C in the dark without shaking. Cells were washed with 1 litre of BY-2 medium (per litre: 4.3 g MS basal salt medium; 30 g sucrose; 10 ml of 1% myo-inositol, 10 ml of 2% KH_2PO_4; 1 ml of 0.1 % thiamine HCl; 2 ml of 0.01 % of 2.4-D; pH=5.8)

using a cell dissociation sieve fitted with a 100 μm mesh (Sigma-Aldrich) and resuspended in 5 ml BY-2 medium containing 250 μg/ml Timenten (Melford Laboratories), 2.5 ml aliquots were then plated onto solidified BY-2 medium (0.8% agar) supplemented with 250 μg/ml Timenten and 80 μg/ml hygromycin (Table 2.1). Plates were sealed with micropore tape and incubated at 27°C in the dark. Hygromycin resistant calli were isolated after 2-4 weeks. Each individual callus was considered as an independent clone, and grown for a further two weeks on fresh plates. Calli were then transferred to 95 ml BY-2 medium supplemented with 250 μg/ml Timenten and 80 μg/ml hygromycin and incubated at 27°C and 130 rpm in the dark until the cultures reached stationary phase (3-5 weeks). Cultures were subsequently maintained as described below section 2.3.1. Cultures were subjected to at least four rounds of subculturing before being used in synchrony experiments.

2.3. Tobacco BY-2 cells synchronization, mitotic index and cell area measurements

2.3.1. Tobacco BY-2 cell culture propagation

The tobacco BY-2 cell line (Nagata et al., 1992; Nagata and Kumagai, 1999) is the most highly synchronizable plant cell system known and is thus ideal for studies of the plant cell cycle. The tobacco BY-2 cell line is cultured at 27°C in darkness in modified Linsmaier and Skoog (1964) medium and subcultured at 7 day intervals by transferring 3 ml of the 7-d-old culture to 95 ml of a new modified Linsmaier and Skoog medium (Fig. 2.3).

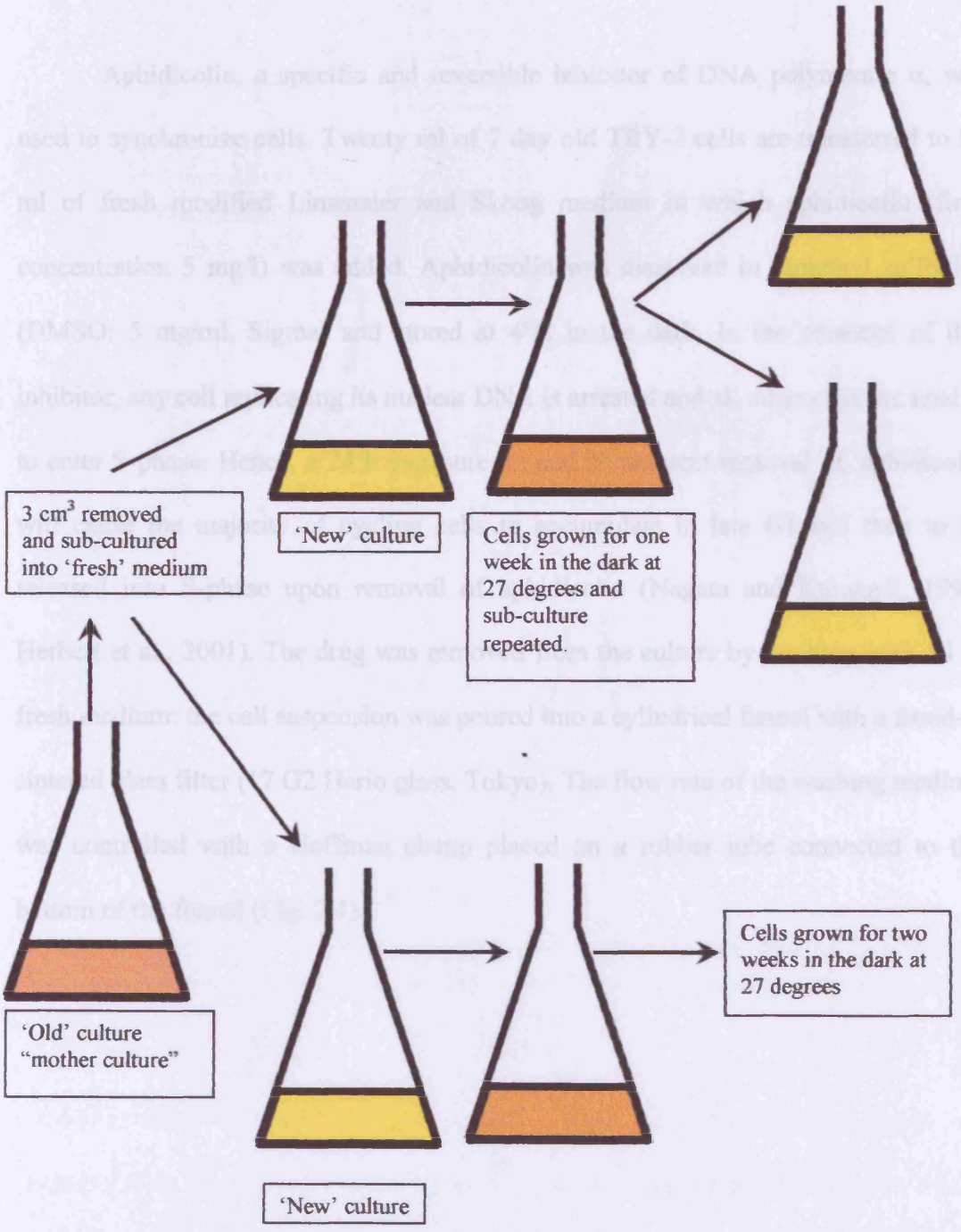


Fig. 2.3: The tobacco BY-2 batch culture method. From each mother culture two new sub-cultures are made every week. After 7 days of growth one culture is used to make a new subculture, whereas the other culture is kept for one more week to be used in case of contamination of the new sub-culture.

2.3.2. Synchronization of tobacco BY-2 cells

Aphidicolin, a specific and reversible inhibitor of DNA polymerase α, was used to synchronize cells. Twenty ml of 7 day old TBY-2 cells are transferred to 95 ml of fresh modified Linsmaier and Skoog medium in which aphidicolin (final concentration 5 mg/l) was added. Aphidicolin was dissolved in dimethyl sulfoxide (DMSO; 5 mg/ml, Sigma) and stored at 4°C in the dark. In the presence of this inhibitor, any cell replicating its nuclear DNA is arrested and all other cells are unable to enter S phase. Hence, a 24 h exposure to, and subsequent removal of, aphidicolin will cause the majority of cycling cells to accumulate in late G1 and then to be released into S-phase upon removal of aphidicolin (Nagata and Kumagai, 1999; Herbert et al., 2001). The drug was removed from the culture by washing with 1 l of fresh medium: the cell suspension was poured into a cylindrical funnel with a fused-in sintered glass filter (17 G2 Hario glass, Tokyo). The flow rate of the washing medium was controlled with a Hoffman clamp placed on a rubber tube connected to the bottom of the funnel (Fig. 2.4).

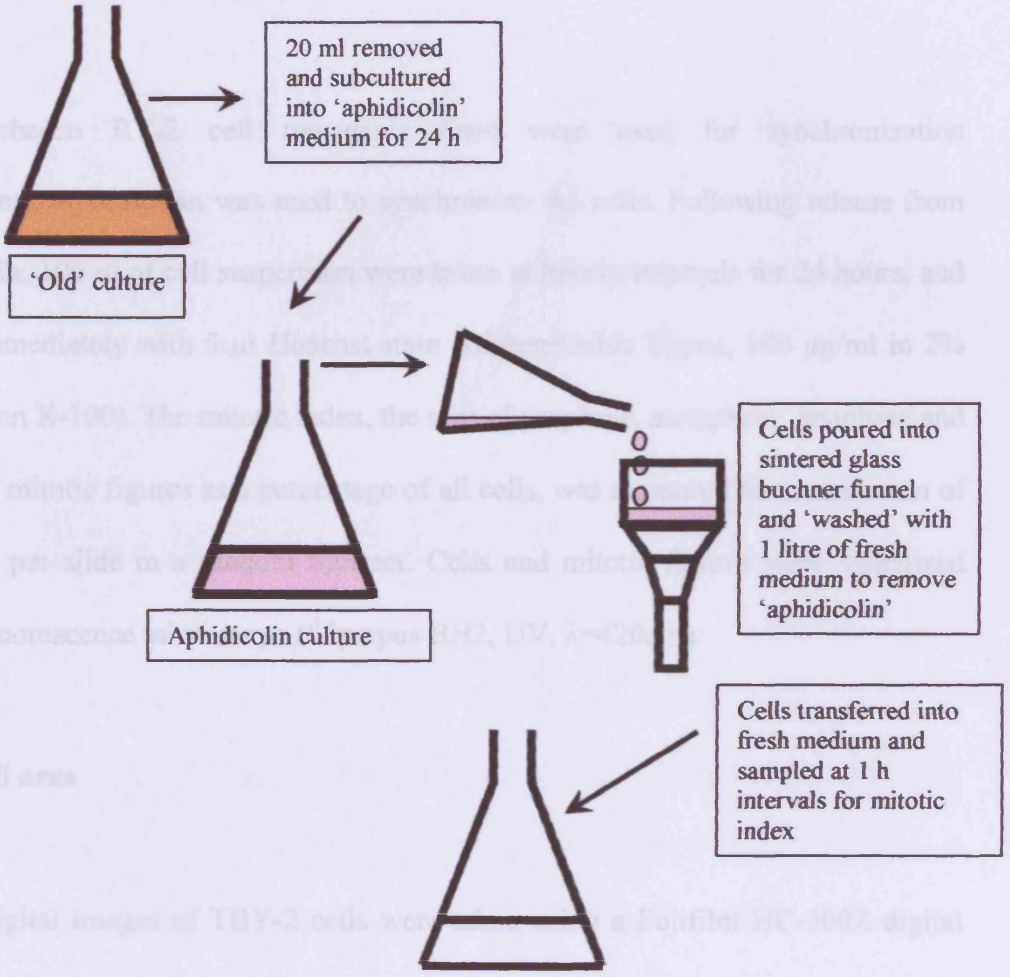


Fig. 2.4 TBY-2 cells can be synchronised in mitosis, which allows for easy study of the plant cell cycle, using the reversible DNA polymerase inhibitor aphidicolin which blocks the cells in S-phase of the mitotic cell cycle.

A total washing time of 15 minutes was optimal for obtaining high levels of synchrony, and the cells were subsequently resuspended in the same volume of fresh medium.

2.3.3. Mitotic index measurements

Tobacco BY-2 cell transgenic lines were used for synchronization experiments. Aphidicolin was used to synchronize the cells. Following release from aphidicolin, 100 µl of cell suspension were taken at hourly intervals for 24 hours, and mixed immediately with 5 µl Hoechst stain (Bisbenzimide Sigma, 100 µg/ml in 2% (v/v) Triton X-100). The mitotic index, the sum of prophase, metaphase, anaphase and telophase mitotic figures as a percentage of all cells, was measured for a minimum of 300 cells per slide in a random transect. Cells and mitotic figures were visualized using a fluorescence microscope (Olympus BH2, UV, λ=420nm).

2.3.4. Cell area

Digital images of TBY-2 cells were taken using a Fujifilm HC-300Z digital camera attachment for the microscope and saved as JPEGs. Digital images of mitotic cells, captured as described above, were measured using SigmaScan® (Sigma), an image analysis program. To take a mitotic cell area measurement, a line was traced around the perimeter of cells undergoing mitosis. To ensure measurements were accurate, a graticule slide was used as a distance reference point from which SigmaScan® could calculate the area of a cell. For each experiment undertaken in this project, mitotic cell area was measured. On average, 150 measurements were made per experiment and the data obtained was stored in Microsoft® Excel.

2.3.5. Growth rate measurement

Tobacco BY-2 cell density was measured for a period of 7 days (from the day at which the new subculture started to the day at which cells reached stationary phase) using a spectrophotometer λ=550nm (Smartspec™3000, Bio-Rad laboratories Ltd). Where absorbance readings were in excess of 0.8, samples were diluted to ensure an accurate measurement.

2.3.6. Tobacco BY-2 cells containing pTA7002

Dexamethasone (final concentration 1 μM) was added three days after subculturing to the TBY-2 cells transformed with pTA7002-*WEE1* vector. Every 12 h, 100 μl of cell suspension were taken and mixed immediately with 5 μl Hoechst stain (Bisbenziamide Sigma, 100 μg cm^{3-} in 2% (v/v) Triton X-100). Samples were collected for a 48 h period and the images were captured and measured using SigmaScan.

2.4. RT-PCR and semi quantitative RT-PCR

2.4.1. RNA extraction

Tobacco BY-2 cells were harvested by centrifugation at 3500 rpm (MSE Centaur 2) for 10 minutes; the pellet was then collected in aluminum foil and frozen in liquid nitrogen, cells were stored at -80°C until required. The frozen cells were ground to a fine powder using liquid nitrogen and a pestle and mortar precooled to -20°C. TRI reagent (2 ml) (Sigma-Aldrich, Gillingam, UK) was added to TBY-2 cells

and ground until a homogenous paste was formed. Equal amounts of paste were transferred to two 1.5 ml microcentrifuge tubes, vortexed and left at room temperature for 5 minutes. Samples were subsequently centrifuged at 12000 rpm (Beckman Coulter Allegra™ 21R, rotor F2402H) at 4°C for 10 minutes. The supernatant was transferred to two new microcentrifuge tubes leaving the solid plant material behind. Two hundred µl of chloroform were added to each tube, vortexed for 15 seconds and left at room temperature for 5 minutes. Samples were then centrifuged at 12000 rpm (Beckman Coulter Allegra™ 21R, rotor F2402H) at 4°C for 15 minutes. The top layer containing RNA was transferred to new 1.5 ml microcentrifuge tubes and 0.5 ml of isopropanol were added. The samples were mixed and left at room temperature for 15 minutes and then centrifuged at 12000 rpm (Beckman Coulter Allegra™ 21R, rotor F2402H) at 4°C for 10 minutes. The supernatant was removed and 1 ml of ethanol was added to wash the pellet. The samples were vortexed for 15 seconds and centrifuged at 12000 rpm (Beckman Coulter Allegra™ 21R, rotor F2402H) at 4°C for 1 minute. The supernatant was carefully removed and the pellet air dried for 30 minutes. The pellet was resuspended in 50 µl UHP H_2O. Samples were stored at -80°C.

2.4.2. DNase treatment of cDNA

Residual genomic DNA was removed from total RNA extracted, by adding 10 µl 10x DNase buffer and 3 µl DNase (Ambion Inc, Austin, USA). Preparations were incubated at 37°C for 1 hour. DNase deactivation reagent (0.2 ml) was added and samples incubated at room temperature for 2 minutes before centrifugation at 13000 rpm (Eppendorf® MiniSpin) for 1 minute. The supernatant was transferred to a new microcentrifuge tube and stored at -80°C.

Quantity and quality of RNA was estimated by running on an agarose gel. Five µl of extracted RNA were mixed with 1 µl of loading buffer (150 mM Tris HCl pH=7.6; 50% glycerol; 0.4% bromophenol blue and dH_2O) and run on an electrophoresis gel (1% agarose, 1 µg/ml EtBr) in TAE buffer (1 l of 50xTAE buffer contains: 242 g of Tris base; 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA). To minimize RNA degradation the gel tank, tray and comb were treated with 0.1 M NaOH and then washed copiously with distilled water. The RNA was visualized under UV light and photographed using a Gene Genius Bioimagining System and Gene Snap® software package (Syngene). RNA concentration was measured using a Gene Quant (Pharmacia) spectrophotometer.

2.4.3. cDNA synthesis

To ensure that comparison between samples could occur, equal amounts of RNA from each sample were reverse transcribed. RNA (5 µg) in a 0.5 ml microcentrifuge tube was made up to 20 µl with sterile dH_2O. One µl of oligo (dt)-15 (50 µg/ml; Promega) was added and samples incubated at 70°C for 10 minutes. Samples were then cooled at 4°C for 10 minutes. Six µl 5x 1st stand buffer (Invitrogen), 2 µl 0.1M DTT (dithiothreitol), and 1 µl 10 mM dNTPs were added and incubated at 42°C for 2 minutes. Superscript II reverse transcriptase (1 µl) (GibcoBRL, Paisley, UK) was added and the samples were incubated for 50 minutes at 42°C. The reactions were heated at 70°C for 15 minutes to inactivate the reverse transcriptase and stored at -20°C. Mock reactions, important to ensure no genomic DNA contamination, were also carried out following the above procedure, but Superscript II reverse transcriptase was not added.

2.4.4. PCR amplification of cDNA

cDNA was subjected to PCR (Polymerase Chain Reaction) amplification using the Reddymix PCR master Mix system (ABgene). This contains all reagents necessary for DNA amplification including dNTPs (0.2 mM); thermoprime plus DNA polymerase (1.25 u); $MgCl_2$ (1.5 mM) and loading buffer for gel electrophoresis. For a total volume of 25 µl, 22 µl of Reddymix, 0.5 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 1 µl of sterile distilled water and 1 µl of DNA was used. Where possible a master mix was used to reduce pipetting error and a negative control (sterile dH_2O substitute to cDNA) was always used to ensure no water contamination, also a positive control was used when available.

Table 2.2 shows the primers used for each gene of interest and the annealing temperatures used. Thermocycle conditions were: 35 cycles of 95°C (1 min), annealing temperature (1 min) and 72°C (1 min) in a PTC100 thermocycler. PCR products were run out on an agarose gel (1 %) with EtBr (1 µg/ml) and visualized under UV light.

2.4.5. Design of degenerate and non-degenerate primers

Available amino acid sequences of homologous *WEE1* plant genes from *Zea mays* (*ZmWEE1*) and *Arabidopsis thaliana* (*AtWEE1*) were aligned and highly conserved regions of at least 6 amino acids were found. Selected from these conserved regions were those with at least the last two amino acids at the 3' end conserved across all genes and coded by as few codons as possible, for example methionine which is encoded by only one codon (best 3' amino acids are M and W,

worst are R, L and S). If there were discrepancies between homologues, degeneracies (wobbles) were introduced but sequences with as few wobbles as possible were selected. Regions that required wobbles at the 3' end were avoided. Melting temperatures (Tm) of the selected regions were calculated using

$$Tm = 2°C \times (A+T) + 4°C \times (C+G)$$

and equalized by the deletion or addition of extra bases if necessary.

Degenerate primers were used to isolate a portion of *Nicotiana tabacum WEE1* (*NtWEE1*). The *NtWEE1* gene fragment was then sequenced and specific (non-degenerate) primers were designed on the basis of its sequence (Table 2.2).

Table 2.2: Primer pairs used for DNA amplification (PCR)

Primer pair	Target gene	Oligonucleotide sequence	Product size (bp)	Tm (°C)
AtWEE1F AtWEE1R	*AtWEE1*	5'-AGCTTGTCAGCTTTGCCT 5'-CGTGCATCCCTCCTTCTTCTACT	229	55
WEE1F WEE1R	*NtWEE1* (degenerate primers used to isolate a portion of the *WEE1* gene from tobacco)	5'-TCKTGGTTYGARAAYGARCA 5'-AGAGAAGATRTCNACYTTRTC	339	60
WEE1TSF WEE1TSR	*NtWEE1* (used to amplify *N. tabacum WEE1*)	5'-GTGACCACAGCTTATCCAAC 5'-GCATCCCCCTCTTCGATC	228	60

2.4.6. Estimation of optimum PCR cycles for semi-quantitative RT-PCR

The optimum number of PCR cycles for semi-quantitative PCR was determined for each of the four specific primer pairs by carrying out PCRs on a set of standards over a range of cycles (Table 2.3). The standards were produced from dilutions of a mixed cDNA made using 1 µl of each cDNA sample. The products were run on electrophoresis gels, stained with ethidium bromide and the intensity of each band measured on a Gene Genius Bioimaging System (Syngene Ltd.).

An appropriate number of PCR cycles, which gave a steep linear plot of band strength against concentration, with an intercept close to zero, was selected for each primer pair for use in semi-quantitative work.

2.4.7. Semi-quantitative RT-PCR.

Samples of cells (5 ml) were taken during synchronization experiments every hour after removal of aphidicolin, and immediately frozen at -80°C. At the same time, measurement of the mitotic index was carried out. RNA was extracted from each sample and used to synthesize cDNA.

PCRs were carried out on the cDNA samples prepared using each of the specific primer pairs at the optimum cycle number as described in section 2.4.4 except that cycles were limited as shown in table 2.3. All PCRs were repeated at least three times to provide replicate results. A set of the standards were included in each PCR run. PCR products were run on ethidium bromide (1 µg/ml) stained agarose (1 %) gels and the band strengths measured using a Gene Genius Bioimaging System and Gene Tools software package (Syngene Ltd.).

The measurement of gel band strength, for all genes of interest, was normalized by dividing the EtBr fluorescence obtained from primers to the gene of interest by the EtBr fluorescence obtained from 18S ribosomal primer set (PUV2 and PUV4).

Table 2.3: Primer details for Gene Expression Analysis

Primer pair	Target gene	Oligonucleotide sequence	Product size (bp)	Tm (°C)	PCR cycles used
PUV2 PUV4	*18 ribosomal RNA*	5'-TTCCATGCTAATGTATTCAGAG 5'-ATGGTGGTGACGGGTGAC	488	60	36
H4F H4R	*Histone H4*	5'-GGCACAGGAAGGTTCTGAGGGA TAACA 5'-TAACCGCCGAAACCGTAGAGAG TCC	320	60	30
AtWEE1F AtWEE1R	*AtWEE1*	5'-AGCTTGTCAGCTTTGCCT 5'-CGTGCATCCCTCCTTCTTCTACT	229	55	30
WEE1TSF WEE1TSR	*NtWEE1*	5'-GTGACCACAGCTTATCCAAC 5'-GCATCCCCCTCTTCGATC	228	60	30

Chapter 3: Expression of *Arabidopsis thaliana WEE1 (AtWEE1)* in TBY-2 cells induces a shortened G2 phase, a premature entry into mitosis and a smaller mitotic cell area

3.1. Introduction

WEE1 kinases function in a highly conserved mechanism that controls the timing of entry into mitosis. Loss of Wee1 and Swe1 function causes fission yeast and budding yeast cells to enter mitosis before sufficient growth has occurred, leading to formation of daughter cells that are smaller than normal (Nurse, 1975; Harvey and Kellog, 2003). Conversely, when WEE1 is over expressed, mitosis is delayed until cells grow to a larger size (Russell and Nurse, 1987). Early work in fission yeast suggested that Wee1 kinase is part of a cell size checkpoint that prevents entry into mitosis before cells have reached a critical size (Nurse, 1975; Fantes and Nurse, 1978). In fission yeast cells, *wee1* is the main genetic element in cell size control (Nurse, 1975; Fantes and Nurse, 1978), Wee1 inhibits CDK1 (encoded by the *cdc2* gene) by phosphorylating a highly conserved tyrosine (Tyr 15) residue near to the N-terminus which results in an inhibition of mitosis (Russell and Nurse, 1987), whereas Cdc25 promotes mitosis by removing the inhibitory phosphate (Russell and Nurse, 1986).

In our lab, the fission yeast *Schizosaccharomyces pombe* was transformed with *AtWEE1* using an inducible promoter (pREP1-*AtWEE1*). Expression of *AtWEE1* in fission yeast cells induced a significant increase in cell length compared with wild type,

yeast cells transformed with uninduced pREP1-*AtWEE1* and pREP1-empty vector (Sorrell *et al.*, 2002). Sorrell and colleagues have also demonstrated that colony formation by cells containing induced pREP1-*AtWEE1* was substantially reduced compared to empty vector. The substantial reduction of colony formation suggested that *AtWEE1* expression was inhibiting cell division (Sorrell *et al.*, 2002). Different results came from the very recent expression of the putative mitotic inducer gene *AtCDC25* in fission yeast using an inducible promoter (pREP1-*AtCDC25*) (Sorrell *et al.*, 2005). Expression of *AtCDC25* in fission yeast induced a reduction in mitotic cell length compared with wild type, empty vector and uninduced pREP1-*AtCDC25* fission yeast cells (Sorrell *et al.*, 2005). The data reported by Sorrell and colleagues indicated that *AtCDC25* can function as a mitotic accelerator in fission yeast (Sorrell *et al.*, 2005). So, over expression of *SpWEE1* and *SpCDC25* in fission yeast resulted in a long and short cell length phenotype, respectively (Russell and Nurse, 1986, 1987), as did expression of *AtWEE1* and *AtCDC25* (Sorrell *et al.*, 2002, 2005).

One of the biggest problems in cell cycle research is how cell size is regulated at mitosis. In this chapter data are reported about tobacco BY-2 cell size at division using *AtWEE1* gene as a tool. To obviate the possibility of gene silencing, or mitotic catastrophe, *AtWEE1* was expressed in the TBY-2 cell line using an inducible system in addition to a constitutive system. The hypothesis was tested that *AtWEE1* induces large cell size in transformed TBY-2 cells.

3.2. Materials and methods

Experimental details are fully described in chapter 2 from section 2.1.1 to 2.4.3.

3.3. Results: Production of tobacco BY-2 cell lines transformed with *AtWEE1* constructs and empty vector controls

Tobacco BY-2 cells were transformed using two different plasmids: 1) a constitutive plasmid BIN HYG TX-*AtWEE1* and the respective empty vector (BIN HYG TX-EV) (see chapter 2 section 2.1.1 for more details) and 2) an inducible plasmid pTA7002-*AtWEE1* and the respective empty vector (pTA7002-EV) (see chapter 2 section 2.1.1 for more details). pTA7002-*AtWEE1* was induced by adding a synthetic glucocorticoid (dexamethasone).

All plasmids were cloned and selected in *Escherichia coli* DH5α (see chapter 2, section 2.2.1). For the constitutive plasmid, one clone (1S) was isolated and sequenced (Appendix I), and there were no base changes. For the inducible construct it was necessary to isolate and sequence three different clones 1X, 2X and 3X (Appendix I), to find a clone with perfect amino acid sequence (clone 2X). The plasmids were then extracted and used to transform *Agrobacterium tumefaciens* LBA4404 cells (see chapter 2, section 2.2.2). *Agrobacterium tumefaciens* was then used to transform the tobacco cells (see chapter 2, section 2.2.3.). For each transformation 12 different clones were isolated and allowed to grow over a period of 3-4 weeks after which just three clones per

transformation were chosen (Table 3.1). The clones used for the experiments were maintained in subculture as described in chapter 2 section 2.3.1.

Table 3.1: tobacco BY-2 cell lines transformed with *AtWEE1* using the constitutive (BIN HYG TX-*AtWEE1*) or the inducible (pTA7002-*AtWEE1*) promoter

Plasmids:	**Clones:**	**Code used in this thesis**
BIN HYG TX -*AtWEE1*	2	WEE1-c-2
	10	WEE1-c-10
	12	WEE1-c-12
BIN HYG TX-empty vector	10	EV-c-10
pTA7002-*AtWEE1*	1	WEE1-i-1
	3	WEE1-i-3
	6	WEE1-i-6
pTA 7002-empty vector	1	EV-i-1
	3	EV-i-3
	4	EV-i-4

Transformation of tobacco BY-2 cells was repeated after one and a half years because TBY-2 cells from the previous transformation began to die. Table 3.2 shows the clones obtained from the new transformation:

Table 3.2: tobacco BY-2 cell lines transformed with BIN HYG TX-*AtWEE1* and pTA7002-*AtWEE1* new transformation

Plasmids:	**Clones:**	**Code used in this manuscript**
BIN HYG TX -*AtWEE1*	1	WEE1-c-1+
	2	WEE1-c-2+
	3	WEE1-c-3+
BIN HYG TX-empty vector	1	EV-c-1+
	2	EV-c-2+
	3	EV-c-3+
pTA7002-*AtWEE1*	1	WEE1-i-1+
	2	WEE1-i-2+
	3	WEE1-i-3+
pTA 7002-empty vector	1	EV-i-1+
	2	EV-i-2+
	3	EV-i-3+

3.4. Results: Analysis of tobacco BY-2 cells transformed with BIN HYG TX-*AtWEE1*

The three independent cell lines carrying *AtWEE1* under the control of an attenuated version of the 35-S cauliflower mosaic virus (CaMV) promoter, BIN HYG TX (Gatz *et al.*, 1992) were investigated in relation to cell size and cell cycle. In conjunction with the *AtWEE1* expressing lines (denoted as shown in table 3.1) one empty vector line (EV-c-10) was used as experimental control.

Forward and reverse AtWEE1 primers were designed specifically to amplify *Arabidopsis thaliana WEE1* (see chapter 2 section 2.4.5 for more details), and were used to confirm *AtWEE1* expression in WEE1-c-2, WEE1-c-10 and WEE1-c-12 (Fig. 3.1 a). Wild type

TBY-2 cDNA was also used as negative control (Fig. 3.1 b), the quality of wild type cDNA was tested using primers PUV2 and PUV4 directed against *18S rRNA* (see chapter 2 section 2.4.5 for more details) (Fig. 3.1 c).

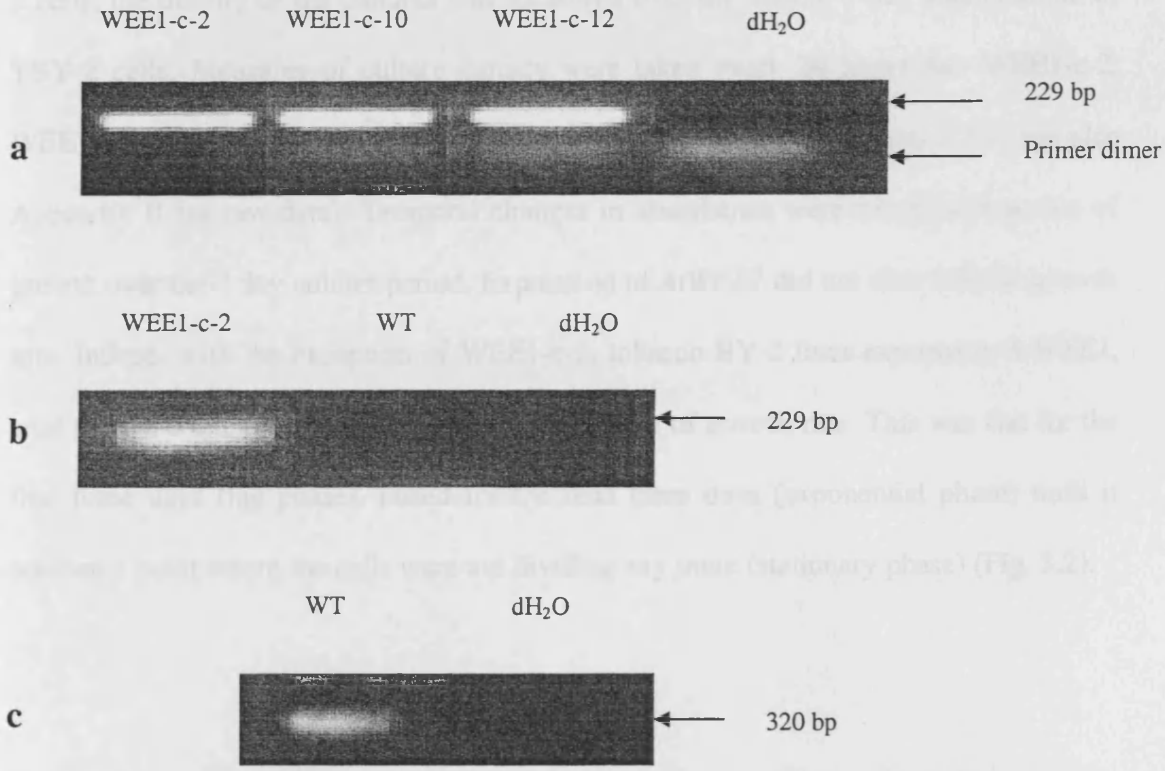


Fig. 3.1: RT-PCR analysis of *AtWEE1* expression in **a)** three independent tobacco BY-2 cell lines expressing *AtWEE1* constitutively (WEE1-c-2, WEE1-c-10 and WEE1-c-12) using AtWEE1 primers, distilled water was used as the control; **b)** wild type (WT) cDNA was also used as negative control with the AtWEE1 primers; **c)** the quality of wild type cDNA was tested using PUV2 and PUV4 primers.

3.4.1. Comparison of growth rate between tobacco BY-2 cell lines expressing *AtWEE1*, wild type and empty vector

To test whether expression of *AtWEE1* under the control of the constitutive promoter or the presence of the promoter itself perturbed the normal growth rate of TBY-2 cells, the density of the cultures was measured over the normal 7 day batch culture of TBY-2 cells. Measures of culture density were taken every 24 hours for: WEE1-c-2, WEE1-c-10, WEE1-c-12, EV-c-10 and wild type (see chapter 2 section 2.3.5; see also Appendix II for raw data). Temporal changes in absorbance were interpreted as rate of growth over the 7 day culture period. Expression of *AtWEE1* did not alter cellular growth rate. Indeed, with the exception of WEE1-c-2, tobacco BY-2 lines expressing *AtWEE1*, wild type and empty vector all had the same pattern of growth rate. This was flat for the first three days (lag phase), raised for the next three days (exponential phase) until it reached a point where the cells were not dividing any more (stationary phase) (Fig. 3.2).

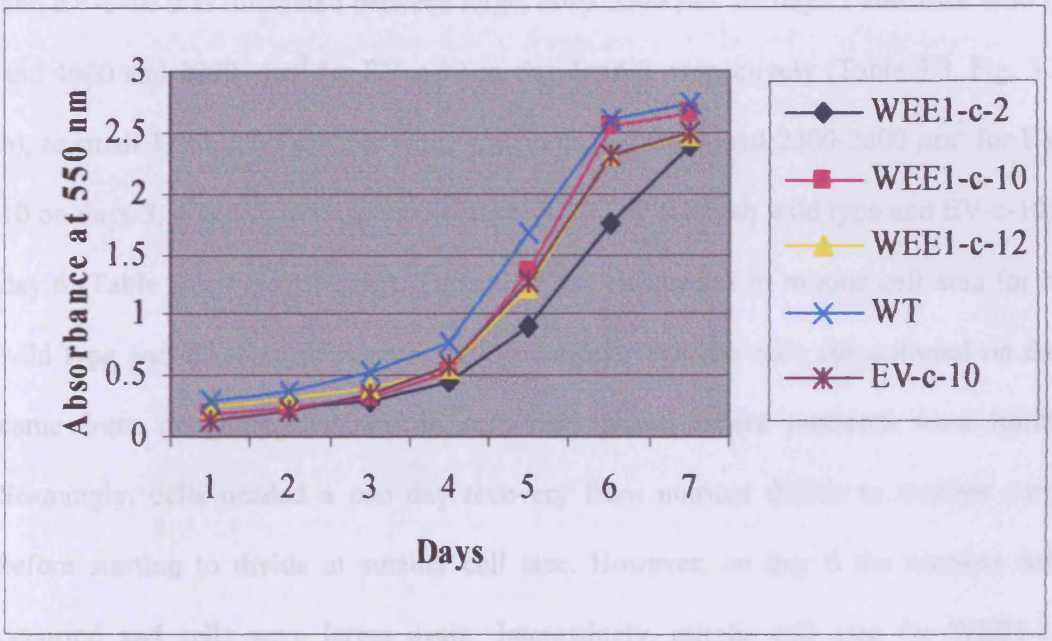


Fig. 3.2: Temporal changes in absorbance at 550 nm in WEE1-c-2, WEE1-c-10, WEE1-c-12, EV-c-10 and wild type.

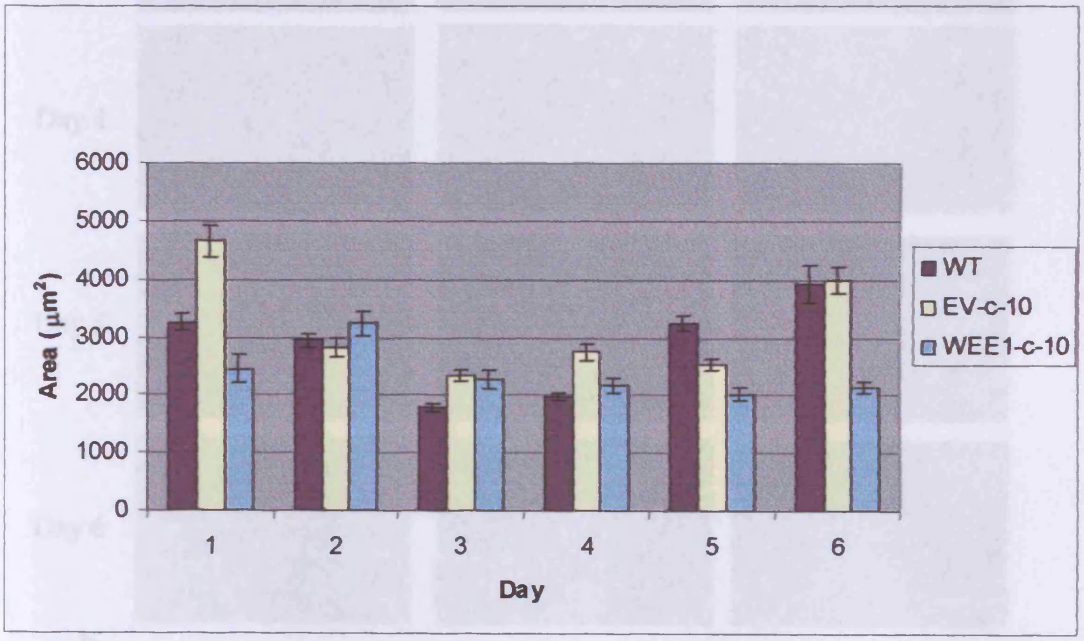
3.4.2. Mitotic cell area is smaller in TBY-2 cells expressing *AtWEE1* over a 6 day period

If AtWEE1 kinase is a negative regulator at the G2/M transition (see Introduction), then increased expression of *WEE1* in tobacco BY-2 cells should cause cells to divide at a large cell size. This hypothesis was investigated by measuring the mitotic cell area of WEE1-c-10, EV-c-10 and wild type cultures every day over a 6 day period (see Appendix II for raw data); on day 7 mitotic cells were not found. The mitotic WEE1-c-10 cells divided, except on day 2, at a constantly smaller cell size circa 2000-2500 μm^2 (Table 3.3, Fig. 3.3 a, b), compared with corresponding data for the wild type

and EV-c-10 that fluctuated between large, circa 3000 μm^2 on days 1 and 2 for wild type and 4600 and 2800 μm^2 for EV-c-10 on day 1and 2, respectively (Table 3.3, Fig. 3.3 a, b), to small 1700-2000 μm^2 for wild type on days 3 and 4 and 2300-2800 μm^2 for EV-c-10 on days 3, 4 and 5, to large again (circa 4000 μm^2 for both wild type and EV-c-10) on day 6 (Table 3.3, Fig. 3.3 a, b,). To explain the fluctuation in mitotic cell area for both wild type and EV-c-10, it is necessary to consider that the cells sub-cultured on day 1 came from a culture that was in stationary phase, where nutrients were limiting. Seemingly, cells needed a one day recovery from nutrient deficit to nutrient surplus before starting to divide at smaller cell size. However, on day 6 the nutrient deficit occurred and cells were larger again. Interestingly, mitotic cell area for WEE1-c-10 remained relatively constant from day 1 to day 6 so that significant differences were evident compared with wild type and EV-c-10. This result showed that either shortage of nutrients or expression of *AtWEE1* were not resulting in an increased mitotic cell area.

Table 3.3: The mean number and standard error (in parenthesis) of WT, EV-c-10 and WEE1-c-10 cell area over 6 days experiment. Levels of significance are indicated by Student's t-test.

day	WT	EV-c-10	WEE1-c-10	P (WT/WEE1-c-10)	P (EV-c-10/WEE1-c-10)
1	3254 (±153)	4658 (±277)	2442 (±234)	= 0.007	= 0.000
2	2948 (±115)	2809 (±162)	3228 (±204)	= 0.239	= 0.115
3	1777 (±60)	2345 (±97)	2267 (±153)	= 0.005	= 0.670
4	1971 (±64)	2747 (±155)	2179 (±130)	= 0.156	= 0.006
5	3244 (±120)	2532 (±91)	2020 (±110)	= 0.000	= 0.001
6	3922 (±314)	3991 (±225)	2150 (±91)	= 0.000	= 0.000



a

Fig. 3.3: a) Comparison between the average mitotic cell area of WEE1-c-10, wild type (WT) and EV-c-10 over a 6 day period (± SE) (n≥15).

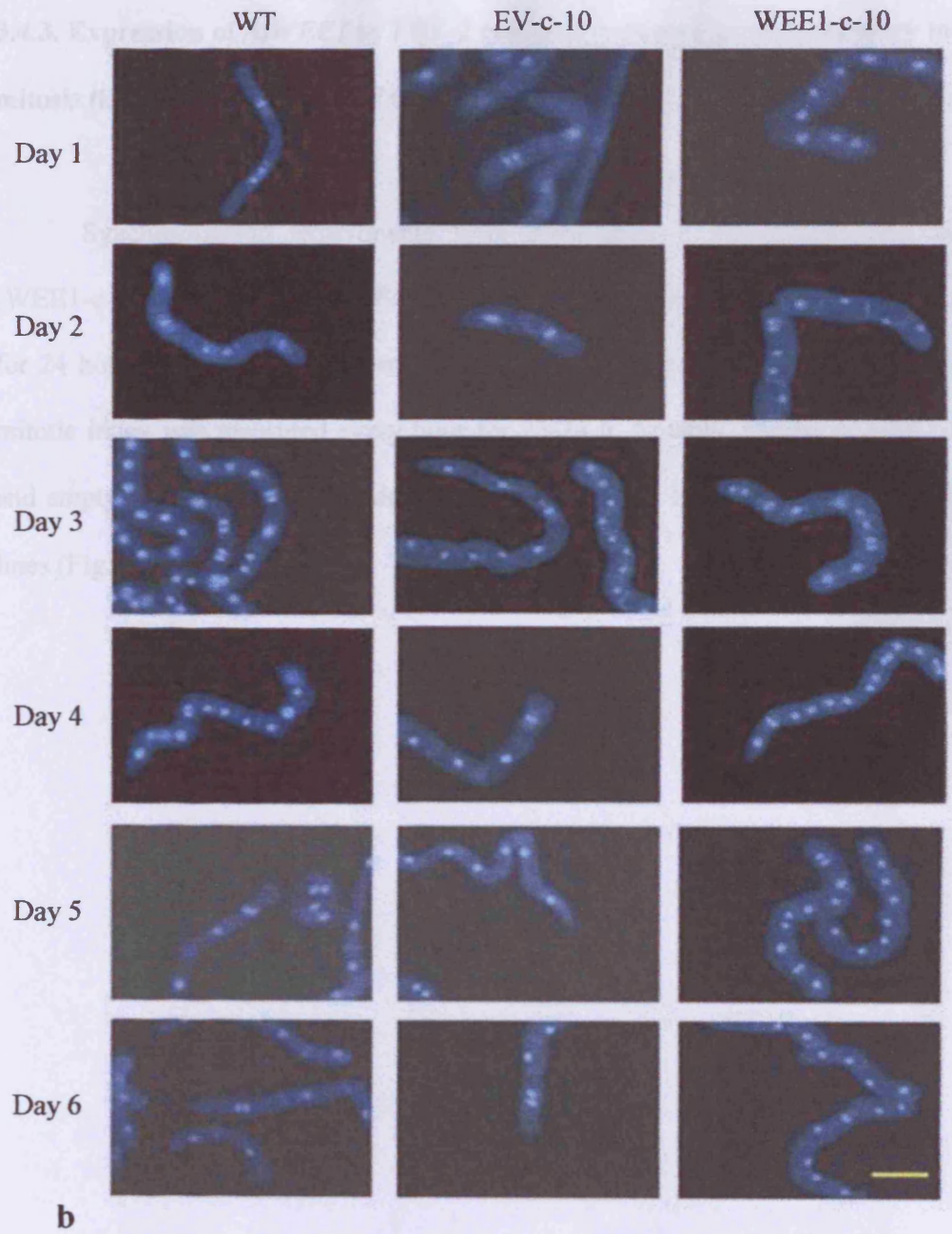
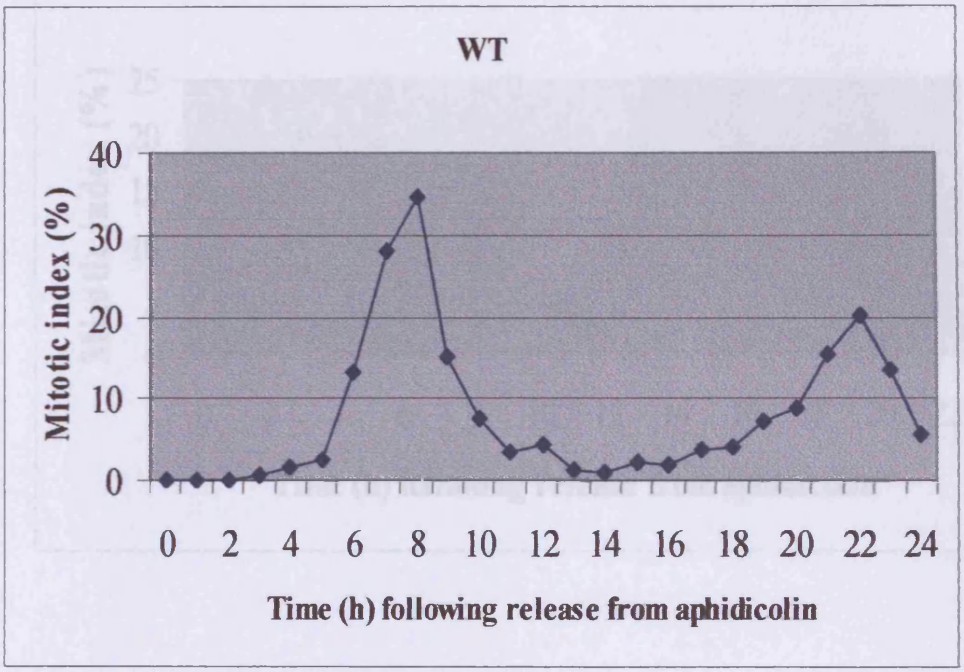


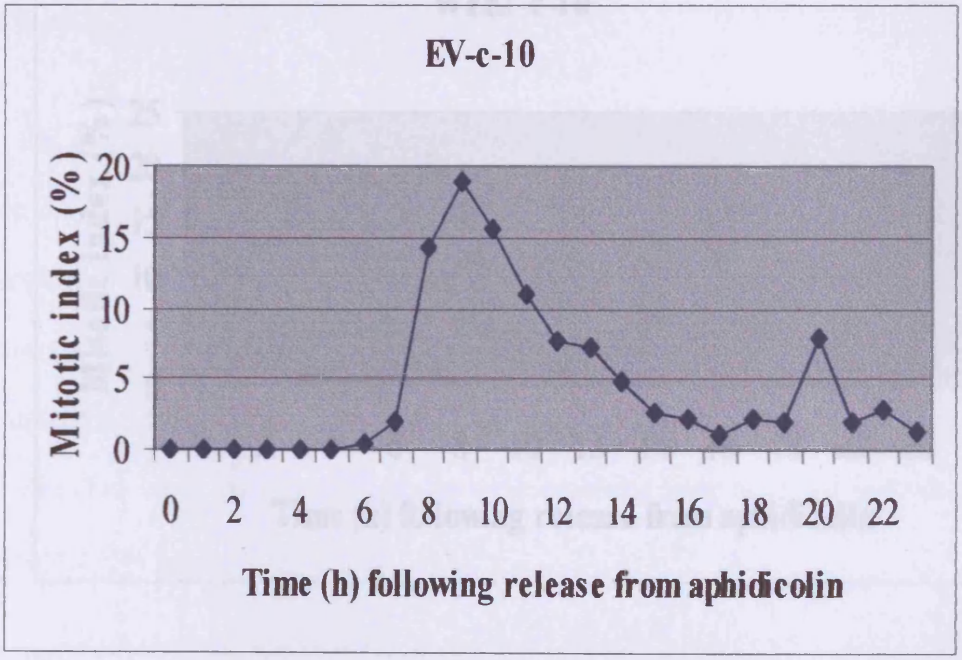
Fig. 3.3: b) Pictures of TBY-2 cells expressing *AtWEE1* compared with wild type and empty vector taken during the 6 day experiment (bars = 100 µm).

3.4.3. Expression of *AtWEE1* in TBY-2 cell lines induces a premature entry into mitosis through a shortening of G2 phase

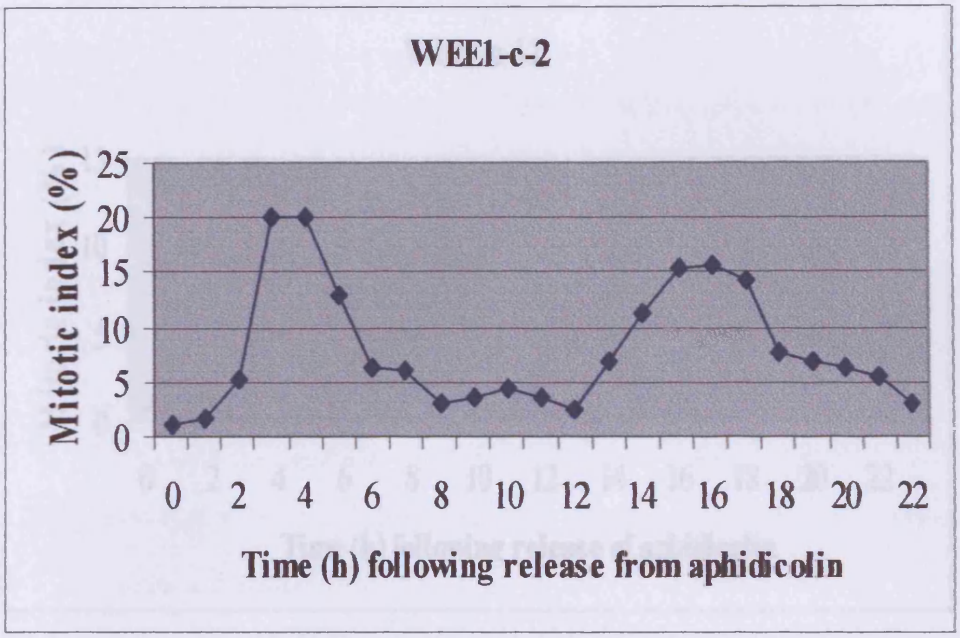
Synchronization experiments were conducted on all transformed cell lines (WEE1-c-2, WEE1-c-10 and WEE1-c-12). Following synchronization with aphidicolin for 24 hours, aphidicolin was removed (see chapter 2 sections 2.3.2 and 2.3.3) and the mitotic index was measured every hour for 23-24 h. Notably, compared with wild type and empty vector, the mitotic index began to rise sooner in the constitutive *AtWEE1* cell lines (Fig. 3.4; Appendix III).


WT
Mitotic index (%)
40
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Time (h) following release from aphidicolin

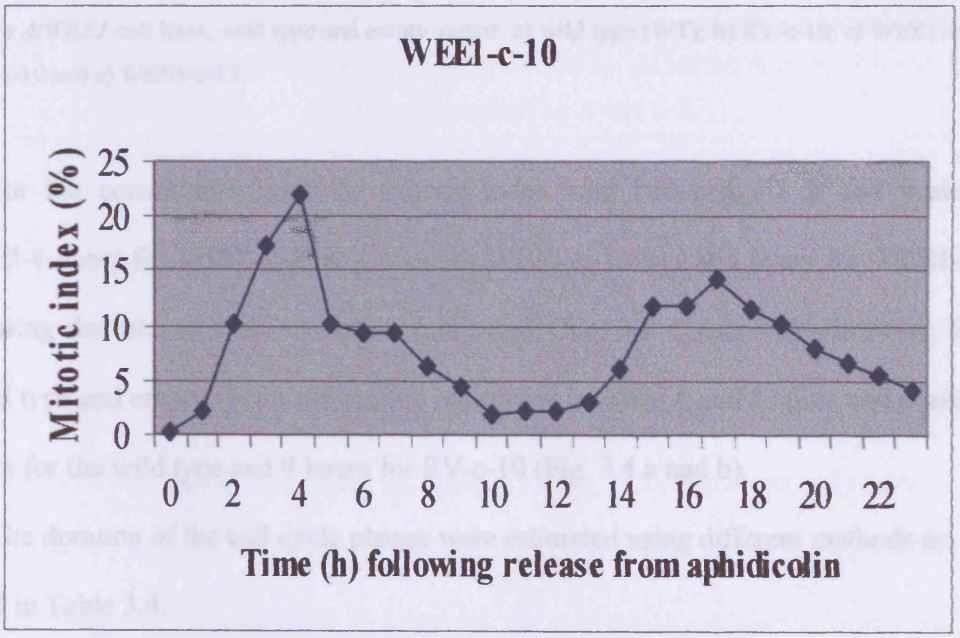
a


EV-c-10
Mitotic index (%)
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h) following release from aphidicolin

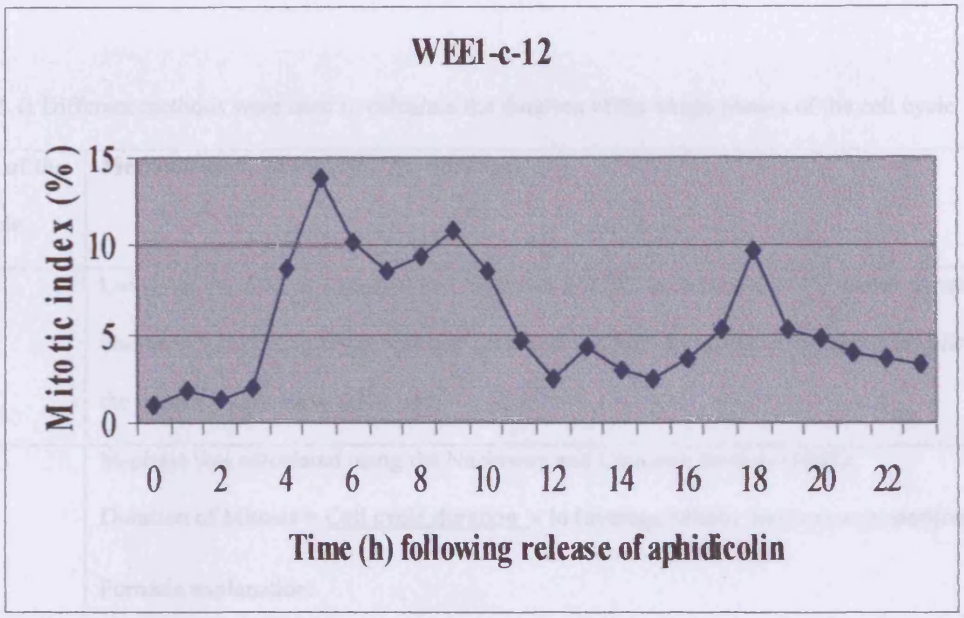
b


WEE1-c-2
Mitotic index (%)
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h) following release from aphidicolin

c


WEE1-c-10
Mitotic index (%)
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h) following release from aphidicolin

d



e

Fig. 3.4: Mitotic index curves obtained following synchronization (with aphidicolin for 24 h) of each of the constitutive *AtWEE1* cell lines, wild type and empty vector. **a)** wild type (WT); **b)** EV-c-10; **c)** WEE1-c-2; **d)** WEE1-c-10 and **e)** WEE1-c-12.

For the constitutive lines the mitotic index rose between 2-3 h and peaked between 3-4 hours for WEE1-c-2, at 4 hour for WEE1-c-10 and at 5 hours for WEE1-c-12 following the release from the aphidicolin block (Fig. 3.4 c, d and e). However, for both wild type and empty vector the mitotic index rose between 6 and 8 hours and peaked at 8 hours for the wild type and 9 hours for EV-c-10 (Fig. 3.4 a and b).

The duration of the cell cycle phases were estimated using different methods as indicated in Table 3.4.

Table 3.4: Different methods were used to calculate the duration of the single phases of the cell cycle

Phases of the cell cycle	**Methods used to calculate the duration**
G2	Using the method of Quastler and Sherman (1959), an estimate of G2 phase duration is obtained from the interval between zero and the 50% intercept of the ascending limb of the mitotic index curve (G2+½M).
M	M-phase was calculated using the Nachtwey and Cameron method (1968): Duration of Mitosis = $\frac{\text{Cell cycle duration}}{\ln 2} \times \ln$ (average Mitotic Index as a proportion + 1) Formula explanation: • Cell cycle duration/ln 2: since the cell cycle time is measured in a population of exponentially growing cells, the cell cycle duration occurs with respect to ln 2 (because an exponential curve increase by the exponent of 2) • ln (Mitotic Index as a proportion+1): Mitotic Index is from exponentially growing population represented by (1). The measurement is as a population (1) that doubles during exponential growth. So, the Mitotic Index value is with respect to a population that is doubling from 1 to 2.
S	S-phase was measured as the 50% intercept of the ascending and descending limbs of the first peak of the mitotic index curve (Quastler and Sherman, 1959).
G1	G1-phase was calculated as difference (ie. C-(S+G2+M)).

The more rapid rise in the mitotic index in the *AtWEE1* expressing lines is consistent with a much shorter G2 phase compared with the EV (Fig. 3.5). However, M phase and S phase are almost unaltered in the *AtWEE1* lines compared with the empty vector or wild type lines (Fig. 3.5). The overall duration of the cell cycle calculated as the interval between peaks is marginally longer in the *AtWEE1* lines compared with empty vector, by 1-2 hours. Since G1 is obtained by difference, the dramatic shortening of G2 phase in the *AtWEE1* lines is compensated by a long G1 phase (Fig. 3.5).

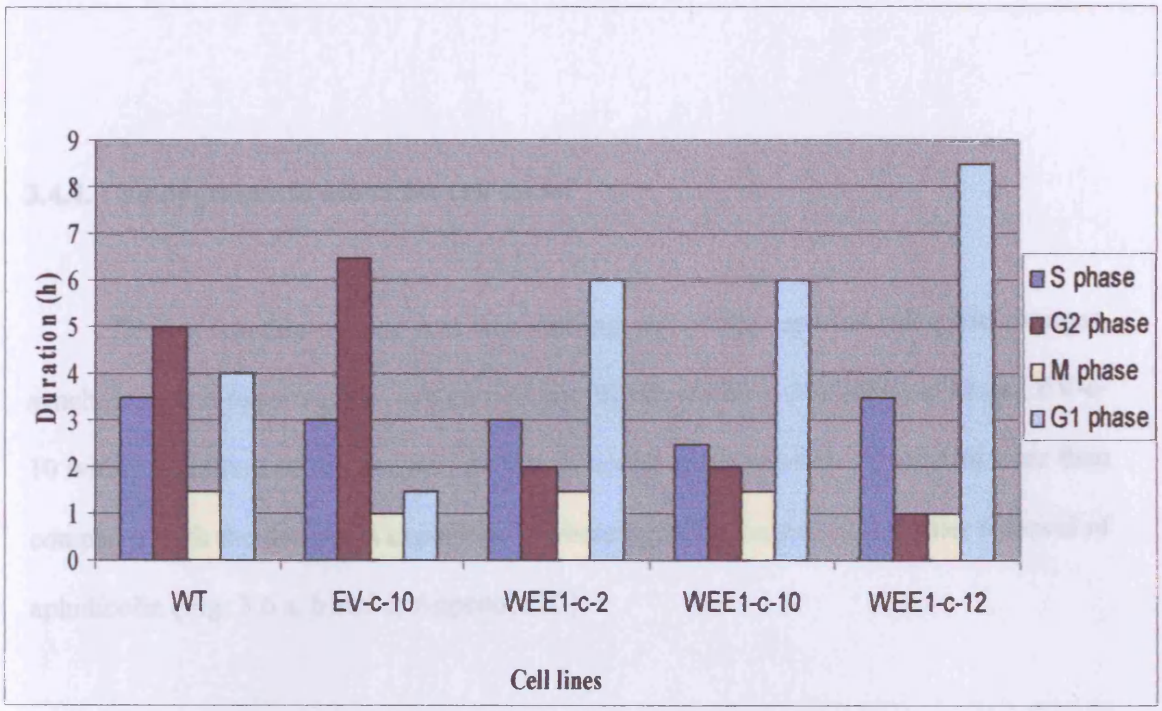
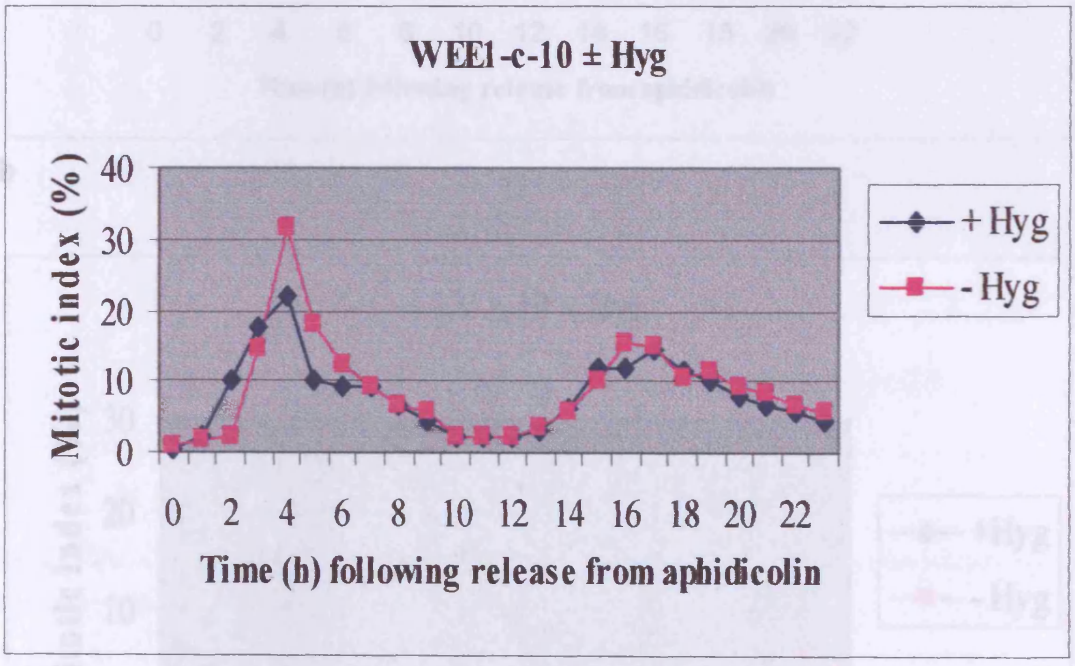


Fig. 3.5: The duration of the cell cycle (C) and its component phases in the constitutive *AtWEE1* cell lines together with the corresponding empty vector following synchronization, wild type is also represented. The duration of the cell cycle is: 14 hours wild type, 12 hours EV-c-10, 12.5 hours WEE1-c-2, 12 hours WEE1-c-10 and 14 hours WEE1-c-12.

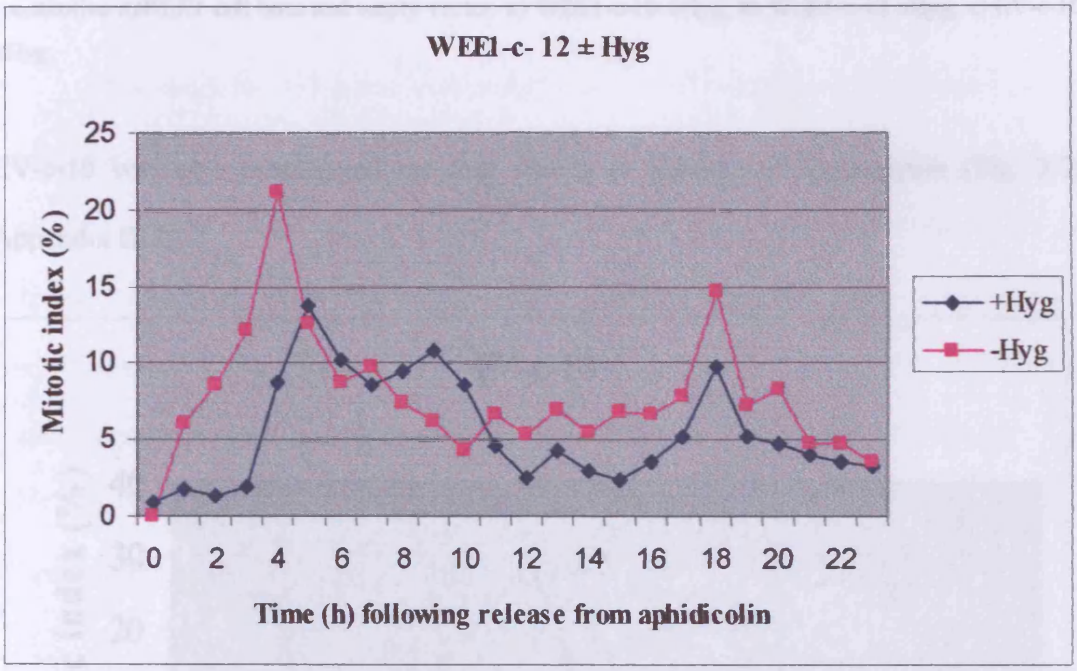
Hence, constitutive expression of *AtWEE1* in the tobacco BY-2 cell line clearly results in premature cell division through a shortening of the G2 phase. Remarkably, this is the exact opposite of the expression of *SpWEE1* and *AtWEE1* in fission yeast, where a delay in mitosis is due to a lengthening of the G2 phase. Clearly, constitutive *AtWEE1* expression led to an unusual and unpredicted cell cycle response in the TBY-2 cell line.

3.4.4. Can hygromycin affect the cell cycle?

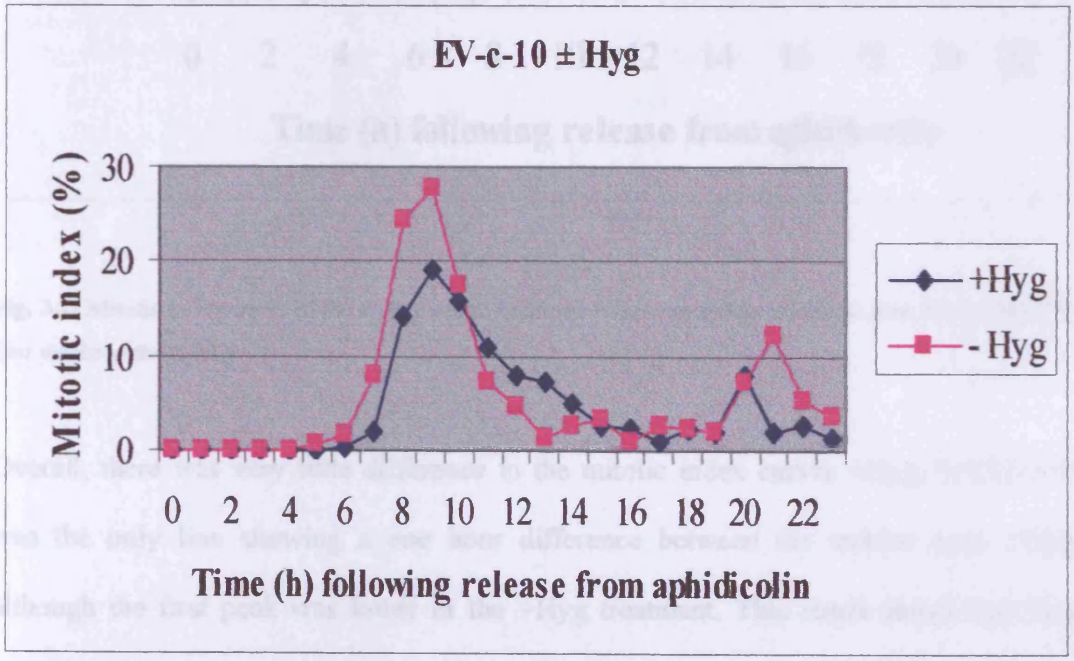
To test whether hygromycin was causing any of the reported cell cycle changes, synchronization experiments were carried out for the WEE1-c-10, WEE1-c-12 and EV-c-10 without addition of hygromycin after aphidicolin was removed. These data were then compared with the data from experiments where hygromycin was added after removal of aphidicolin (Fig. 3.6 a, b and c; Appendix III).



a


WEE1-c- 12 ± Hyg
Mitotic index (%)
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h) following release from aphidicolin
+Hyg
-Hyg

b


EV-c-10 ± Hyg
Mitotic index (%)
30
20
10
0
0 2 4 6 8 10 12 14 16 18 20 22
Time (h) following release from aphidicolin
+Hyg
- Hyg

c

Fig. 3.6: Mitotic index curves obtained following synchronization (with aphidicolin for 24 hours) of the constitutive *AtWEE1* cell lines and empty vector. **a)** WEE1-c-10 ±Hyg; **b)** WEE1-c-12 ±Hyg; **c)** EV-c-10 ±Hyg.

EV-c-10 was also subcultured for four weeks in absence of hygromycin (Fig. 3.7; Appendix III).

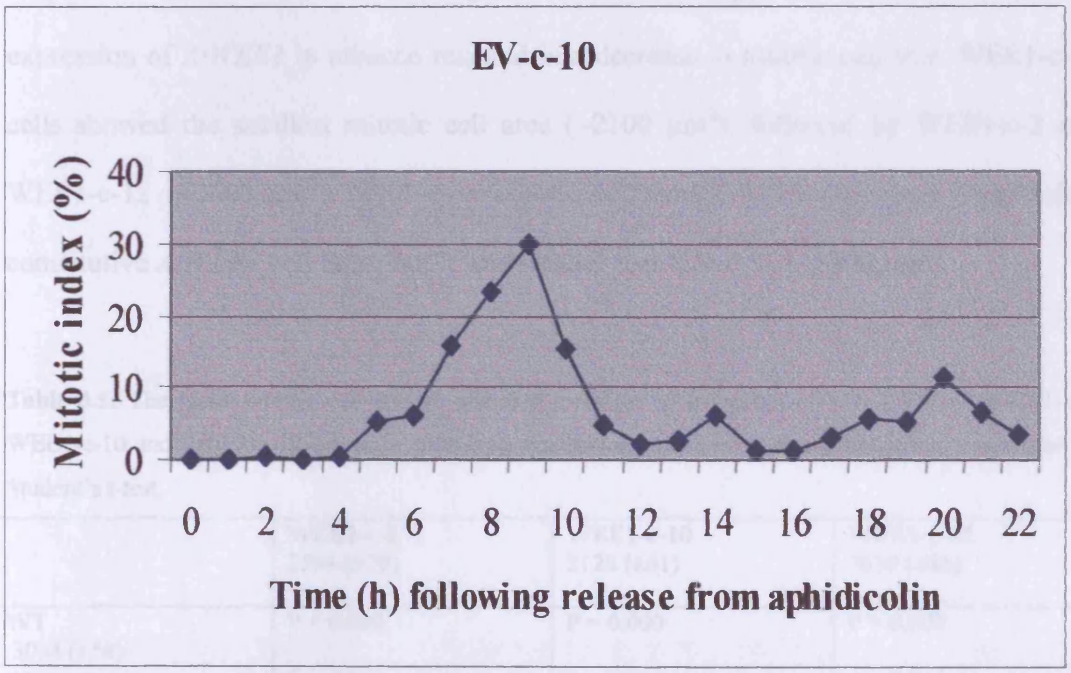


Fig. 3.7: Mitotic index curve of the empty vector obtained following synchronization after subculturing for four weeks without Hyg.

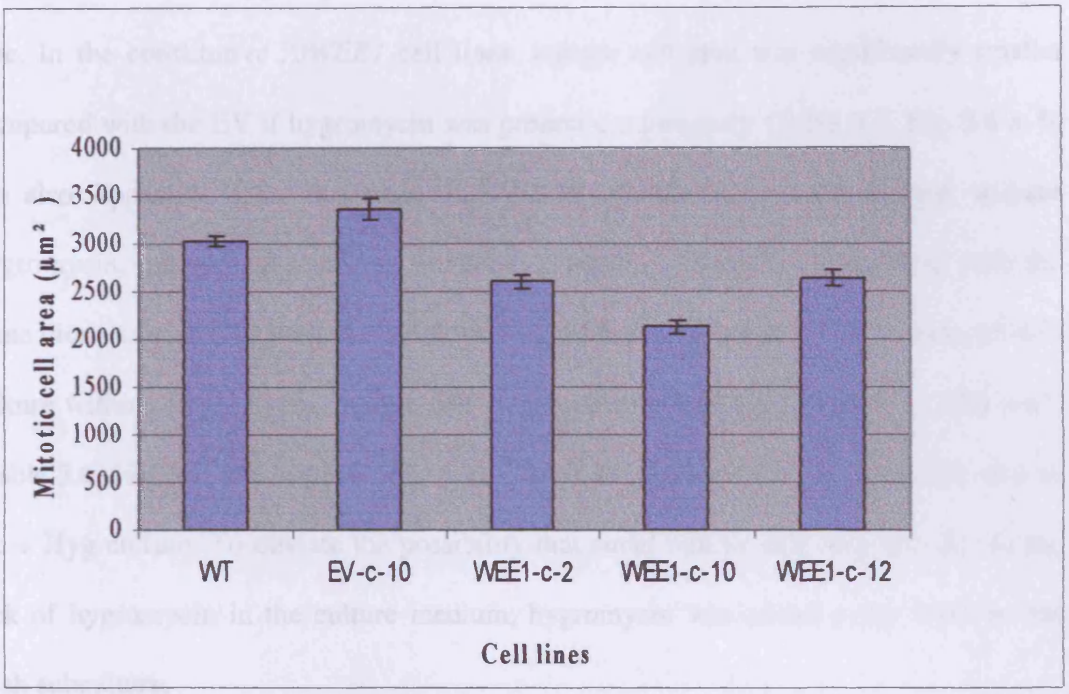
Overall, there was very little difference in the mitotic index curves ±Hyg, WEE1-c-12 was the only line showing a one hour difference between the mitotic peak ±Hyg, although the first peak was lower in the +Hyg treatment. This result shows that Hyg treatment does not have an effect on the timing of the component phases of the cell cycle, but it did result in a slight reduction in the percentage of cycling cells.

3.4.5. Mitotic cell area is smaller in the constitutive *AtWEE1* expressing lines

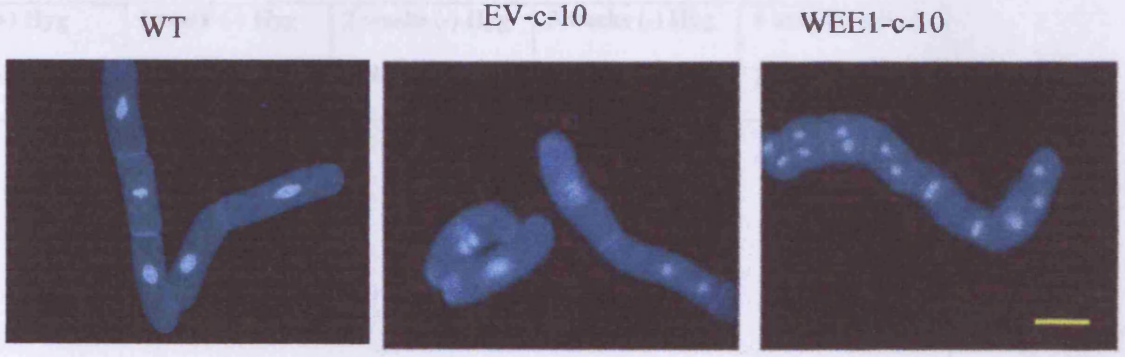
The shortened G2 phase obtained in the *AtWEE1* expressing lines would suggest premature cell division at a reduced cell size. This was confirmed when I analyzed mitotic cell area following synchronization in the various lines by image analysis (see chapter 2 section 2.3.4) (Table 3.5, Fig. 3.8 a, b; see also Appendix II for raw data). Thus expression of *AtWEE1* in tobacco resulted in a decrease in mitotic cell size. WEE1-c-10 cells showed the smallest mitotic cell area (~2100 μm^2), followed by WEE1-c-2 and WEE1-c-12 (~2600 μm^2). Wild type mitotic cell area (~3000 μm^2) was bigger than constitutive *AtWEE1* cell lines, but it was smaller than EV-c-10 (~3300 μm^2).

Table 3.5: The mean mitotic cell area (± standard error (in parenthesis)) of WT, EV-c-10, WEE1-c-2, WEE1-c-10 and WEE1-c-12 cell area, following synchronization. Levels of significance are indicated by Student's t-test.

	WEE1-c-2 2598 (±76)	**WEE1-c-10** 2128 (±61)	**WEE1-c-12** 2630 (±86)
WT 3034 (±58)	P = 0.000	P = 0.000	P = 0.000
EV-c-10 3350 (±112)	P = 0.000	P = 0.000	P = 0.000



a



b

Fig. 3.8: a) Mean (± SE) mitotic cell area in the wild type, constitutive *AtWEE1* cell lines and the corresponding EV. Measurements were taken following release from aphidicolin (n≥ 150). **b)** Examples of mitotic cells in wild type (WT), EV-c-10 and one representative *AtWEE1* expressing line (WEE1-c-10) (bars = 100 µm).

The effect of hygromycin on mitotic cell area was investigated using EV-c-10 cell line. In the constitutive *AtWEE1* cell lines, mitotic cell area was significantly smaller compared with the EV if hygromycin was present continuously (Table 3.5, Fig. 3.8 a, b; see also Appendix II for raw data). If EV-c-10 was subcultured for 1 week without hygromycin, mitotic cell area was smaller (~2500 μm^2) (Table 3.6) compared with the same clone subcultured with the antibiotic (~3500 μm^2). However, over 4 weeks of sub-culture without hygromycin, mitotic cell area became progressively larger (~2700 μm^2) (Table 3.6, Fig. 3.9; see Appendix II for raw data), but did not reach the same cell area as the + Hyg culture. To obviate the possibility that small mitotic cell area was due to the lack of hygromycin in the culture medium, hygromycin was added every week to the fresh subculture.

Table 3.6: The mean mitotic cell area (± standard error (in parenthesis)) of EV-c-10 subcultured with and without hygromycin over 4 weeks.

(+) Hyg	**1 week (-) Hyg**	**2 weeks (-) Hyg**	**3 weeks (-) Hyg**	**4 weeks (-) Hyg**
3537 (±103)	2515 (±118)	2045 (±69)	1916 (±81)	2687 (±67)

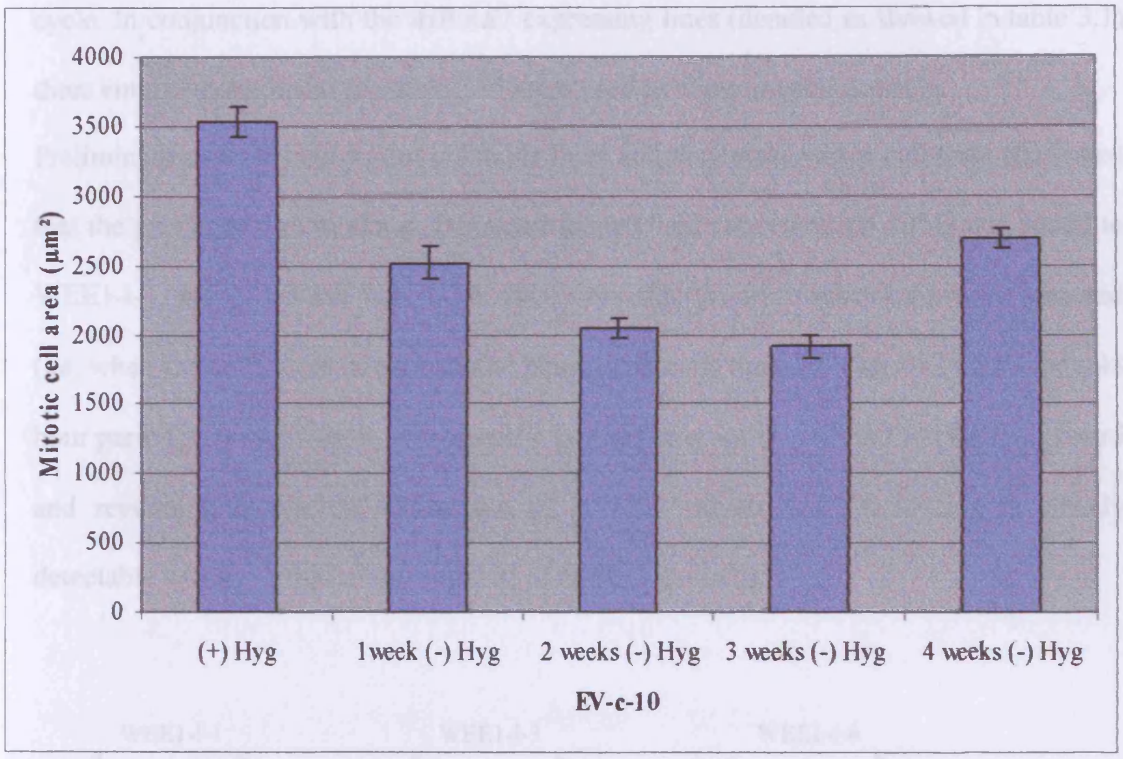


Fig. 3.9: Mean (± SE) mitotic cell area for EV-c-10 subcultured with hygromicin ((+) Hyg) or without hygromicin ((-) Hyg) ($n \geq 50$) for 1,2 ,3 and 4 weeks.

3.5. Results: TBY-2 cells transformed with pTA7002-*AtWEE1*

To examine whether TBY-2 cells "adapted" to the transgene over several generations or whether the changes to the cell cycle were rapid, tobacco BY-2 cells were transformed with *AtWEE1* using a chemical induction system (pTA7002). To induce expression of *AtWEE1*, dexamethasone (DEX) a strong synthetic glucocorticoid is required.

Three independent cell lines carrying *AtWEE1* under the control of the DEX-inducible promoter (Aoyama and Chua., 1997) were investigated in relation to cell size and cell

cycle. In conjunction with the *AtWEE1* expressing lines (denoted as showed in table 3.1) three empty vector lines (see table 3.1) were used as experimental controls. Preliminary experiments on the inducible lines and the empty vector cell lines confirmed that the promoter was working. Dexamethasone (final concentration 1μM) was added to WEE1-i-1, WEE1-i-3 and WEE1-i-6, three days after the fresh subcultures were prepared (i.e. when the cells were in exponential phase) and cells sampled every 12 hours for a 48 hour period. Using RT-PCR, with specific primers to amplify *AtWEE1* (AtWEE1 forward and reverse primers), the expression of *AtWEE1* at the mRNA level was already detectable within 15 min of the addition of DEX (Fig. 3.10).

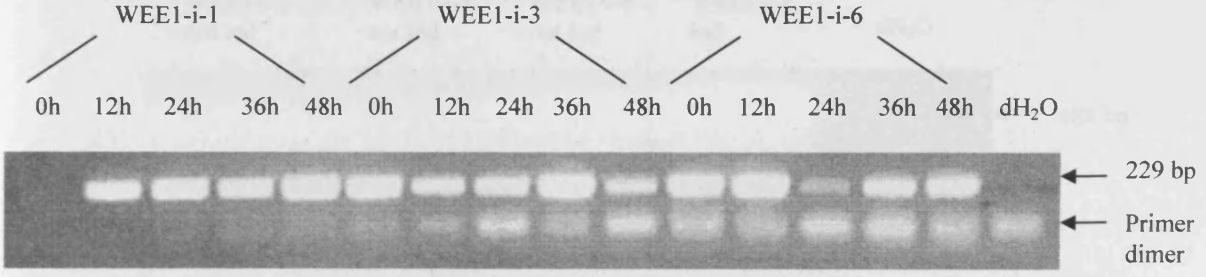


Fig. 3.10: RT-PCR analysis of *AtWEE1* expression in three independent tobacco BY-2 cell lines induced to express *AtWEE1* (WEE1-i-1, WEE1-i-3 and WEE1-i-6) distilled water (dH_2O) was used as the control. Cells were induced at time 0 h (within 15 min of the addition of DEX) and sampled every 12 hours for a 48 hour period.

AtWEE1 expression was also checked by RT-PCR in non-induced pTA7002-*AtWEE1* cell lines in the same experimental conditions except for the absence of the inducer, and in this case *AtWEE1* expression was not detected (Fig 3.11 a). However, to ensure that cDNA was synthesized successfully and the lack of expression of *AtWEE1* was due to the

lack of induction of the pTA7002-*AtWEE1* promoter, the same cDNA was used in PCR with PUV primers directed against *18S rRNA* (Fig 3.11 b). PCR product of the correct size was obtained for all the lines indicating that mRNA extraction and cDNA synthesis were successful.

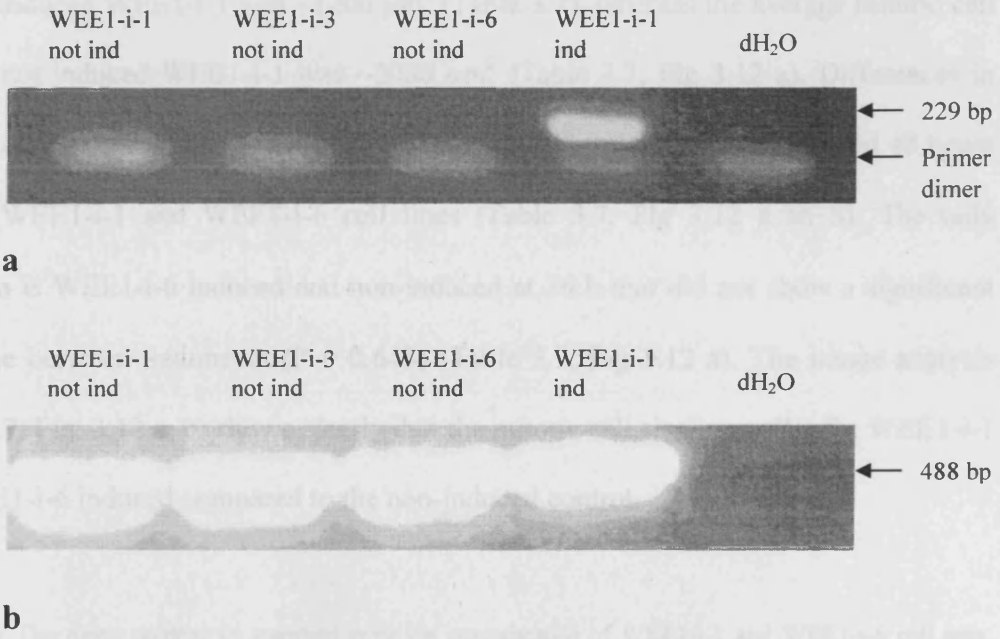


Fig. 3.11: a) cDNA from three independent inducible tobacco BY-2 cell lines without addition of the DEX inducer showing lack of expression of *AtWEE1* compared to the induced WEE1-i-1 cell line (positive control). The arrow is pointing to the *AtWEE1* band, the lower band is primer dimers. **b)** not induced WEE1-i-1, WEE1-i-3 and WEE1-i-6 cDNA amplified using primers directed against 18S rRNA, WEE1-i-1 induced cDNA was used as a positive control. In both cases the negative control is distilled water.

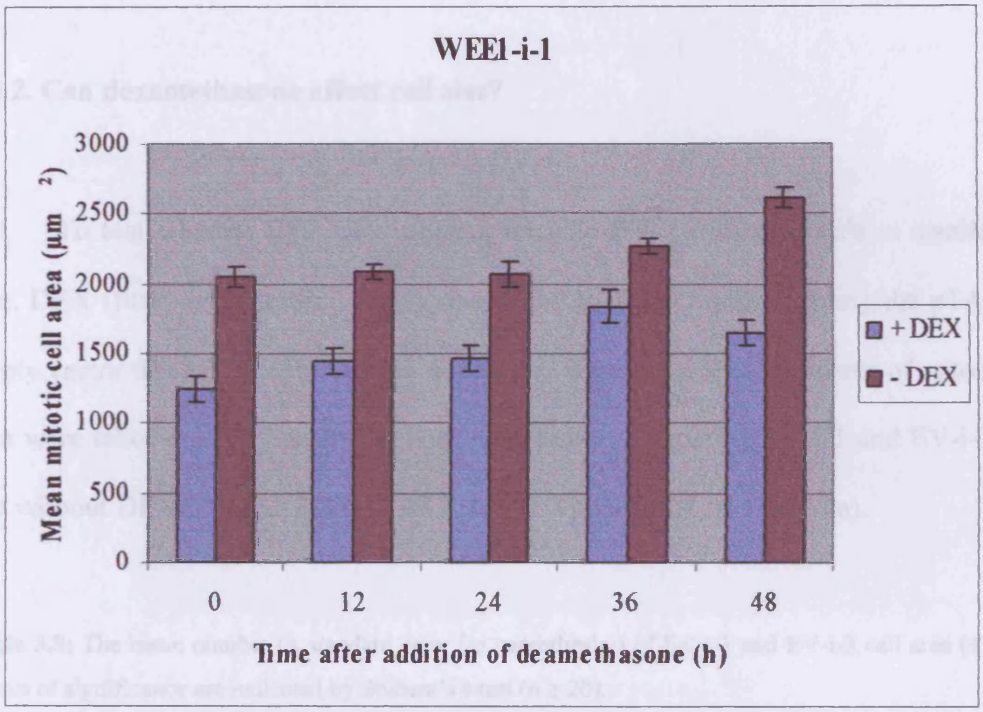
3.5.1. Mitotic cell area in the inducible *AtWEE1* cell lines

Measurements of mitotic cell area were made in tobacco BY-2 cell lines transformed with *AtWEE1* under the control of the inducible promoter. Three days after

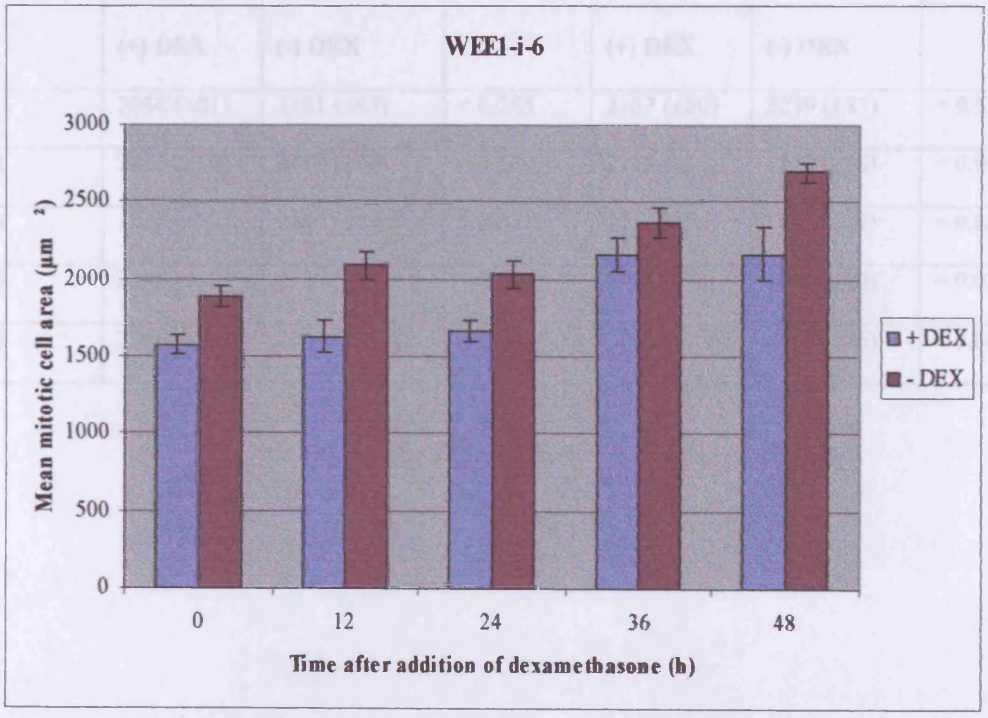
the fresh subcultures were made; mitotic cell size was measured every 12 hours for a 48 hour period following induction (see Appendix II for raw data). The 0 h sample for induced pTA7002-*AtWEE1* cells was collected 15 min after addition of DEX. Mitotic cell area for induced lines (WEE1-i-1 and WEE1-i-6) was compared with the same cell lines which had not been induced (Table 3.7, Fig. 3.12 a, b). At 0 h the average mitotic cell area for induced WEE1-i-1 was ~1200 μm^2 (Table 3.7), whereas the average mitotic cell area for not induced WEE1-i-1 was ~2000 μm^2 (Table 3.7, Fig 3.12 a). Differences in mitotic cell area dimension between treatments were observed at 12, 24, 36 and 48 hours in both WEE1-i-1 and WEE1-i-6 cell lines (Table 3.7, Fig 3.12 a an b). The only exception is WEE1-i-6 induced and non-induced at 36 h that did not show a significant difference between treatments (P = 0.643) (Table 3.7, Fig 3.12 a). The image analysis (Table 3.7, Fig. 3.12 a, b) shows clearly that the mitotic cell size is smaller for WEE1-i-1 and WEE1-i-6 induced compared to the non-induced control.

Table 3.7: The mean number (± standard error (in parenthesis)) of WEE1-i-1 and WEE1-i-6 cell area, induced and non-induced. Levels of significance are indicated by Student's t-test ($n \geq 15$).

Time (h)	WEE1-i-1 induced	WEE1-i-1 non-induced	P	WEE1-i-6 induced	WEE1-i-6 non-induced	P
0	1239 (±95)	2048 (±82)	= 0.000	1575 (±66)	1886 (±72)	= 0.000
12	1446 (±85)	2085 (±59)	= 0.000	1627 (±101)	2088 (±91)	= 0.000
24	1463 (±91)	2069 (±86)	= 0.000	1667 (±73)	2029 (±90)	= 0.001
36	1838 (±113)	2271 (±60)	= 0.002	2029 (±512)	2367 (±90)	= 0.643
48	1650 (±89)	2613 (±75)	= 0.000	2167 (±171)	2694 (±62)	= 0.023


WEE1-i-1
Mean mitotic cell area (µm ²)
3000
2500
2000
1500
1000
500
0
0
12
24
36
48
Time after addition of deamethasone (h)
+ DEX
- DEX

a


WEE1-i-6
Mean mitotic cell area (µm ²)
3000
2500
2000
1500
1000
500
0
0
12
24
36
48
Time after addition of dexamethasone (h)
+ DEX
- DEX

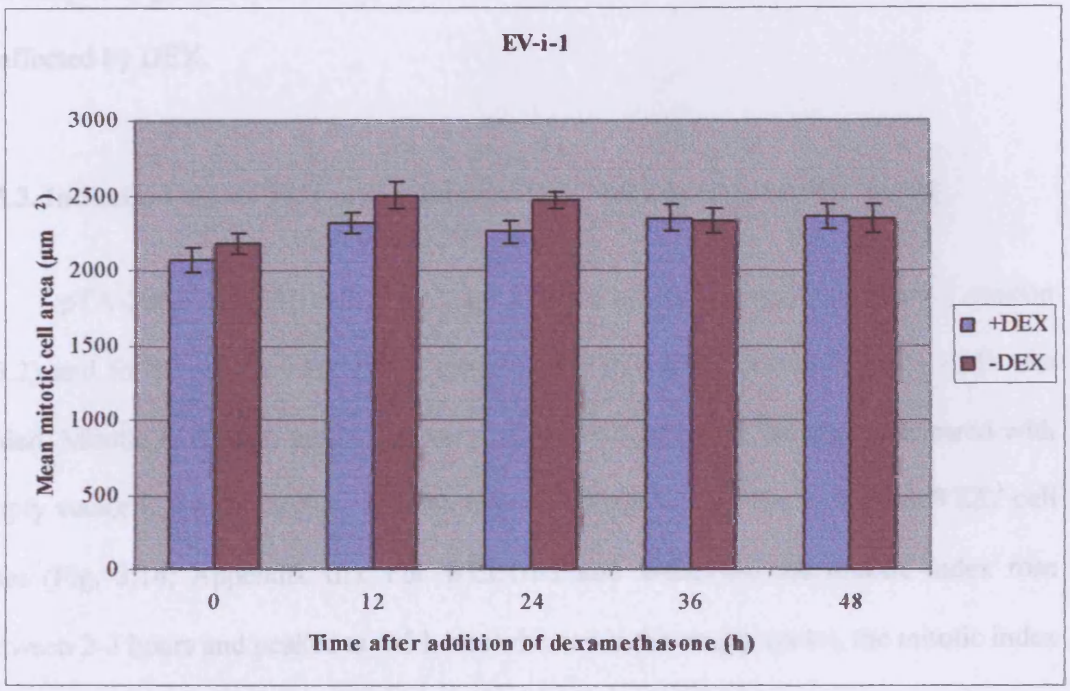
b

3.5.2. Can dexamethasone affect cell size?

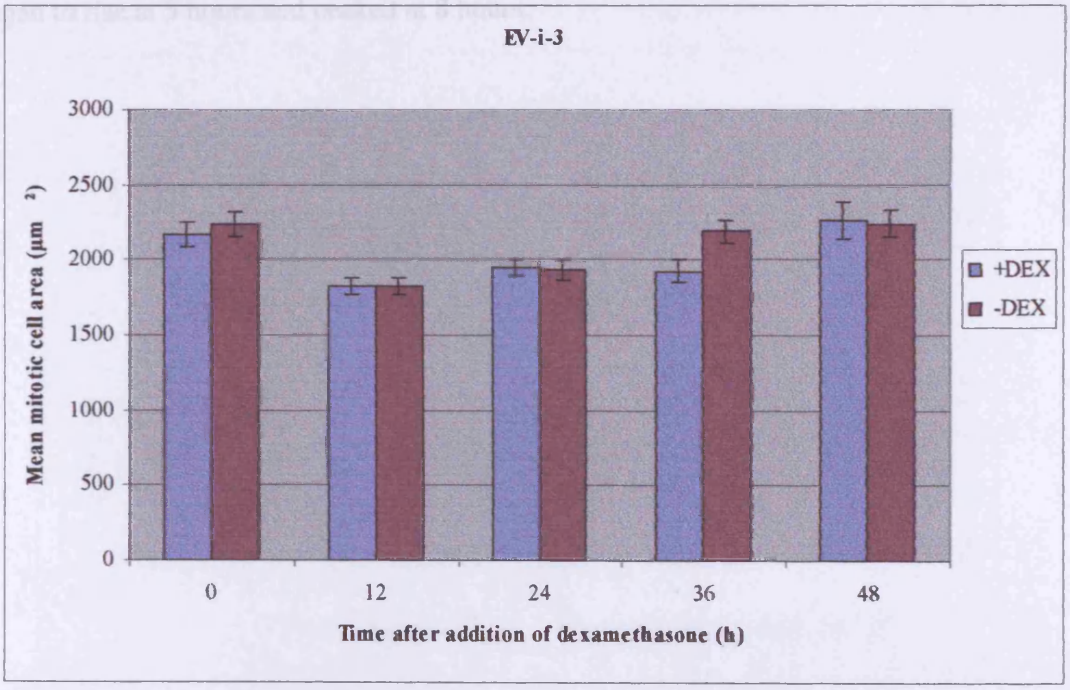
To test whether DEX was causing tobacco BY-2 cells to divide at smaller cell size, DEX (final concentration 1μM) was added to TBY-2 cells carrying the pTA7002-empty vector three days after the new subculture was made. Measurements of mitotic cell area were taken every 12 hours for a 48 hour period comparing EV-i-1 and EV-i-3 with and without DEX (Table 3.8, Fig. 3.13 a, b; see Appendix II for raw data).

Table 3.8: The mean number (± standard error (in parenthesis)) of EV-i-1 and EV-i-3 cell area (±) DEX. Levels of significance are indicated by Student's t-test ($n \geq 20$).

Time (h)	EV-i-1 (+) DEX	EV-i-1 (-) DEX	P	EV-i-3 (+) DEX	EV-i-3 (-) DEX	P
0	2068 (±81)	2181 (±67)	= 0.285	2167 (±80)	2239 (±83)	= 0.534
12	2315 (±69)	2499 (±90)	= 0.109	1829 (±58)	1830 (±56)	= 0.990
24	2253 (±72)	2464 (±53)	= 0.021	1952 (±54)	1939 (±71)	= 0.888
36	2348 (±88)	2325 (±82)	= 0.846	1929 (±75)	2190 (±80)	= 0.020
48	2360 (±84)	2344 (±101)	= 0.902	2267 (±125)	2241 (±86)	= 0.862



a



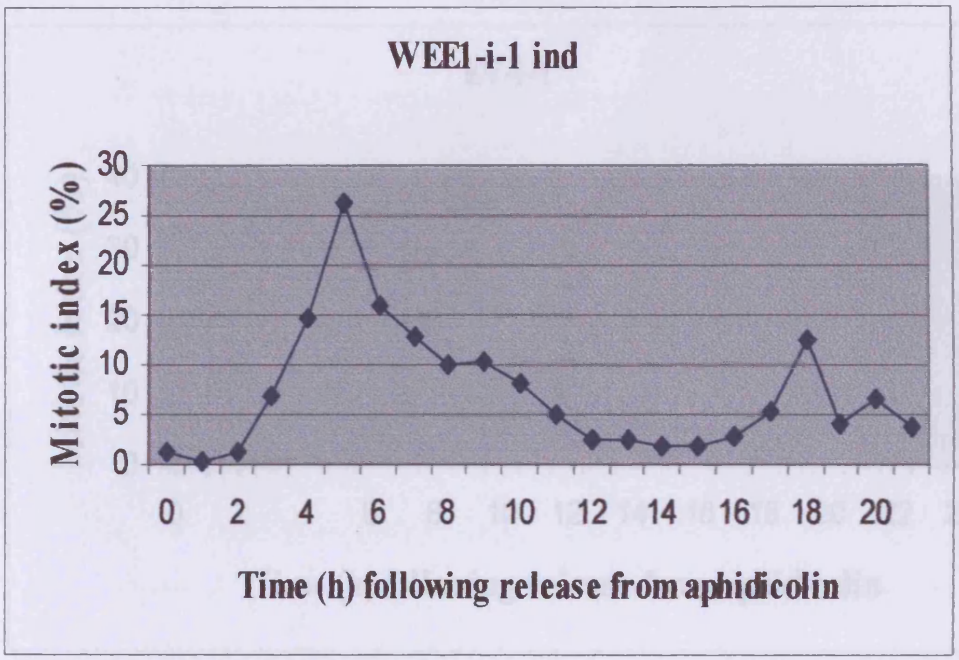
b

Fig. 3.13: Mean mitotic cell area (± SE) in: **a)** EV-i-1 (+) DEX and (-) DEX and **b)** EV-i-3 (+) DEX and (-) dex ($n \geq 20$).

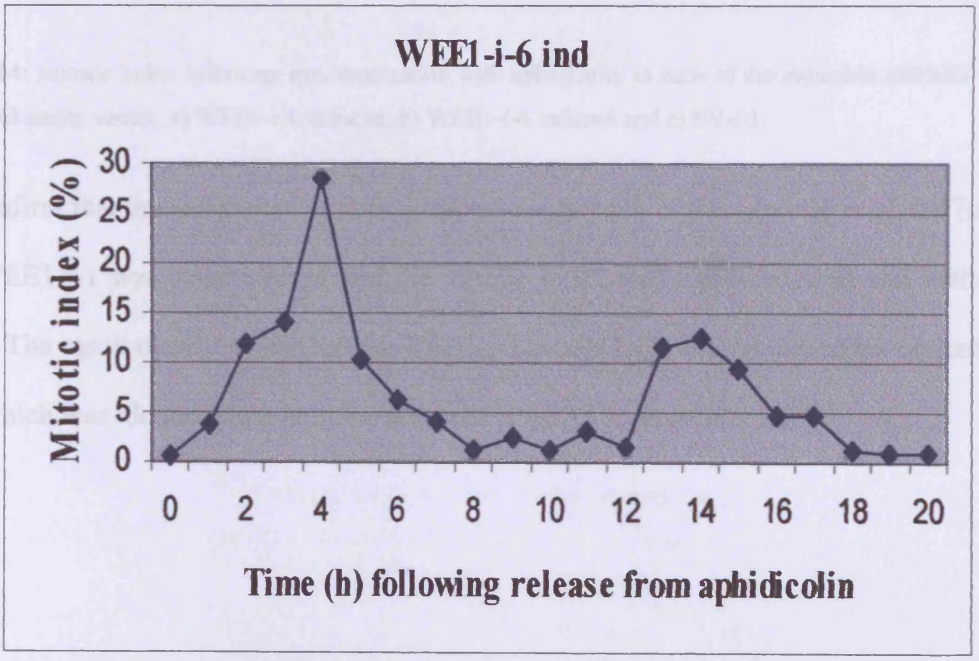
The image analysis showed clearly that the mitotic cell size in empty vector lines is unaffected by DEX.

3.5.3. Induction of *AtWEE1* causes tobacco BY-2 cells to divide prematurely

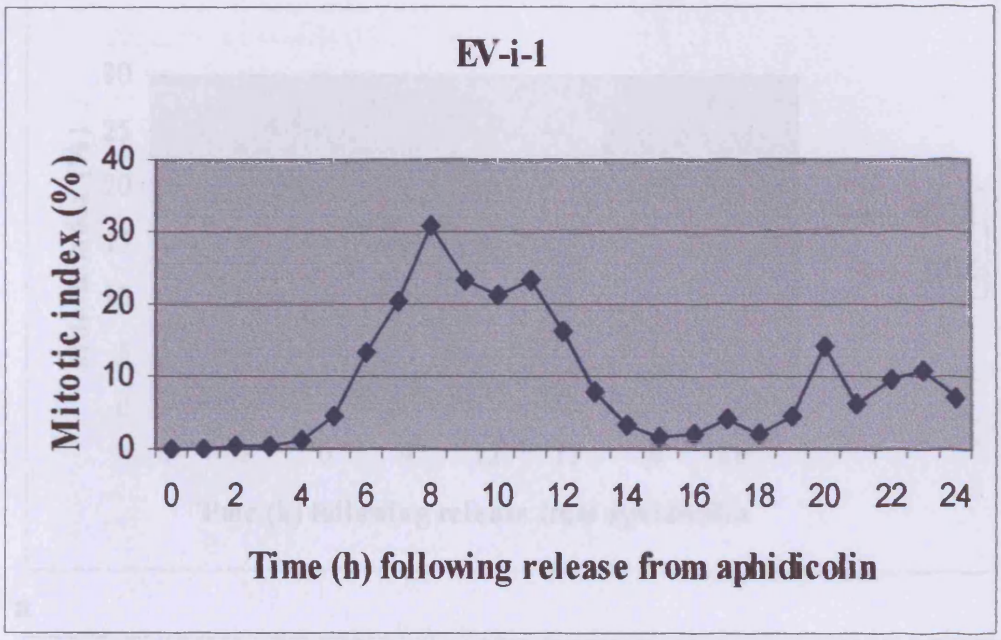
pTA-7002-*AtWEE1* cell lines 1 and 6 were synchronized (see chapter 2 section 2.3.2) and following the removal of aphidicolin, DEX (final concentration 1 μM) was added. Mitotic index was measured every hour for 21-24 hours. Notably, compared with empty vector (EV-i-1) the mitotic index began to rise sooner in the induced *AtWEE1* cell lines (Fig. 3.14; Appendix III). For WEE1-i-1 and WEE1-i-6 the mitotic index rose between 2-3 hours and peaked at 4-5 hours whereas in the empty vector, the mitotic index began to rise at 5 hours and peaked at 8 hours.


WEE1-i-1 ind
Mitotic index (%)
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
Time (h) following release from aphidicolin

a


WEE1-i-6 ind
Mitotic index (%)
30
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
Time (h) following release from aphidicolin

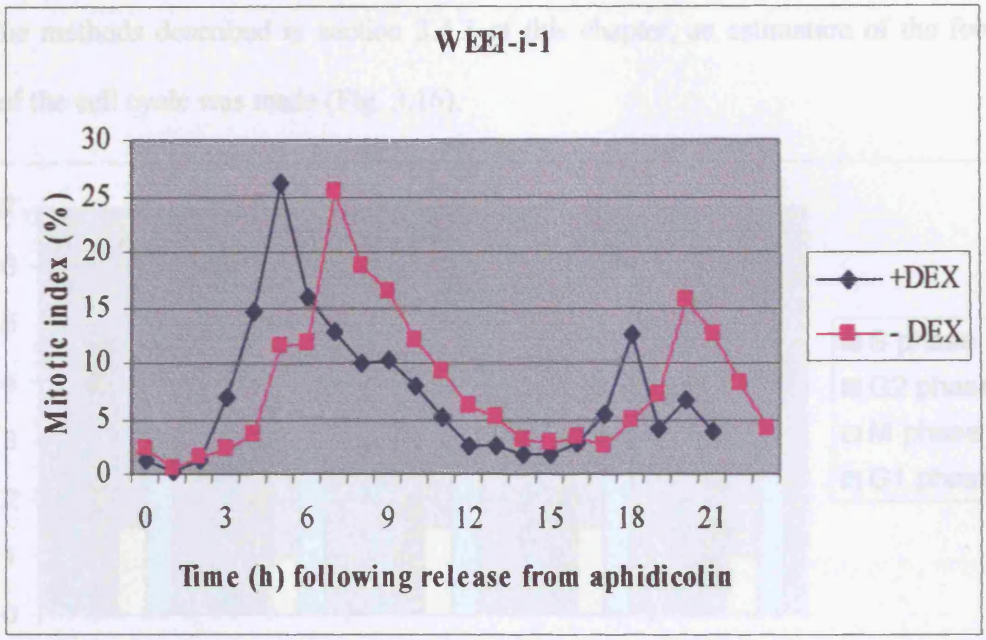
b



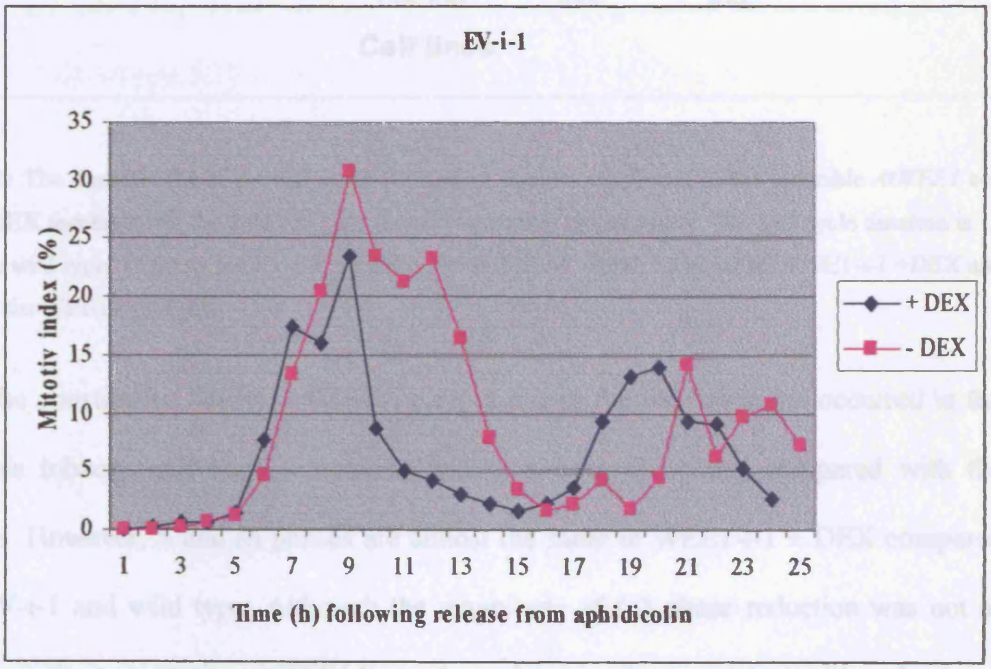
c

Fig. 3.14: Mitotic index following synchronization with aphidicolin in each of the inducible *AtWEE1* cell lines and empty vector. **a)** WEE1-i-1 induced; **b)** WEE1-i-6 induced and **c)** EV-i-1.

To confirm that the earlier rise of mitotic index was a result of the expression of *AtWEE1*, the WEE1-i-1 was synchronized and the mitotic index was measured with and without DEX. The result clearly shows that in WEE1-i-1 minus DEX the mitotic index peaked at 8 h, which was identical to that in the EV cells (Fig. 3.15; Appendix III).



a



b

Fig. 3.15: The mitotic index following synchronization with aphidicolin in **a)** WEE1-i-1 line ± DEX and **b)** EV-i-1 ± DEX.

Using the methods described in section 3.4.3 of this chapter, an estimation of the four phases of the cell cycle was made (Fig. 3.16).

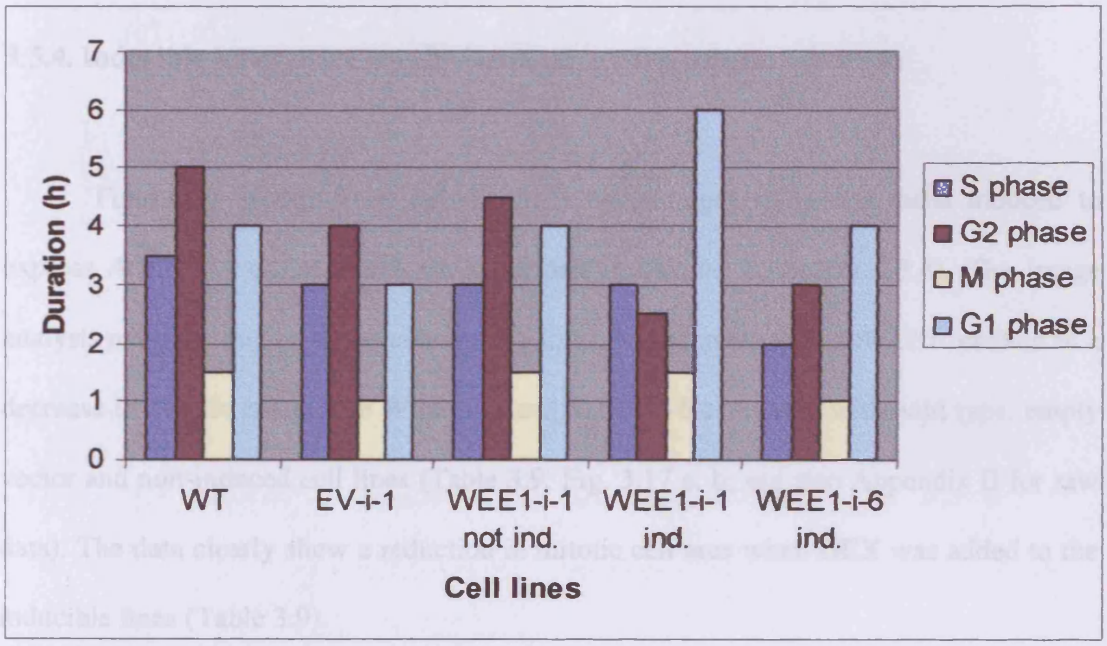


Fig. 3.16: The duration (h) of the cell cycle (C) and its component phases in the inducible *AtWEE1* cell lines ± DEX together with the wild type and the corresponding empty vector. The cell cycle duration is 14 hours for wild type; 11 hours for EV-i-1; 13 hours for WEE1-i-1 -DEX; 13 hours for WEE1-i-1 +DEX and 10 hours for WEE1-i-6 +DEX.

As in the constitutive WEE1 cell lines, a rapid rise in the mitotic index occurred in the inducible tobacco cell lines, consistent with a shorter G2 phase compared with the controls. However, S and M phases are almost the same in WEE1-i-1 ± DEX compared with EV-i-1 and wild type. Although the magnitude of G2 phase reduction was not as great as in the constitutive *AtWEE1* lines, these data are also consistent with a shortened G2 and a premature entry of cells into mitosis compared with the controls. However, a compensatory lengthening of G1 phase was only evident in the induced line 1 but not line

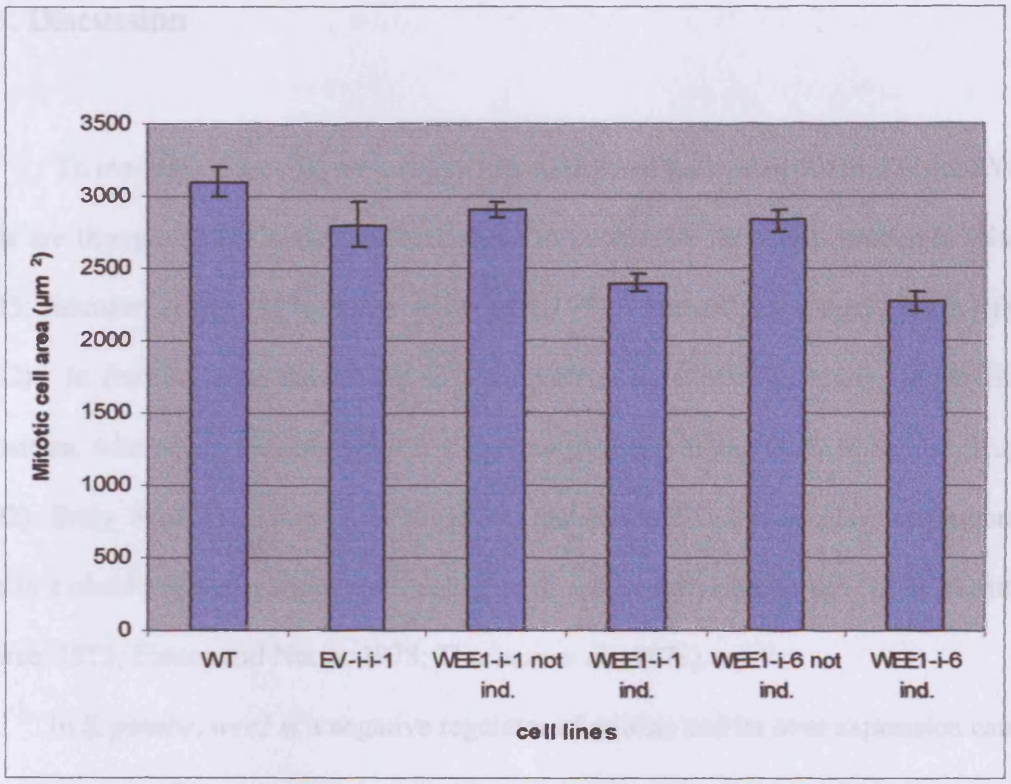
6. Line 6 exhibited one of the fastest cell cycles (10 hours) ever recorded for TBY-2 cells in Cardiff.

3.5.4. Inducible tobacco BY-2 cells divide at smaller mitotic cell areas

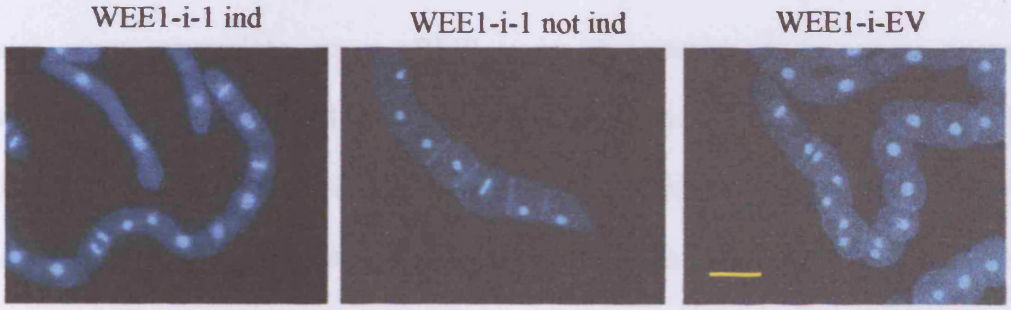
Following release from aphidicolin block, images of mitotic cells induced to express *AtWEE1* were recorded (as described in chapter 2 section 2.3.4). The image analysis revealed that as in the constitutive lines, the expression of *AtWEE1* resulted in a decrease in mitotic cell size in WEE1-i-1 and WEE1-i-6 compared with wild type, empty vector and non-induced cell lines (Table 3.9, Fig. 3.17 a, b; see also Appendix II for raw data). The data clearly show a reduction in mitotic cell area when DEX was added to the inducible lines (Table 3.9).

Table 3.9: Comparison of mean mitotic cell area (μm^2) (± standard error (in parenthesis)) in WT, EV-i-1, WEE1-c-1 and WEE1-c-6 cell area (induced and non-induced) following a synchronization experiment. Levels of significance are indicated by Student's t-test (n ≥150).

	WEE1-i-1 induced 2395 (±58)	**WEE1-i-6 induced** 2266 (±67)
WT 3034 (±58)	P = 0.000	P = 0.000
EV-c-10 2803 (±156)	P = 0.015	P = 0.002
WEE1-i-1 non-induced 2903 (±53)	P = 0.000	
WEE1-i-6 non-induced 2831 (± 71)		P = 0.000



a



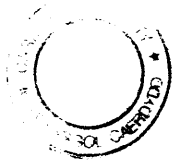
b

Fig. 3.17 a) Mean (± SE) mitotic cell area in the wild type; EV-i-1; WEE1-i-1 ±DEX and WEE1-i-6 ±DEX. Measurements were taken following release from aphidicolin ($n \geq 150$). **b)** Examples of mitotic cells that were smaller in WEE1-i-1 +DEX compared with the same line –DEX and the empty vector (bars = 100 µm).

3.6. Discussion

To maintain a specific size, cells must coordinate their growth and division. Yeast cells are thought to use cell-size checkpoints to coordinate these two processes (Nurse, 1975; Johnston *et al.*, 1977; Fantes and Nurse, 1997; Hartwell and Unger, 1997; Rupes, 2002). In fission yeast, the critical size requirement is exerted primarily at the G2/M transition, whereas in budding yeast it is exerted primarily at the G1/S transition (Rupes, 2002). Early work in fission yeast suggested that the WEE1 kinase plays an important role in a checkpoint that coordinates cell growth and cell division at the G2/M transition (Nurse, 1975; Fantes and Nurse, 1978; Thuriaux *et al.*, 1978).

In *S. pombe*, *wee1* is a negative regulator of mitosis and its over expression causes cells to arrest in G2, to grow but not divide resulting in very elongated cells (Russell and Nurse, 1987). *AtWEE1* was used in a functional assay by cloning the ORF into the fission yeast expression vector, pREP1 under the control of the potent thiamine-repressible *nmt1* promoter (Maundrell, 1993; Sorrell *et al.*, 2002). Colony formation by cells containing pREP1-*AtWEE1* was substantially reduced when the promoter was activated by removal of thiamine from the medium. However, the cells containing empty vector formed colonies regardless of thiamine supply. The substantial reduction of colony formation suggests that *AtWEE1* expression was inhibiting cell division. The cells containing pREP1-*AtWEE1* grown in the absence of thiamine were elongated and exhibited a 3.9-fold increase in length compared with empty vector (Sorrel *et al.*, 2002). Notably, this long cell phenotype was also obtained when human and maize homologues were expressed in *S. pombe* and are indicative of G2 arrest (Sun *et al.*, 1999). Hence,



AtWEE1 exhibits functional properties that are characteristic of WEE1 kinase expression. In *Arabidopsis* plants, over-expression of *AtWEE1* led to the same results observed previously in other organisms; the average of meristematic epidermis cells size was 1.41 fold greater than wild type (Cardiff cell cycle laboratory, unpublished data). Recent work has shown that Swe1, the budding yeast homolog of WEE1, also delays entry into mitosis and is required for cell size control (Jorgensen *et al.*, 2002; Harvey and Kellogg, 2003).

Interestingly, the experimental results reported in this chapter are in contrast to the data reported above and show that constitutive and inducible expression of *AtWEE1* in tobacco BY-2 cells induces a reduction in mitotic cell size. This reduction was observed in the constitutive *AtWEE1* expressing cell line compared with the wild type and the empty vector on day 1 and day 6 during the normal 7 days batch culture. A significant reduction in mitotic cell size was also obtained in three independent constitutive and two independent inducible *AtWEE1* expressing lines following synchronization.

TBY-2 *AtWEE1* expressing cell lines also exhibited unusual cell morphology. Normally, TBY-2 cells are observed to be 2-6 times longer than wide, in *AtWEE1* expressing cell lines, reduction in cell size is mainly achieved by the reduction in cell length, resulting in isodiametric cells.

To confirm that the small mitotic cell size and the abnormal cell cycle was neither a response to Hyg to select transformed tobacco BY-2 cells nor the glucocorticoid used to induce *AtWEE1*, in the inducible cell lines, different experiments were carried out showing that these chemicals did not play any role in causing a small mitotic cell phenotype and a shortened G2 phase.

To obviate the possibility of non-cell cycle effects as a result of the promoter used, tobacco BY-2 cells were also transformed with *AtWEE1* under the control of a dexamethasone inducible promoter/vector system (Aoyama and Chua, 1997). To investigate whether induction of *AtWEE1* expression would also result in small mitotic cell size, I measured mitotic cell size in inducible *AtWEE1* cell lines that were synchronized using aphidicolin. The results showed that induction of *AtWEE1* results in a small mitotic cell area, confirming the results for the constitutive lines.

In both constitutive and inducible *AtWEE1* expressing cell lines, cell size is approximately two-thirds of that observed in wild type and empty vectors. Notably, these data are very similar to those reported by Russell and Nurse (1986): over expression of *SpCdc25* in fission yeast induces fission yeast cells to divide at approximately two-thirds of that observed in wild type. Experiments carried out in our lab have also confirmed that expression of *SpCdc25* in TBY-2 cell lines reduce cell size at division (Orchard *et al.*, 2005). Taken together, these results indicate that cells do not continue to divide at smaller sizes, resulting in mitotic catastrophe, but instead establish a new threshold size for cell division. Although the existence of cell-size checkpoints was proposed over 20 years ago (Fantes and Nurse, 1977, 1978), the underlying molecular mechanisms have remained elusive, and how exactly cells monitor cell size or cell growth is still unknown.

To test how *AtWEE1* expression resulted in small mitotic cell size, cell cycles were assessed by charting the mitotic index following synchronization with aphidicolin. For both the constitutive and the inducible cell lines the mitotic index curve rose sooner and peaked earlier compared with all the controls (wild type, empty vectors and not induced lines). Hence the data are indeed consistent with a short G2 phase. The

shortening of G2 phase fits with both premature cell division and small cell size at division. Interestingly, the shorter G2 phase was compensated by a longer G1 phase in tobacco BY-2 cells expressing *AtWEE1* compared with the controls, so that the total duration of the cell cycle was not shorter than 12 hours. An exception was induced WEE1-i-6 that had a very unusual short cell cycle (10 hours). The data about a compensatory variation in duration of G1 phase are supported by the data reported by Russell and Nurse (1986) who demonstrated that fission yeast exhibited a compensatory decrease in duration of G1 phase due to the longer G2 phase. Compared with the empty vector, cell cycle duration was 0.5 hour longher for WEE1-c-2 and 2 hours longher for WEE1-c-12. Compared with wild type, cell cycle duration in EV-c-10 and EV-i-1 was reduced by 2 hours and 3 hours, respectively. However, G2 is longer in the empty vector compared with *AtWEE1* transformed cell lines, whereas G1 is shorter. Note that the cell cycle was two hours shorter in the EV-i-1 compared with the WEE1-i-1 cell line. The only exception was WEE1-i-6, which was one hour shorter, compared with the empty vector line.

3.7. Summary

The data reported in this chapter are exactly the opposite of what is known about *AtWEE1* expression in *S .pombe* (Sorrell *et al.*, 2002). Expression of *AtWEE1* in tobacco BY-2 cells induces small mitotic cell area and accelerates the entry into mitosis. Premature cell division occurs through a reduction in the duration of G2 phase. However,

cell cycle duration was very similar to that in wild type with a lengthening of G1 phase compensating for the shortening of G2 phase.

Chapter 4: In tobacco BY-2 cells, induction of *AtWEE1* delays expression of the endogenous *Nicotiana tabacum WEE1 (NtWEE1)* gene

4.1. Introduction

Transgenic techniques have become a powerful tool to address important biological problems in the study of the cell cycle. Many approaches that were impossible to implement by traditional genetics can now be achieved by transgenic techniques, including the introduction of homologous or heterologous genes into cells. The ability to introduce foreign genes into the nuclear genome of plants has provided the methodology to analyse molecular mechanisms that lead to co-ordinate expression of genes (Schell, 1987). It also serves to express alien genes or to modulate the expression of endogenous ones (Sonnewald *et al.*, 1991). Usually, in most experiments, the transgenes are transcribed via a strong promoter, such as the 35S promoter from the cauliflower mosaic virus (CaMV). However, a more flexible gene expression system is needed to extract greater benefits from transgenic technology. Good inducible transcription systems are desired because transgenic cells with an inducible phenotype are as useful as conditional mutants isolated by traditional genetics. Also a regulated promoter is often desirable for example to induce expression at defined time points during the cell cycle, in addition, a tightly repressed promoter is required if the expression of the gene product of interest interferes with the regeneration process.

Several induction systems have been reported and successfully used. One of the most commonly used is the glucocorticoid receptor (GR) which is a member of the family of vertebrate steroid hormone receptors. The GR is not only a receptor molecule but also a transcription factor which in the presence of a glucocorticoid such as dexamethasone, activates transcription from promoters containing glucocorticoid response elements (GREs) (Beato, 1989: Picard, 1993). The GR system was chosen as a good induction system in plant cells because it is simple, and the glucocorticoid itself does not cause any pleiotropic effects in plant cells (Schena *et al.*, 1991).

My research has focused on the effect of *AtWEE1* on the plant cell cycle and in particular its effect on cell size in BY-2 cells. To obviate the possibility of gene-silencing, a heterologus approach was taken by expressing *AtWEE1* in the tobacco BY-2 cells. Clearly, the induction of a small cell size was surprising, so it was hypothesised that *AtWEE1* perturbed the native tobacco *WEE1* (*NtWEE1*). To test this hypothesis, the pattern and timing of expression of *AtWEE1* and *NtWEE1* genes were studied in synchronized tobacco BY-2 cells in which *AtWEE1* could be induced by dexamethasone. Cells were sampled every hour for a 12-14 hour period (see chapter 2 sections 2.3.2 and 2.3.6). RNA was extracted and cDNA synthesized from each sample (see chapter 2 sections 2.4.1, 2.4.2 and 2.4.3). Expression was estimated in a semi-quantitative manner by measuring the ethidium bromide fluorescence after gel electrophoresis of the products from limited cycle PCRs using specific primer pairs designed from *18S rRNA, H4, AtWEE1* and *NtWEE1* genes (see chapter 2 sections 2.4.4 and 2.4.5).

Hence the aims of the work reported here were to examine whether expression of *AtWEE1* results in a change in pattern and timing of *NtWEE1* expression during the

tobacco BY-2 cell cycle. To do this, a portion of the *NtWEE1* gene was cloned by RT-PCR using degenerate primers.

4.2. Materials and methods

Details of Materials and Methods are fully described in chapter 2 from section 2.3.2 to section 2.4.5.

4.3. Results

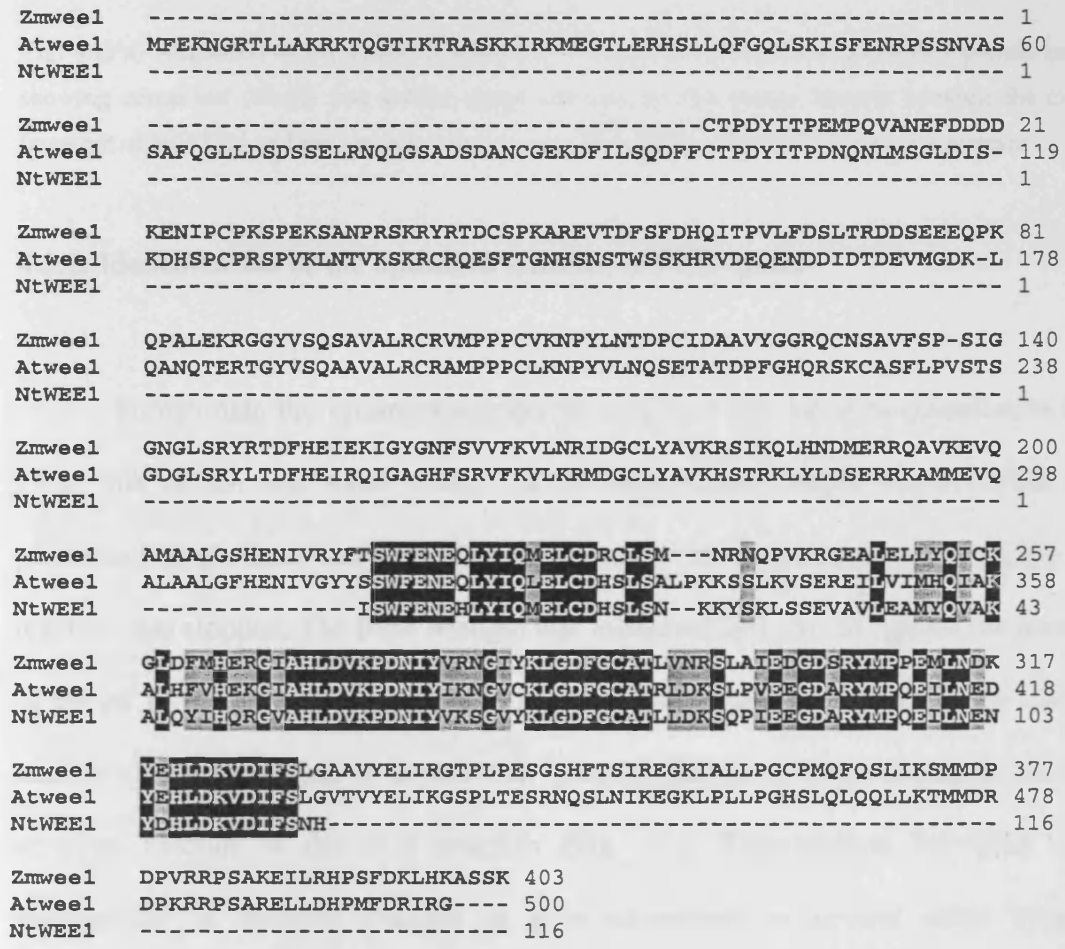
Following synchronization with and after removal of aphidicolin, samples were collected and RNA extraction was successfully carried out from wild type cells and the WEE1-i-1 ± DEX cells. This enabled an analysis of *NtWEE1* expression in its native background, or in the presence of *AtWEE1* transcripts. Semi-quantitative RT-PCR was carried out to investigate the expression of *AtWEE1* and *NtWEE1* in the transgenic TBY-2 cell lines. These results were normalized against *18S rRNA*, the expression of *H4* was investigated as a control since histone H4 expression is a sensitive marker of S phase (Sorrell *et al.*, 1999, Herbert *et al.*, 2001).

4.3.1. Isolation of a homologue tobacco *WEE1* (*NtWEE1*)

Using RT-PCR and two degenerate primers, it was possible to amplify a 348 bp region of the tobacco *WEE1* (*NtWEE1*) open reading frame. From a comparison of the amino acid sequence of NtWEE1 with AtWEE1 and ZmWEE1 kinase proteins

(Fig. 4.1) the percentage of homology at the amino acid level was 70.8% and 61.8%, respectively, indicating that a tobacco homologue had been amplified.

The *NtWEE1* sequence was then used to design specific primers which were used for semi-quantitative RT-PCR investigation of the native tobacco *WEE1* gene.



a

Percent Similarity

	AtWEE1	ZmWEE1	NtWEE1
AtWEE1		62.7	70.8
ZmWEE1	34.9		61.8
NtWEE1	25.5	34.3	

Percent Divergence

b

Fig. 4.1: a) Alignment of the cloned fragment of NtWEE1 to AtWEE1 and ZmWEE1 protein kinase, showing conserved (black) and similar (grey) residues. **b)** Percentage identity between the cloned fragment of NtWEE1 and the corresponding regions of AtWEE1 and ZmWEE1 kinase proteins.

4.3.2. Identification of the optimum number of PCR cycles

To estimate the optimum number of cycles to use for semi-quantitative RT-PCR, mix cDNA was made using 1 μl of each cDNA sample and RT-PCR was performed. Eight reactions were then set up and every 2 cycles (from 24 to 38) one reaction was stopped. The band strength was measured and plotted against the number of cycles to identify the linear phase of the RT-PCR reaction which is the correct number of cycles to use to ensure that product amount is proportional to starting template amount in the PCR reaction (Fig. 4.2). This method has been used successfully to measure changes in gene expression in several other systems (Wagstaff *et al.*, 2002; Parfitt *et al.*, 2004).

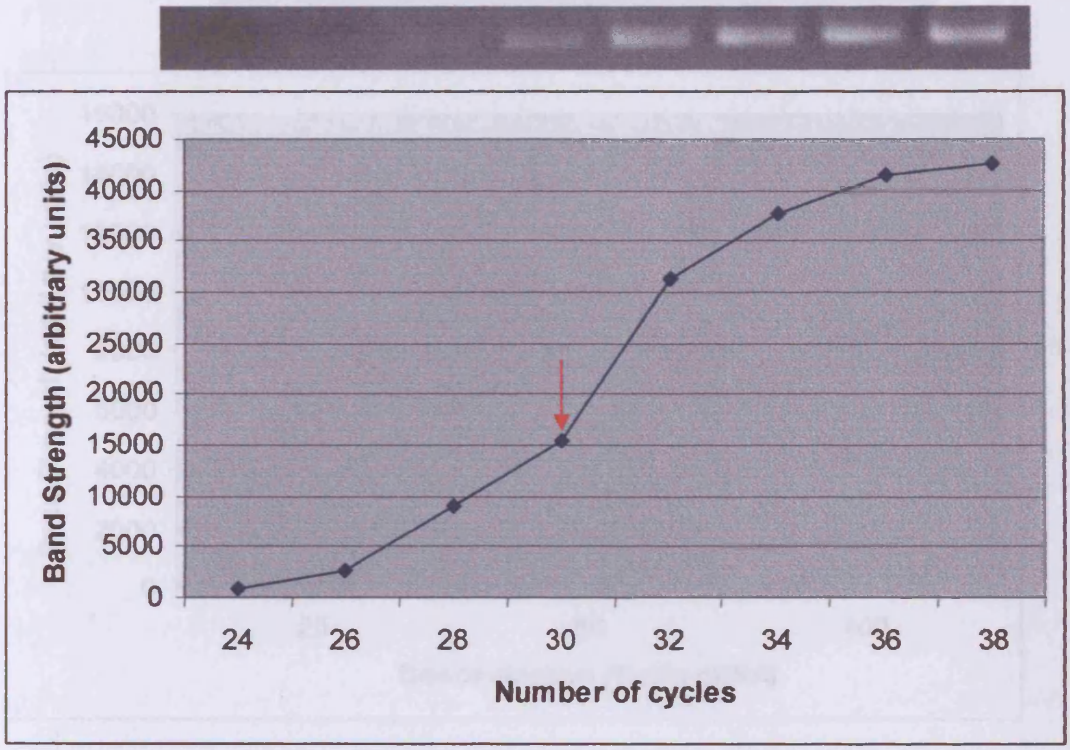


Fig. 4.2: Example of estimation of optimum cycle number to use for semi-quantitative RT-PCR for histone H4 from WEE1-i-1 cDNA. Arrow indicates cycle number chosen for further experiments.

To ensure that semi-quantitative RT-PCR was performed to produce a linear relationship between product band strength and template concentration, each of the primers for *18S rRNA, H4, AtWEE1* and *NtWEE1* genes were tested on dilutions of a mix cDNA. These reactions were included in each semi-quantitative RT-PCR experiment performed. An example of a calibration plot using dilutions of the mix cDNA from WEE1-i-1 induced using an optimum number of cycles for the *H4* primer pair is shown in Figure 4.3.

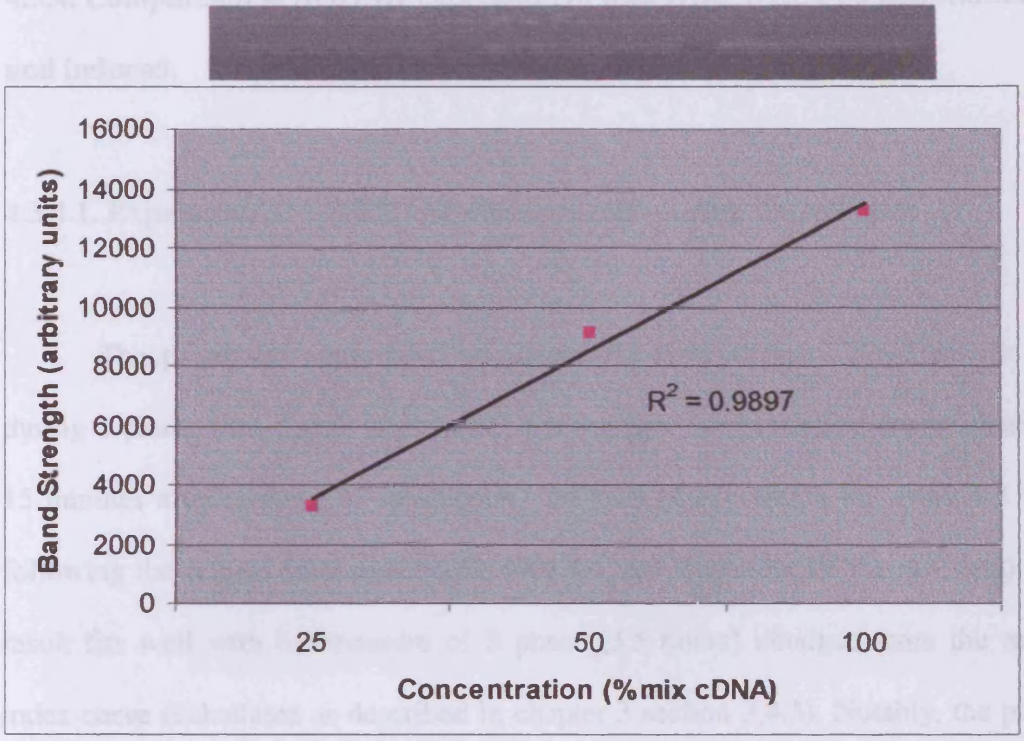


Fig. 4.3: Calibration plots using dilutions of WEE1-i-1 mix cDNA sample and histone H4 primers at an optimum number of cycles.

4.3.3. Statistical measure of semi-quantitative RT-PCR results

To obtain a formal statistical measure of the significance of results obtained by semi-quantitative RT-PCR, means of three replicate PCRs, were used. The means were normalized against two correction methods: assuming constant *18S rRNA* expression and assuming constant total RNA concentration. The results for each putative gene are displayed as histograms (Figs 4.4 to 4.10). Each column in the histogram represents a mean value for the target gene product for each hour, divided by the corresponding mean for *18S rRNA*. The ratio of the target gene to *18S rRNA* was multiplied by 1000 to provide a more easily readable scale.

4.3.4. Comparison of *NtWEE1* expression in wild type, WEE1-i-1 non-induced and induced.

4.3.4.1. Expression of *NtWEE1* in wild type cells during the cell cycle

The pattern of expression for *histone H4* in wild type TBY-2 cells is high during S phase with a peak of expression at 0 hours (corresponding approximately to 15 minutes after removal of aphidicolin). Its level of expression fell away 3-4 hours following the release from aphidicolin (Fig.4.4; see Appendix IV for raw data). This result fits well with the measure of S phase (3.5 hours) obtained from the mitotic index curve (calculated as described in chapter 3 section 3.4.3). Notably, the pattern of histone *H4* expression is similar to that previously reported in synchronized tobacco BY-2 cells using northern blot analysis which also showed an S phase of 3.5 hours (Herbert *et al.*, 2001).

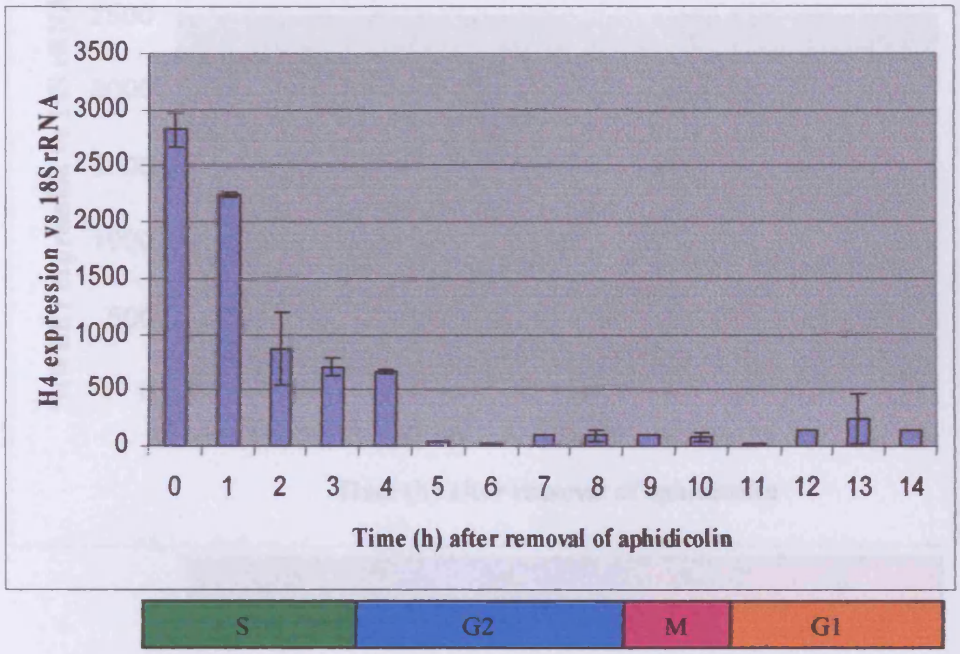


Fig. 4.4: Semi-quantitative RT-PCR analysis of *H4* expression (normalised mean ± standard error) during the cell cycle of synchronized wild type TBY-2 cells following release from aphidicolin. The duration of the cell cycle component phases are reported below the histogram (see chapter 3 section 3.4.3) (n=3).

The level of *NtWEE1* expression fluctuated during the cell cycle (Fig. 4.5; see Appendix IV for raw data). Maximum expression was observed at 4 hours after removal of aphidicolin (corresponding to the transition point between S and G2 phase), its expression then decreased to reach its minimal level in mid G2 phase. Low levels of *NtWEE1* expression were also detected throughout mitosis and early G1 phase. In late G1 phase of the subsequent cell cycle *NtWEE1* expression rose again.

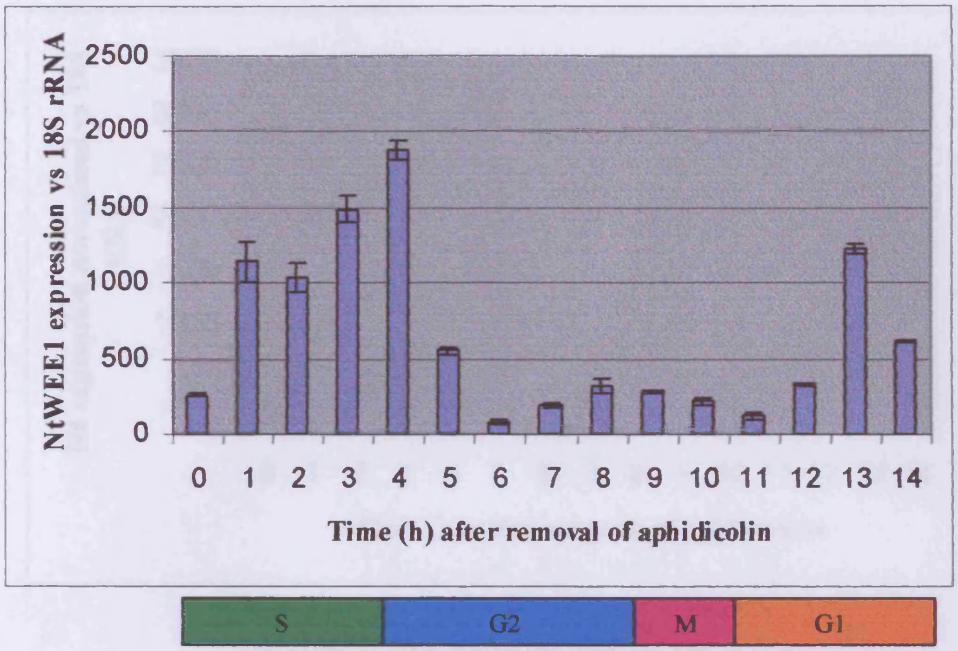


Fig. 4.5: Semi-quantitative RT-PCR analysis of *NtWEE1* expression (normalised mean ± standard error) during the cell cycle of synchronized wild type TBY-2 cells following release from aphidicolin. Below the histogram is reported the duration of the TBY-2 cell cycle phases derived from the mitotic index (see chapter 3 section 3.4.3) (n=3).

4.3.4.2. Expression of *NtWEE1* in non induced WEE1-i-1 cells during the cell cycle

Expression of *H4* in non induced WEE1-i-1 cells rose to a maximum 1 to 2 hours following the release from aphidicolin (Fig. 4.6; see Appendix IV for raw data). The expression level decreased after 3 hours when the cells were going into G2-phase in agreement with the measure of S phase (3 hours) obtained from the mitotic index curve (calculated as described in chapter 3 section 3.4.3, see also section 3.5.3).

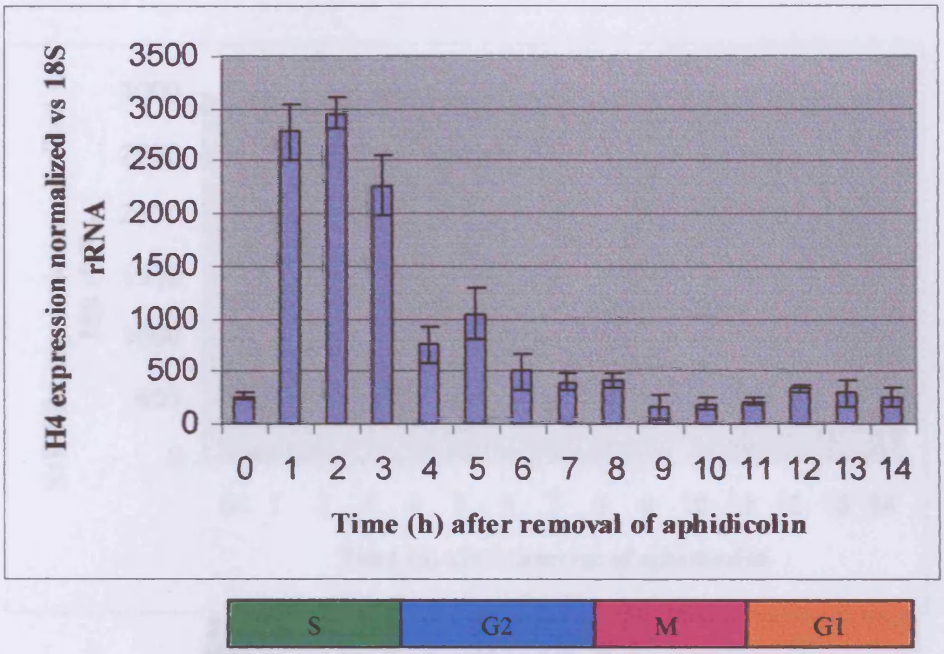


Fig. 4.6: Semi-quantitative RT-PCR analysis of *H4* expression (normalised mean ± standard error) during the cell cycle in synchronized non induced WEE-1-i-1 cells following release from aphidicolin. The duration of the cell cycle component phases derived from the mitotic index are reported below the histogram (see chapter 3 section 3.5.3) (n=3).

The pattern of expression of *NtWEE1* for non induced WEE1-i-1 was very similar to that in wild type tobacco BY-2 cells with a maximum of expression in S-phase; the only difference is that the peak of *NtWEE1* expression in wild type was at hour 4 (Fig. 4.5) whereas the peak of expression for *NtWEE1* in non induced WEE1-i-1 was at hour 3 (Fig. 4.7; see Appendix IV for raw data). Nevertheless, hour 3 represents the border point between S and G2 phase, as was found in the wild type TBY-2 cells.

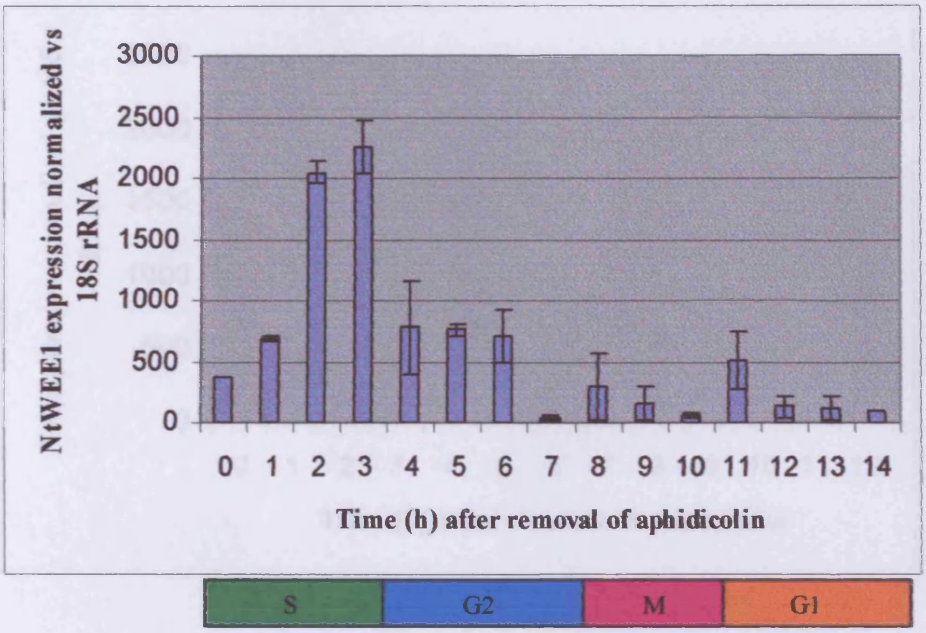


Fig. 4.7: Semi-quantitative RT-PCR analysis of *NtWEE1* expression (normalised mean ± standard error) during the cell cycle in synchronized non induced WEE-1-i-1 cells following release from aphidicolin. The duration of the cell cycle component phases derived from the mitotic index are reported below the histogram (see chapter 3 section 3.5.3) (n=3).

4.3.4.3. Expression of *NtWEE1* and *AtWEE1* in induced WEE1-i-1 cells during the cell cycle

The pattern of expression for *H4* in the induced WEE1-i-1 cell line is high during S phase with a peak at 1 hour following the release from aphidicolin. Its level of expression decreases by ~35% at 4 hours following the release from aphidicolin and remains low thereafter (Fig. 4.8; see Appendix IV for raw data). This result fits well with the measure of S phase (3 hours) obtained from the mitotic index curve (calculated as described in chapter 3 section 3.4.3, see also section 3.5.3).

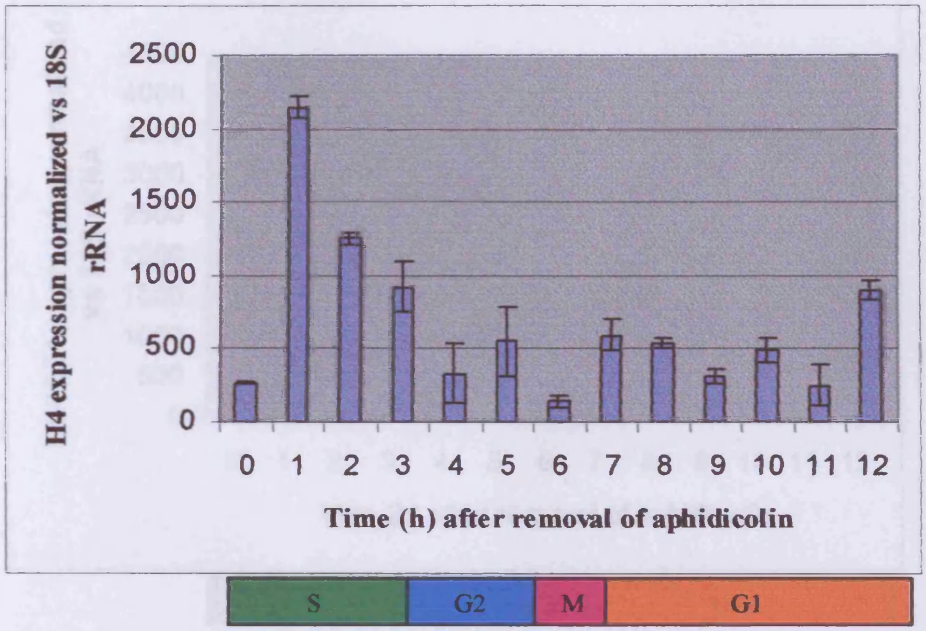


Fig. 4.8: Semi-quantitative RT-PCR analysis of *H4* expression (normalised mean ± standard error) during the cell cycle in synchronized induced WEE-1-i-1 cells following release from aphidicolin. The duration of the cell cycle component phases derived from the mitotic index are reported below the histogram (see chapter 3 section 3.5.3) (n=3).

However, the pattern of expression of *NtWEE1* in induced WEE1-i-1 cells was different from the expression of the same gene in wild type and non induced WEE1-i-1 cells. In contrast to these controls, the expression of *NtWEE1* in induced WEE1-i-1 began to rise in late G2 phase and peaked in the middle of M-phase (at 6 hours following release from aphidicolin) decreasing again in late mitosis (Fig.4.9; see Appendix IV for raw data).

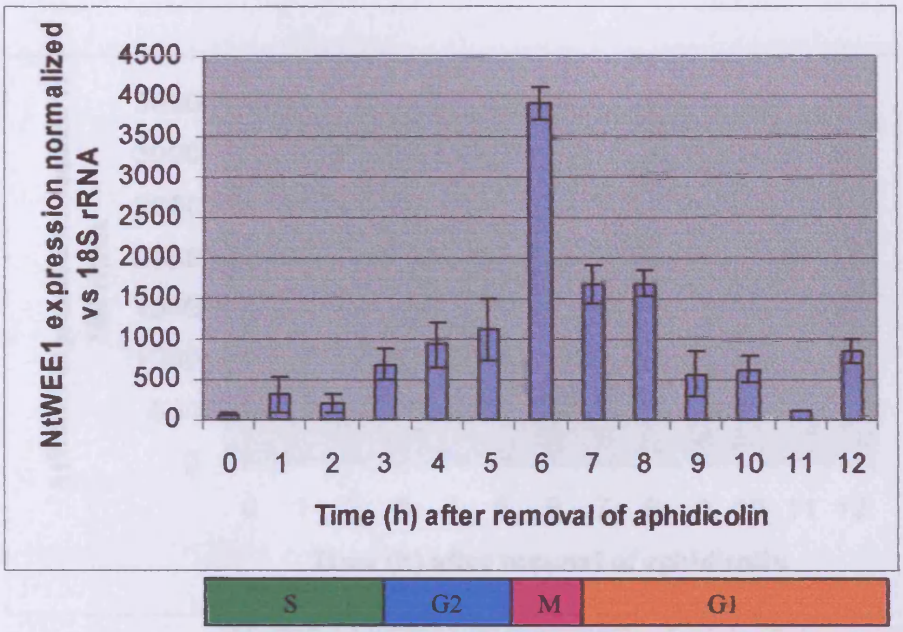


Fig. 4.9: Semi-quantitative RT-PCR analysis of *NtWEE1* expression (normalised mean ± standard error) during the cell cycle in synchronized and induced WEE-1-i-1 cells following release from aphidicolin. The duration of the component phases derived from the mitotic index are reported below the histogram (see chapter 3 section 3.5.3) (n=3).

Induction of the pTA7002 promoter resulted in an increased level of *AtWEE1* at 1 hour following release from aphidicolin with a transcriptional peak at 3 h which, equates with late S-phase/beginning of G2 phase. In G2, M and G1 phases the expression of *AtWEE1* decreased to a more or less constant level. The expression of *AtWEE1* increased again in G1/S-phase of the next cell cycle (Fig. 4.10, see Appendix IV for raw data).

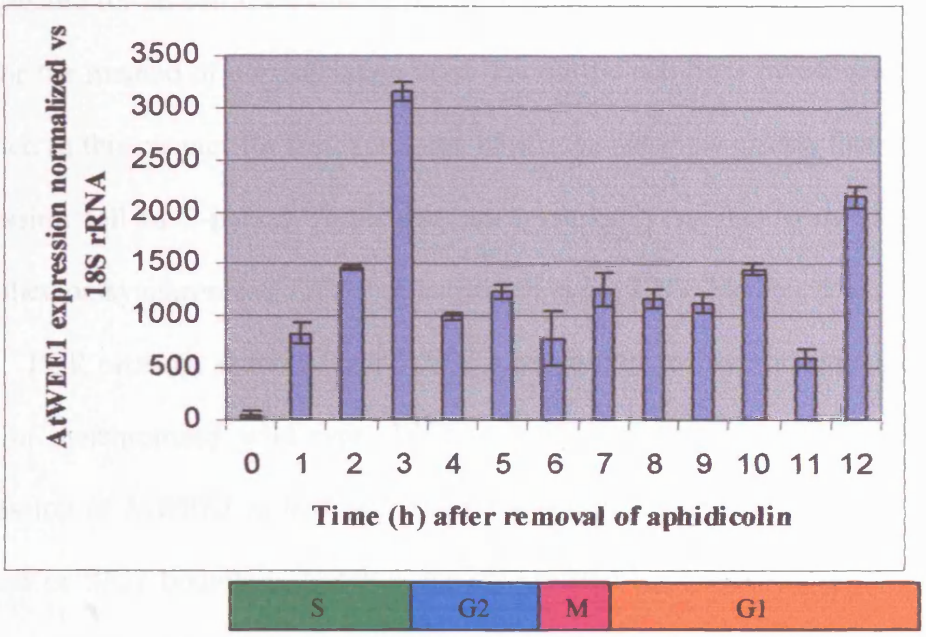


Fig. 4.10: Semi-quantitative RT-PCR analysis of *AtWEE1* expression (normalised mean ± standard error) during the cell cycle in synchronized induced WEE-1-i-1 cells following release from aphidicolin. The duration of the cell cycle component phases derived from the mitotic index are reported below the histogram (see chapter 3 section 3.5.3) (n=3).

4.4. Discussion

Calibration plots were used to demonstrate that a linear relationship exists between agarose gel band strengths of semi quantitative RT-PCR products and starting cDNA concentrations. This is important since PCR reactions need to be stopped during the linear phase of amplification if the assumption is made that PCR product yield is proportional to starting cDNA concentration. Normalization against a standard such as *18S rRNA* is also important to ensure that variations in total levels of cDNA in the different samples is taken into account when estimating the relative amounts of a particular message in different samples.

The pattern of expression of *histone H4* which typically peaks in S-phase was investigated for all cell lines studied, as a control for the quality of cDNA synthesized and for the method of normalization used. For all the cell lines investigated, the data reported in this chapter for the expression of *histone H4* show clearly that the level of expression fell in S-phase. These data are remarkably similar to those previously published on synchronized TBY-2 cells (Sorrell *et al.*, 1999; Herbert *et al.*, 2001).

PCR primers, specific for *NtWEE1* were used to analyse the expression of this gene in synchronised wild type, WEE1-i-1 non induced and induced cell lines. Expression of *NtWEE1* in both wild type and non induced WEE1-i-1 peaked during S-phase or S/G2 boundary. Notably, the data related to *NtWEE1* expression in wild type and WEE1-i-1 non induced are in complete agreement with each other. These data are also in complete agreement with the recently published analysis of *NtWEE1* expression during the TBY-2 cell cycle by Gonzalez and colleagues (2004). In their studies the authors investigated by semi-quantitative RT-PCR the pattern of expression of *NtWEE1*, and showed that *NtWEE1* was also strongly expressed during the S-phase and its expression decreased during mitosis and early G1-phase (Gonzalez *et al.*, 2004).

In molecular terms, the premature entry into mitosis at reduced cell size, observed for TBY-2 cell lines expressing *AtWEE1*, could be regulated at the transcriptional, translational or protein level (or all three). My hypothesis was that transcription of the *Nicotiana tabacum WEE1* was inhibited or suppressed in the presence of *AtWEE1* transcripts. Remarkably, when *AtWEE1* was induced in WEE1-i-1 cell line the major peak of *NtWEE1* expression was shifted by 4 hours from S phase to middle M phase. The delayed increase in the level of expression of *NtWEE1* in the induced WEE1-i-1 cell line, compared to non-induced and wild type,

did not confirm the initial hypothesis that transcription of the *Nicotiana tabacum WEE1* was inhibited. The timing of its expression was perturbed but not inhibited in the presence of *AtWEE1* transcripts so that a negative regulation of CDKs, normally imposed by NtWEE1, could be removed, enabling *AtWEE1*-expressing cells to enter into mitosis at a smaller cell size. Loss of *wee1* activity in fission yeast causes cells to enter mitosis before sufficient growth has occurred and therefore results in two abnormally small daughter cells (Nurse, 1975). Conversely, increasing the gene dosage of *wee1* causes delayed entry into mitosis and an increase in cell size, indicating that the levels of Wee1 kinase activity determine the timing of entry into mitosis and can have major effects on cell size (Russell and Nurse, 1987). Recent work has shown that Swe1, the budding yeast homologue of WEE1, also delays entry into mitosis and is required for cell size control (Jorgensen *et al.*, 2002; Harvey and Kellog, 2003). *Swe1*Δ cells undergo premature entry into mitosis before sufficient growth of the daughter bud has occurred; producing abnormally small cells (Harvey and Kellogg, 2003). Loss of function of WEE1 kinases in *Xenopus* and *Drosophila* also causes premature entry into mitosis; however, a requirement for WEE1 itself in cell size control has not yet demonstrated in animal cells (Walter *et al.*, 2000).

In addition, PCR primers specific for *AtWEE1* enabled comparative measurements of *AtWEE1* and *NtWEE1* expression in the inducible WEE1-i-1 cell line. Hence this experimental design enabled a comparison of *NtWEE1* expression alone or in the presence of *AtWEE1* transcripts in the different genetic backgrounds of wild type, induced and non-induced WEE1-i-1. Induction of *AtWEE1* resulted in a transcriptional peak of *AtWEE1* at 3 hours which corresponds to late S-phase/beginning of G2 phase. Transcription of the transgene depends on the activation of the promoter used in the construct. In the experiments reported in this

chapter the promoter was activated by the addition of DEX soon after removal of aphidicolin when the cells were entering into S phase (Aoyama and Chua 1997). Note that the transcriptional peak for *AtWEE1* occurred at the same time as the *NtWEE1* peak in non-induced WEE1-i-1. Thus, these data suggested that rather than a transcriptional inhibition of *NtWEE1 per se*, *AtWEE1* transcript affected the timing of *NtWEE1* transcription. The *AtWEE1* data suggest that replacement of *NtWEE1* expression in S phase by *AtWEE1* expression is insufficient to regulate cell size at division, suggesting that *AtWEE1* is not fully recognized in the tobacco cells.

4.5. Summary

Expression of *AtWEE1* in the induced WEE1-i-1 TBY-2 cell line results in an alteration of the timing of the endogenous *WEE1* gene during the cell cycle. In wild type the expression profile of *NtWEE1* revealed a major peak of transcription in late S-phase/beginning of G2-phase. In the non-induced *AtWEE1* cell line, *NtWEE1* transcript also peaked in mid-to-late S-phase. Remarkably, when *AtWEE1* was induced, the major peak of *NtWEE1* expression was shifted to M phase. Induction of *AtWEE1* resulted in a transcriptional peak at late S-phase.

Chapter 5: CDKA and CDKB1 kinase activity through the cell cycle in synchronized TBY-2 cells expressing *AtWEE1*

5.1. Introduction

The events of the cell cycle usually occur in a fixed sequence, and if an early event such as S-phase is incomplete then a later event such as mitosis becomes blocked. There are two general types of mechanism that could link S-phase and mitosis. There could be a hard wiring of the two events such that the later event is unable to occur physically or chemically without the early event. Alternatively the two events could be linked by a regulatory loop that inhibits the second until the first is complete. Hartwell and Weinert (1989) developed the second of these two events into the idea of checkpoints where the cell monitors or "checks" cell cycle progression at certain points during the cell cycle, and if the events prior to that point are incomplete, further progression is delayed (Hartewell and Weinert, 1989). Cyclin dependent kinases (CDKs) are recognized as key players in checkpoint controls of the eukaryotic cell cycle. One of the most important roles for CDKs is to ensure that there is only one S phase in the normal cell cycle. When a proliferative cell completes S phase and enters G2, another S phase does not take place until the mitosis of that cell cycle is complete (Broeck *et al.*, 1991).

In fission yeast, there is a dependency of mitosis upon completion of S phase, this dependency is lost in cells with specific *cdc2* mutations or in mutants with a high level of

the CDK activator, Cdc25 (Enoch and Nurse, 1990) (for more details see chapter 1 section 1.4).

In contrast to the yeast cell which has only one type of CDK gene (*CDC28* for *Saccharmoyces cerevisiae* and *cdc2* for *Schizosaccharomyces pombe*), higher eukaryotic cells have many different types of CDKs (for more details see chapter 1 section 1.1.2). Recently, the tobacco BY-2 cell line was used for studies of plant CDKs and their kinase activity during the cell cycle. A cDNA of a PSTAIRE (CDKA) gene *cdc2Nt1* (renamed Nicta;*CdkA;3* by Joubes *et al.*, 2000) has been cloned from tobacco, and RNA gel-blot analysis showed this gene to be preferentially expressed in dividing TBY-2 cells but did not show significant cell cycle regulation of transcript abundance (Setiady *et al.*, 1996). Sorrell *et al.* (2001) reported the isolation, from a TBY-2 cell cDNA library, of a PSTAIRE CDKA (Nicta; *CdkA;4*) closely related to cdc2Nt1 (Setiady *et al.*, 1996) and to other novel tobacco CDKs (Nicta;*CdkB1;1* and Nicta;*CdkB1;2*) containing the PPTALRE sequence and belonging to the CDK-b1 subgroup. Sorrell *et al.* (2001) demonstrated that CDKA and CDKB1 have a different level of expression during the tobacco BY-2 cell cycle. *CDKA* transcript levels remained at an approximately constant level during the cell cycle, whereas *CDKB1* transcript levels varied with the lowest levels in G1 and the highest levels in S, G2 and M phases (Sorrell *et al.*, 2001). CDKA protein levels, like the RNA levels, were relatively constant throughout the cell cycle except for a decline during G1 phase, but the CDKA kinase activity was higher during the S and G2 phases of the cell cycle (Sorrell *et al.*, 2001). CDKB1 protein levels showed a gradual accumulation from S phase until mitosis, followed by a gradual decline, but the CDKB1 kinase activity showed a sharp peak at the G2/M boundary, followed by an abrupt decline

(Sorrell *et al.*, 2001). These data suggested a differential role for CDKB1 relative to CDKA during the tobacco cell cycle (Sorrell *et al.*, 2001).

In fission yeast, negative (Wee1/Mik1) and positive (Cdc25) regulation of Cdc2 interact to regulate cell size at mitosis (for more details see chapter 1 section 1.4). In data reported in chapter 3, *AtWEE1* expression induced a small cell size in TBY-2 cells. This was surprising given that *AtWEE1* expression induced a long cell length in fission yeast (Sorrell *et al.*, 2002). The *AtWEE1* induction of a small cell size in TBY-2 cells (for more details see chapter 3) was a result of a premature onset of mitosis through a shortening of G2 phase. The data led to the hypothesis that the biochemical basis of this response would be increased CDKA or CDKB (or both) kinase activity, to drive cells into an "early" mitosis.

The aims of the work reported in this chapter were to examine whether expression of *AtWEE1* in transformed TBY-2 cells resulted in a change in CDKA and CDKB1 kinase activity during the cell cycle.

5.2. Materials and methods

5.2.1. Protein extraction

Following synchronization and release from aphidicolin, 5 ml of TBY-2 cell cultures were collected into polypropylene tubes (Falcon) every hour for a 12 h period. The samples were centrifuged at 3500 rpm (MSE Centaur 2) for 5 minutes to form a pellet. The supernatant was discarded and the pellet ground to a fine powder in liquid

nitrogen using a pestle and a mortar precooled at -20°C. Powdered cells were transferred to 1.5 ml microcentrifuge tube and resuspended in 1 ml of lysis buffer (0.5 M Tris-HCl pH 7.5, 0.75 M NaCl, 0.15 M $MgCl_2$, 1 M dithiothreitol, 0.1% Tween 20, 1x complete Tm protease inibitors (Roche), 50 mM NaF, 10 mM NaV, 100 mM Na-pyrophosphate, 0.6 M β-glycerophosphate) on ice. The suspension was homogenized by vortexing for 4 x 30 seconds with 1 min on ice between homogenizations and centrifuged at 13000 rpm (Beckman Coulter Allegra ™ 21R, rotor F2402H) for 30 minutes at 4°C; the supernatant was transferred to a new microcentrifuge tube on ice. In order to measure the concentration of total proteins extracted using a spectrophotometer λ=595nm (Smartspec™3000, Bio-Rad laboratories Ltd) one µl of extract was transferred to 1 ml Coomassie® Protein Assay Reagent (Bradford assay; Sigma) and the absorbance was read against a standard curve (Fig. 2.5). Samples were stored at -80°C until further use.

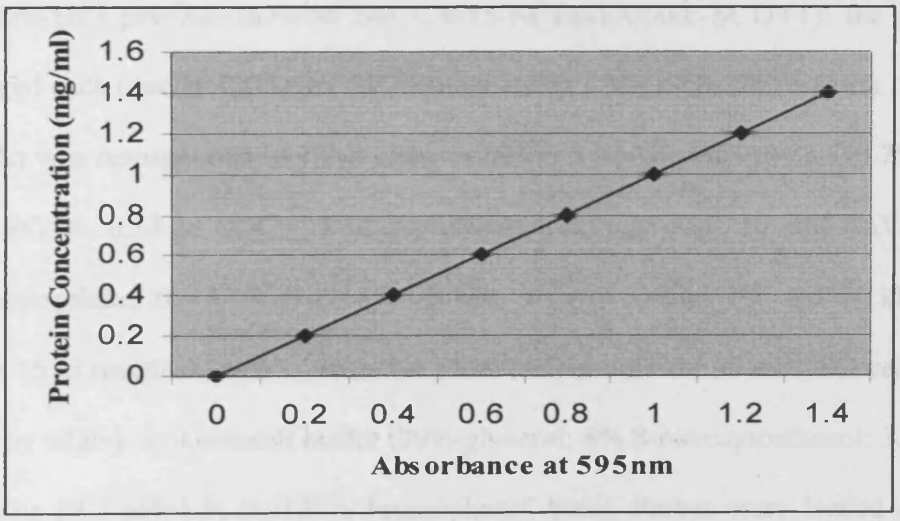


Fig. 2.5: Standard curve for proteins concentration measured against absorbance using known amounts of bovine serum albumen (BSA).

5.2. 2. Kinase Activity

For immunoprecipitations and kinase assays, 250 µg of proteins extracted from TBY-2 cell cultures were pre-incubated with 20 µl of protein A-Sepharose and an appropriate volume of lysis buffer to a final volume of 250 µl. The proteins were rotated for 30 min at 4°C and centrifuged at 13000 rpm (Beckman Coulter Allegra ™ 21R, rotor F2402H) for 10 minutes at 4°C. The supernatant was transferred into a new microcentrifuge tube and incubated with 2 µl of specific antiserum (NtCDKA or NtCDKB) for 2 h on ice. Twenty µl of protein A-Sepharose and 150 µl of lysis buffer were added to a final volume of 400 µl. The samples were rotated at 4°C for 1 h and centrifuged at 3000 rpm (Beckman Coulter Allegra ™ 21R, rotor F2402H) for 1 minute. The pellet was washed 4 times with 1 ml of Suc1-buffer (0.5 M Tris-HCl pH=7.5; 0.75 M NaCl; 0.5 M EDTA; 0.5 M NaF; 0.1% Tween 20) and 2 times with 1 ml of kinase buffer (0.5M Tris-HCl pH=7.5; 0.75 M NaCl; 0.15 M EGTA; 0.1 M DTT); the pellet was centrifuged each time at 3000 rpm for 1 minute using a MiniSpin (Eppendorf).

The pellet was resuspended in 15 µl assay buffer (0.5 M Tris-HCl pH 7.5, 0.75 M NaCl, 0.15 M EGTA, 0.15 M $MgCl_2$, 1 M dithiothreitol, 50 mM NaF, 10 mM NaV, 100 mM Na-pyrophosphate, 0.6 M β-glycerophosphate, 5 µg/µl histone H1 and 74 kBq (γ-P^{32}) ATP per 15 µl reaction), and incubated at room temperature for 30 min. The reaction was stopped by adding 2x Laemmli buffer (20% glycerol; 8% β-mercaptoethanol; 3.2 % SDS; 0.1 M Tris-HCl pH=6.8; 0.0125% bromophenol blue). Probes were loaded on a 10% SDS-PAGE minigel composed of two phases: 1) resolving gel (where the proteins are resolved: 1.25 ml 10% acrylamide; 0.81 ml resolving gel buffer (1.5 M Tris-HCl pH=8.8;

0.4% SDS); 1.61 ml UHP water; 37.5 µl 12% ammonium persulphate; 3.75 µl TEMED) and 2) stacking gel (where samples are loaded: 0.325 ml 10% acrylamid; 0.5 ml stacking buffer (0.5 M Tris-HCl pH=6.8; 0.4% SDS); 2 ml UHP water; 12.5 µl 12% ammonium persulphate; 2.5 µl TEMED). Samples were run at 150 mV with 1x running buffer (1.5% Tris-base; 7.2% glycine; 0.5% SDS) until the dye front was running over the bottom of the gel. For detection of CDK activity the SDS-PAGE gel was wrapped in Saran Wrap™ (DOW Chemical Company) and exposed to radiograph film (Hyperfilm ECL; Amersham) in the dark room. The orientation of the film compared to the SDS-PAGE gel was marked. After exposure, the film was developed and fixed using the developer machine Curix 60 (Agfa). Samples were analyzed and quantified using a Gene Genius Bioimaging System and Gene Tools software package (Syngene Ltd.).

Since the total number of samples was 11 for non-induced WEE1-i-1, and the SDS-gel contained only eight lanes, it was necessary to run samples on different SDS-gels. However, each sampling time comprised at least two replicates.

5.3. Results

5.3.1. Kinase activity of CDKs in TBY-2 cells expressing *AtWEE1*

CDKA and CDKB1 kinase activity were investigated during the cell cycle in synchronously cycling WEE1-i-1 induced and non-induced cells. The cells were synchronized by blocking the cell cycle in early S-phase with aphidicolin. After the aphidicolin block was released, dexamethasone (DEX) was added only to one flask of the

WEE1-i-1 cell line to induce the expression of *AtWEE1*, whereas a second culture of WEE1-i-1 was not induced and served as the control. The progress of cells through the cell cycle was followed by monitoring changes in mitotic index, and cell samples were harvested every hour for 13 hours.

5.3.1.1. Kinase activity of CDKA and CDKB1 in non-induced WEE1-i-1 cells

Following a synchronization experiment, proteins were extracted from non-induced WEE1-i-1 cells choosing specific time points during the cell cycle (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12 hours). Based on the mitotic index curve, these sampling times represented S, G2, M and G1-phase of the non-induced *AtWEE1* line cell cycle (Fig. 5.1).

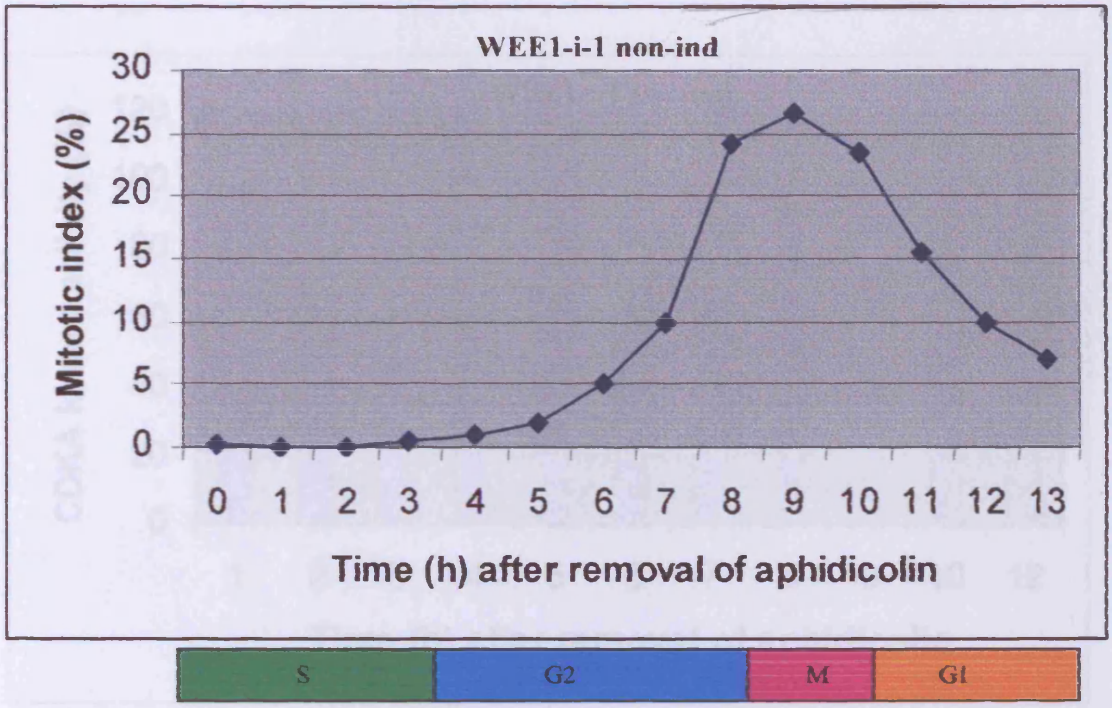


Fig. 5.1: Mitotic index curve, of synchronized non-induced WEE1-i-1 cells, generated from the same experiment used to collect cell samples for protein extractions used for CDK kinase activity assays. The corresponding cell cycle component phases are shown below the graph.

The level of CDKA kinase activity was maximal 1 hour following the release from aphidicolin (early S-phase, Fig. 5.2; see Appendix V for raw data). Subsequently its activity dropped at S/G2 but never fell below 50% compared with the maximal kinase activity level. However, CDKA kinase activity then increased in late G2/beginning of M-phase.

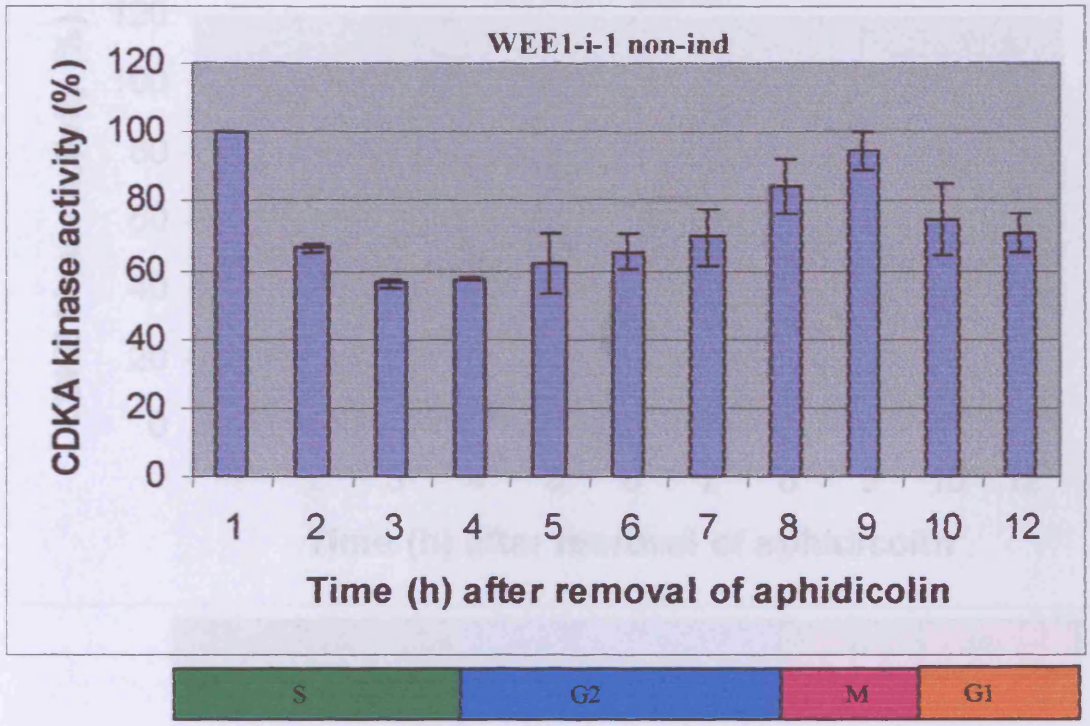


Fig. 5.2: Mean (± S.E.) of CDKA kinase activity in synchronized non-induced WEE1-i-1 cells as percentages of maximum activity (n≥2). The corresponding cell cycle component phases are shown below the graph.

In contrast to CDKA, the kinase activity of CDKB1 peaked at 7 hours (late G2 phase) (Fig. 5.3; see Appendix V for raw data); it started to increase in early G2-phase and peaked in the late G2-phase. The activity then decreased during mitosis and the following G1 phase back to 50% of maximal levels of activity. This pattern of CDKB1 activity is very similar to that for wild type cells (Sorrell *et al.*, 2001).

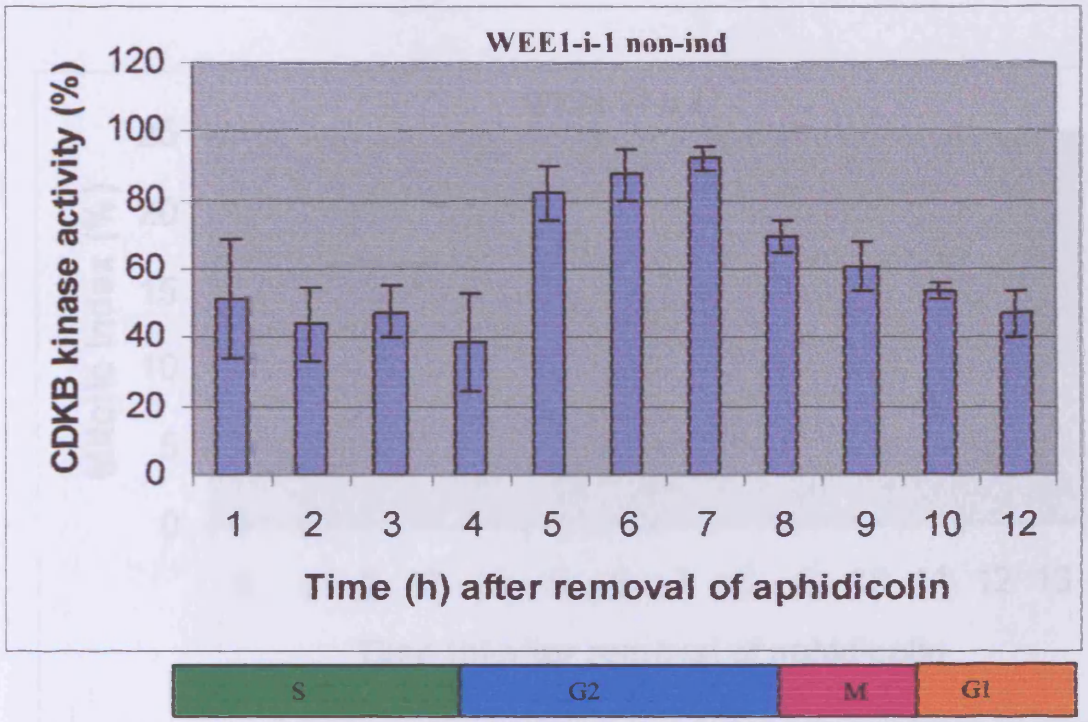


Fig.5.3: Mean (± S.E.) of CDKB1 kinase activity in synchronized non-induced WEE1-i-1 cells as percentages of maximum activity (n≥2). The corresponding cell cycle component phases are shown below the graph.

5.3.1.2. CDKA and CDKB1 kinase activity in induced WEE1-i-1 cells

Following synchronization, *AtWEE1* expression was induced in theWEE1-i-1 cell line by adding dexamethasone immediately after removal of aphidicolin. Every hour for 13 hours, cell samples were harvested and proteins extracted at specific time points during the cell cycle (1, 3, 4, 5, 6, 7 and 9 hours). Based on the mitotic index curve, these samples represented S, G2, M and G1-phase of the induced *AtWEE1* line cell cycle (Fig. 5.4).

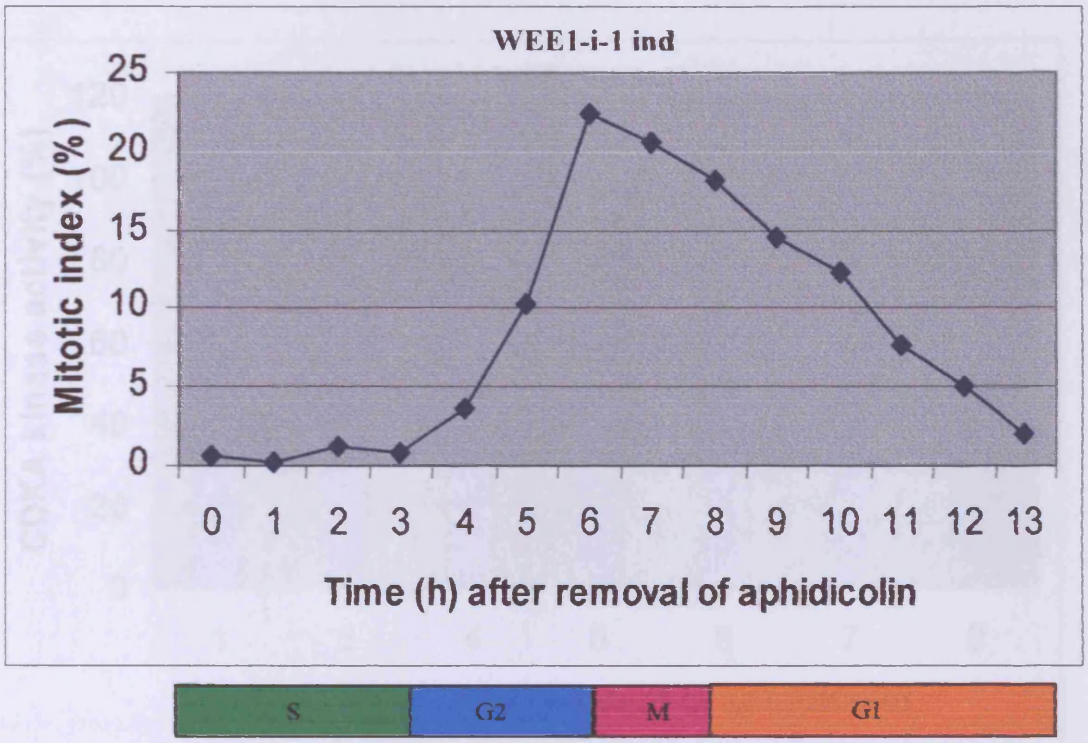


Fig. 5.4: Mitotic index curve, of synchronized induced WEE1-i-1 cells, generated from the same experiment made to collect cell samples for protein extractions used for CDK kinase activity asaays. The corresponding cell cycle component phases are shown below the graph.

The highest CDKA kinase activity for induced WEE1-i-1 cells was 1 hour following the release from aphidicolin which corresponds to early S-phase (Fig. 5.5; see Appendix V for raw data). Subsequently its activity dropped reaching a more or less constant level during S and G2 phase, without falling significantly below 60% compared to the maximum activity level in S phase.

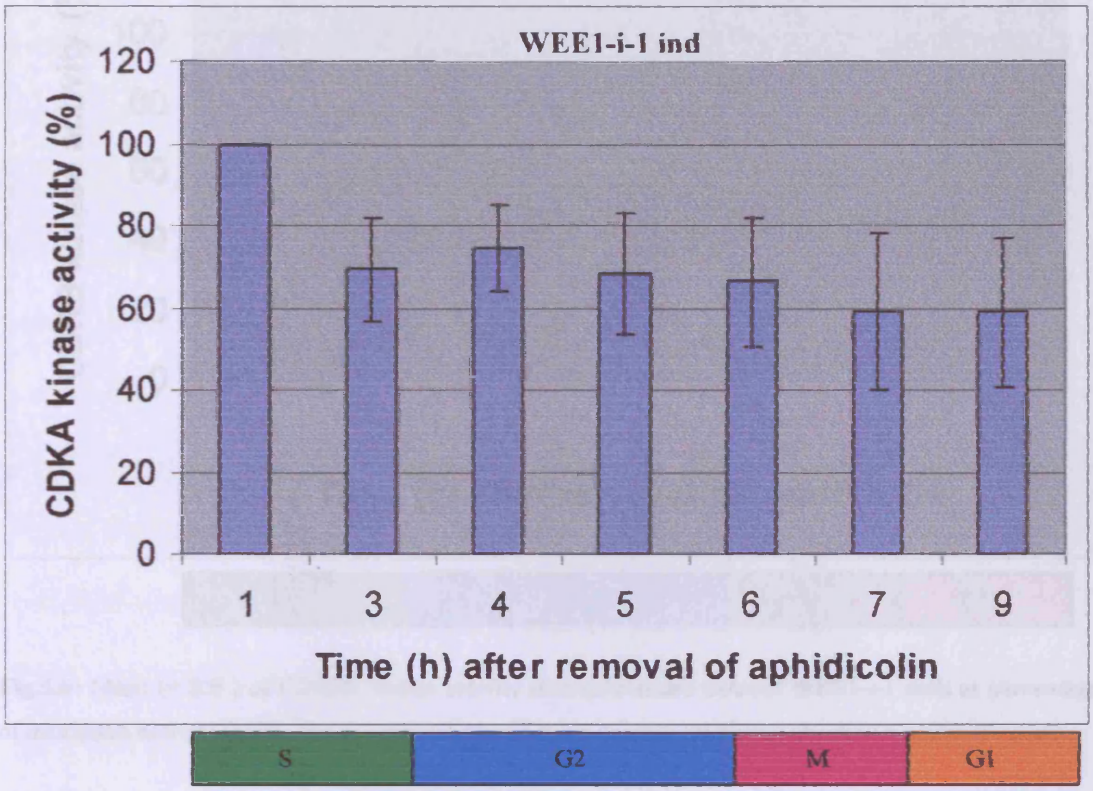


Fig.5.5: Mean (± S.E.) of CDKA kinase activity in synchronized induced WEE1-i-1 cells as percentages of maximum activity (n≥2). The corresponding cell cycle component phases are shown below the graph.

CDKB1 kinase activity showed a very similar pattern to that of CDKA (Fig 5.5). CDKB1 was, indeed, very high 1 hour following the release from aphidicolin. Its activity, like CDKA, then dropped in mid S-phase and remained low at 50-60% of maximal level in late S, G2 and M phase (Fig. 5.6; see Appendix V for raw data).

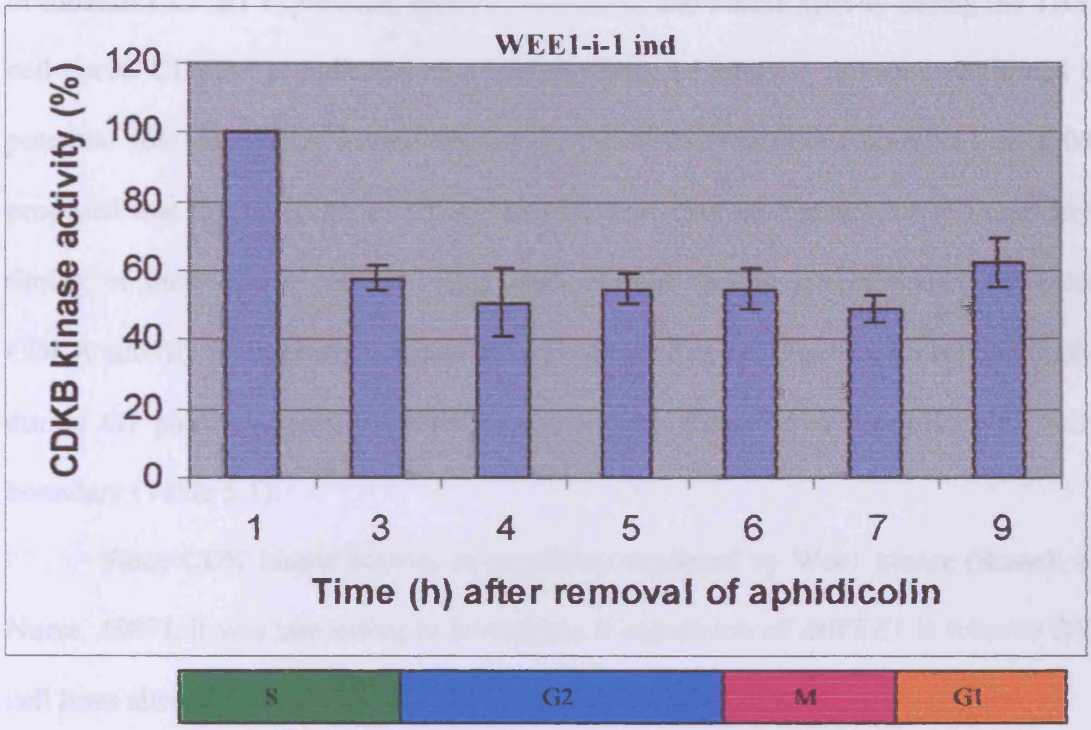


Fig.5.6: Mean (± S.E.) of CDKB1 kinase activity in synchronized induced WEE1-i-1 cells as percentages of maximum activity (n≥2). The corresponding cell cycle component phases are shown below the graph.

5.4. Discussion

Previous publications (Mironov *et al.*, 1999; Joubes *et al.*, 2000) have reported CDK kinase activity in plant cells during the cell cycle. According to these publications CDKA kinase activity starts to rise in late G1 phase and peaks at the G1/S border (Table 5.1). In late S phase the level of CDKA activity decreases until late G2 phase and a second peak occurs in mitosis (Table 5.1). Interestingly, tobacco CDKB1 kinase has a constantly flat level of activity until late G2 phase then its activity starts to increase peaking in mitosis (Table 5.1). Recently, Sorrell *et al.* (2001) carried out the first analysis

of tobacco CDKB1 expression, protein abundance, and kinase activity during the TBY-2 cell cycle. CDKB1 is indicated as a key regulator of mitosis, also they confirmed the potential role of CDKA kinase activity in the G2/M transition. Sorrell *et al.* (2001) proposed that the functions of CDKA and CDKB1 may be distinct due to the different timing of their kinase activity. They also reported that in synchronous TBY-2 cells CDKA activity is relatively constant throughout the cell cycle except for a slight decline during G1 phase, whereas CDKB1 kinase activity showed a sharp peak at the G2/M boundary (Table 5.1).

Since CDK kinase activity is negatively regulated by Wee1 kinase (Russell and Nurse, 1987), it was interesting to investigate if expression of *AtWEE1* in tobacco BY-2 cell lines altered the normal level of CDK kinase activity.

Previous experiments (described in chapter 4) had shown that expression of *AtWEE1* in the tobacco BY-2 cell line results in a delayed expression of the endogenous *NtWEE1* gene. This might enable tobacco CDKs to exhibit an earlier kinase activity. This hypothesis was tested by measuring kinase activity of both CDKA and CDKB1 in the induced WEE1-i-1 cell line. As a control CDKA and CDKB1 kinase activity were tested in non-induced WEE1-i-1 cell line.

In the induced WEE1-i-1 cell line both CDKA and CDKB1 kinase activity peacked 1 hour after removal of aphidicolin which corresponds to early S phase (Figs 5.5 and 5.6) (Table 5.1). Subsequently their activities dropped at the S/G2 border, but never went below 50% compared to maximum. In non-induced WEE1-i-1 cell line, CDKA kinase activity, in agreement with Mironov *et al.* (1999) and Joubes *et al.* (2000), was high at 1 hour following release from aphidicolin (early S phase) and exhibited a second peak at

the G2/M boundary (Table 5.1). However, CDKB1 kinase activity was low from S phase to the beginning of G2 phase, where its level of activity started to increase until it peaked at 7 hour after removal of aphidicolin, which corresponds to late G2 phase (Figs 5.2 and 5.3) (Table 5.1). These data are remarkably similar to those from Sorrell *et al.* (2001) who also reported a peak of CDKB1 kinase activity at G2 phase. These results demonstrate clearly that expression of *AtWEE1* delays expression of *NtWEE1*, but does not affect CDKA kinase activity, in the induced WEE1-i-1 cell line. However, the expression of *AtWEE1* had a profound effect on CDKB1 kinase activity which peaks in M phase for non-induced WEE1-i-1 but in early S phase for induced WEE1-i-1 cells.

In tobacco BY-2 cells expressing *AtWEE1,* the earlier entry into mitosis can be interpreted as the response to an earlier activation of CDKB1 in the cell cycle. Interestingly, expression of *Spcdc25* in the tobacco BY-2 cell line lead tobacco cells to divide prematurely as a direct result of increased Cdc25 phosphatase activity. This resulted in a higher activity of CDKB1 at earlier samples time compared with the control (empty vector). CDKB1 kinase activity was consistently high in S and early G2 phase (Orchard *et al.*, 2005).

As already reported, CDKA and CDKB1 have distinct roles during the cell cycle due to the different timing of their activity (Sorrell *et al.*, 2001). CDKB1 is thought to be more impotant at G2/M transition (Sorrell *et al.*, 2001). The data reported in this chapter confirm an involvement of CDKB1 in regulating entry into mitosis.

Table 5.1: Summary of peaks of CDKs activity in the data presented in this chapter compared with published data in plants. Note the premature CDKB1 activity in the transgene expressing lines.

CDKA		CDKB1		
G1/S	**G2/M**	**G1/S**	**G2/M**	**Source**
+	+	-	+	Plants (Mironov *et al.*, 1999; Joubes *et al.*, 2000)
Constant through the cell cycle		-	+	Tobacco BY-2 cells (Sorrell *et al.*, 2001)
+	-	+	-	WEE1-i-1 ind cells
+	+	-	+	WEE1-i-1 non-ind cells

5.5. Summary

Expression of *AtWEE1* in tobacco BY-2 cells results in an alteration of the timing of *NtWEE1* expression, and as a consequence unlocks the negative regulation activity normally imposed by NtWEE1 kinase on CDKB1. The net result is a premature increase in CDKB1 kinase activity.

Chapter 6: Over expression of *AtWEE1* alters root growth and development in *Arabidopsis thaliana*

6.1. Introduction

Experimental evidence suggests that regulatory proteins such as WEE1 play a very important role during the cell cycle and they might have an important part in regulating plant growth and development. Altered cell cycle duration can influence growth in a direct manner. For example, in plant roots cell division takes place in the root apical meristem (RAM). Cell division in the RAM contributes to primary root growth by providing the root with the new cells it needs in order to grow. In theory, the faster the rate of cell division, the faster the rate of primary root elongation. Investigations into the primary root growth rates of several ecotypes of *Arabidopsis* confirmed this hypothesis: the ecotypes with the fastest growth rates exhibited short cell cycle duration times and elevated rates of cell division (Beemster *et al.*, 2002). In addition, CDKs and other cell cycle control factors such as cyclins are clearly capable of altering plant growth rates by modulating the cell cycle duration (Doerner *et al.*, 1996; Doonan, 1996; Cockcroft *et al.*, 2000; De Veylder *et al.*, 2001a-b; Beemster *et al.*, 2002). However, cell elongation is also very important in controlling plant growth rate; both elongation and cell division make equally important contributions to growth rate (Beemster *et al.*, 2000, 2003). The cell cycle is not only important in controlling growth, it may also play a complex role in the control of plant organ development. For example, lateral roots form from the pericycle, the outer-most layer of the vascular cylinder. Clusters of pericycle cells, act as founder cells of lateral root morphogenesis. In order for primordia to form, the founder cells of the pericycle must divide first transversely and then longitudinally (Dubrovsky et al., 2001). Thus, the

formation of root organs is highly dependent on the rate, site and plane of cell division (Meyerowitz, 1997). Interference with cell cycle control often leads to changes in plant development. The over-expression of ICK1 (a CDK inhibitor) in *Arabidopsis* altered root, leaf and petal shape (Wang *et al.*, 2003). In addition, the expression of the fission yeast homologue to *cdc25* (*Spcdc25*) in tobacco plants led to small mitotic cell size and an increased frequency in lateral root initiation. In these experiments, the expression of *Spcdc25* led to an increase in the number of lateral roots formed per unit length of primary root (McKibbin *et al.*, 1998). *Spcdc25* expression is also capable of stimulating tobacco shoot development, even in the absence of the plant growth regulators and in high auxin and cytokinin treatment that should favour root formation (Suchomelova *et al.*, 2004). Control genes such as *AtWEE1* may also have an important role to play in the development of plant organs.

The aims of the work reported in this chapter, were to check the possible role of *AtWEE1* in the control of primary root growth and lateral root initiation in *Arabidopsis* plants.

6.2. Materials and methods

Arabidopsis thaliana (L. Heynh. ecotype Columbia) was independently transformed using two different constructs containing respectively a constitutive promoter (BIN HYG TX) and an inducible promoter (pER8) both driving the *AtWEE1* gene. The BIN HYG TX vector was already discussed in chapter 2 section 2.2.1. The inducible promoter vector (pER8) allowed the expression of *AtWEE1* only when oestradiol was added to the culture (Fig. 6.1). The *AtWEE1* ORF was amplified using PCR primers incorporating *XhoI* and *SpeI* restriction sites. The PCR product was

cloned into pER8 by cutting and ligating at the *XhoI* and *SpeI* restriction sites in the vector (see chapter 2 section 2.1.1). Both vectors also contained a hygromycin resistance gene, so that plants containing the constructs could be selected.

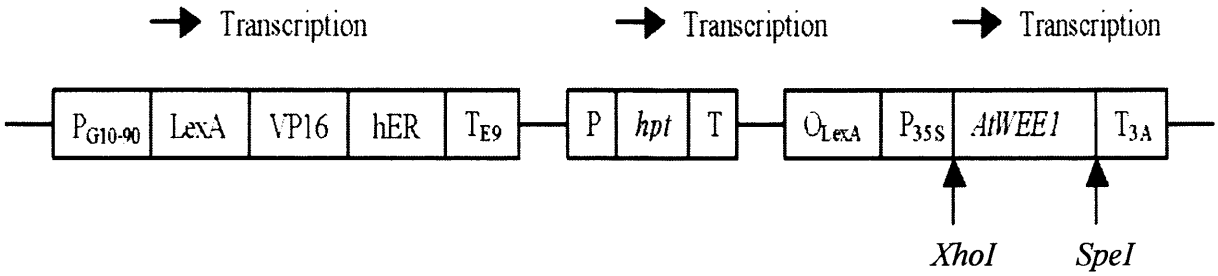


Fig. 6.1: The pER8-*AtWEE1* construct was based on the system developed by Professor N.H. Chua (Zuo *et al.*, 2000), consisting of a synthetic promoter driving the transcription of an estradiol-inducible transcriptional factor, composed by the DNA binding domain of the bacterial repressor LexA, the transcription activation domain of VP16 and the regulatory region of the human estrogen receptor (hER). The terminator sequence E9, encodes a poly-A tail addition site that terminates the production of mRNA by the transcription factor. The *hpt* gene encodes hygromycin phosphotransferase II – required for hygromycin resistance. This gene was under the control of its own promoter and terminator. The *AtWEE1* open reading frame (ORF) was inserted under the control of the minimal 35S promoter fused with 8 copies of the LexA operator sequence. Translation of this sequence was terminated by the 3A terminator.

6.2.1. Isolation of transgenic lines

Both BIN HYG TX-*AtWEE1* and pER8-*AtWEE1* constructs were transformed first into *Escherichia coli* (see chapter 2 section 2.2.1) and then into *Agrobacterium tumefaciens* GV1301 (see chapter 2 section 2.2.2). The presence of the plasmids in *Agrobacterium* was checked by PCR before they were used as vectors to transform *Arabidopsis* plants.

To transform *Arabidopsis thaliana* plants, *Agrobacterium* cells were cultured over-night in 500 ml of 2xYT-medium (per litre: 16 g bacto-tryptone, 10 g bacto-yeast extract, 5 g NaCl, pH=7) at 30°C with gentle shaking. The bacterial cells were then centrifuged at 5500 g for 20 min at room temperature (Beckman Coulter J-E centrifuge, rotor JA-14). *Agrobacterium* cells were then resuspended in a solution of 5% sucrose and 0.05% Silwet L-77 (Lehle Seeds) in 500 ml distilled water. The *Agrobacterium* cells were placed in a metal bowl and *Arabidopsis* plants were inverted and dipped into the bacterial suspension for approximately 10 seconds with agitation. The plants were then covered with a plastic bag over-night to retain humidity.

A few weeks following the transformation, seeds were collected from the transformed plants and stored for two weeks at room temperature. Seeds were then sterilized by first immersing them in bleach/water mix (1:10 with water) for 5 minutes, followed by a 5 minute immersion in an ethanol/water/bleach mix (7:2:1 parts respectively). The seeds were then washed three times with sterile distilled water for 5 minutes each time. After the third wash, the seeds were added to a solution of water and agar (0.8%) and sown into Petri dishes containing Murashige and Skoog medium (1962) (M&S, basal salt mixture, Duchefa Biochemie, Netherlands) supplemented with 3% (w/v) sucrose, 1% agar and 30 mg/l hygromycin at a density of approx 10,000 seeds per plate. Transformants were clearly identifiable as they grew on and were green, while untransformed seeds bleached at the cotyledon stage and did not grow any further. At the stage of 3-4 small leaves, the plants were transferred to soil to form the first generation. At this stage, the presence of the construct in the plants was confirmed by PCR. Approximately 200 mg of leaf tissue were placed in a 1.5 ml microfuge and ground using an microfuge grinder; 200 µl of DNA extraction buffer

(0.5% sodium dodecyl sulphate, 250 mM NaCl, 100 mM Tris-HCl (at pH=8) and 25 mM EDTA) were added to the homogenate and the mix was centrifuged for 5 minutes at 12,000 rpm in a microcentrifuge (Eppendorf MiniSpin). The resulting supernatant (150 µl) was removed from the microfuge tube and transferred to a fresh one containing an equal volume of isopropanol. This mix was incubated on ice for 5 minutes before another centrifugation at 12000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was removed and the pellet was air dried at room temperature for 10 minutes and then resuspended in 100 µl of Tris-EDTA (10 mM Tris , 1 mM EDTA, pH=8) buffer.

The plant DNA extracts were used in PCR reactions with primers designed to target the BIN HYG TX-*AtWEE1* construct (BHTXF 5'– GTTAACGGTACCCGGGCTCG and P39 5'– CATGGGATGGTCCAGTAATTC) or the pER8-*AtWEE1* construct (primers 35STRS 5'– ACGCTGAAGCTAGTCGACTC and P61 – GTAATGCCTTTGCTATC) respectively. The PCR reactions were as follows: 1 µl of DNA extract was added to 24 µl of PCR mix, composed of 1 µl of the respective primers (10 µM of forward, 10 µM of reverse primer), and 22 µl of Reddy Mix™. Each mix was subjected to 28 cycles in a PTC100 thermocycler consisting of 95°C for 1 minute, then 55°C (for BHTXF and P39 primers) or 60°C (for 35STRS and P61 primers) for 1 minute and finally 72°C for 1 minute. A final incubation of 72°C was carried out for 15 minutes before the PCR mixtures were cooled to 15°C. Five µl of each PCR product were run on a 1% agarose gel containing 1µg/ml ethidium bromide. Positive bands indicated the presence of the constructs in the transgenic plants which were selected for further studies. Subsequently, seeds from the first generation of successfully transformed plants were collected and used to form the second generation, which was used for the

experiments described in this chapter.

6.2.2. RNA extraction, cDNA synthesis and RT-PCR

DNA extractions were performed to confirm the successful incorporation of the construct into *Arabidopsis* plants, but they did not prove that the transgenes were being expressed. RT-PCR was used to demonstrate that the transgenic lines were expressing *AtWEE1* at the mRNA level. Total RNA was extracted from two to three leaves of the second plant generation. Leaf material (equivalent to about 200 mg), was collected from plants, wrapped in aluminium foil and immediately frozen in liquid nitrogen. Leaves were then removed from the aluminium foil and ground to a powder in liquid nitrogen using a sterile pestle and mortar (pre-cooled to -20°C). Details of mRNA extractions, cDNA synthesis and cDNA amplification are fully described in chapter 2 sections 2.4.1, 2.4.2 and 2.4.3.

BIN HYG TX-*AtWEE1* and pER8-*AtWEE1* cDNA samples were subjected to PCR using the primers described in section 6.2.1 of this chapter. Since BHTXF and 35STRS were designed to be directed against the vectors BIN HYG TX and pER8 respectively, they can be used to discriminate between the transgene and the endogenous *AtWEE1*. Each of the cDNA samples was used in a RT-PCR reaction mix consisting of 1 µl cDNA, 22 µl of Reddy Mix™, 1 µl of 10 µM forward primer and 1 µl of 10 µM reverse primer. The total number of cycles in a PTC100 thermocycler were 40 consisting of 95°C for 1 minute, 55°C for 1 minute and finally 72°C for 1 minute. Five µl of each of the PCR reaction products were run on a 1% agarose gel containing 1 µg/ml ethidium bromide. Positive bands indicated the presence of *AtWEE1* mRNA in leaf material, where it is not normally expressed at high levels.

6.2.3. Seed sterilization, growth conditions and methods of fixation

Seeds from both BIN HYG TX-*AtWEE1* and pER8-*AtWEE1* transgenic lines were planted alongside the wild type seeds for comparison. All seeds were sterilised (as described in section 6.2.1 of this chapter) and plated individually onto round Petri dishes containing a suitable growth medium. In the case of the wild type, the growth medium consisted of just the basal media: 1% Murashige and Skoog medium at pH 5.7, supplemented with 30 g/l of sucrose and 10 g/l of agar. Transgenic lines were grown on the same basal medium (M&S), which contained 30 mg/l hygromycin used to select for recombinant seedlings. All seeds were arranged in rows, with approximately 20-25 seeds per Petri dish placed more or less 1 cm apart. The plates containing the seedlings were then sealed using porous tape (Micropore™) and incubated at 4°C for 48 hours to coordinate and stimulate germination. All seedlings were then grown in 18 hours light / 6 hours dark per day at 22°C, usually for 2 weeks, but different experiments required different incubation times. The plates were placed at an angle of approximately 45°.

6.2.4. *AtWEE1* lines: growth conditions and sampling methods

Samples, consisting of germinated seedlings, were taken at time points ranging from 15 to 19 days post-incubation, typically every two days (i.e. day 15, 17 and 19). All samples were removed from the agar and fixed in 70 % ethanol:glacial acetic acid (3:1).

Fixed samples were stored at 4°C until removed and stained with Feulgen as follows: each sample was transferred from the ethanol:glacial acetic acid fixative to a

fresh 1.5 ml microfuge tube and rinsed in approximately 1 ml of distilled water for 5 minutes at room temperature. The samples were then added to 1 ml of 5 M HCl for 25 minutes at 25°C and then subjected to three separate 5 minute rinses with distilled water at 4°C. All samples were then immersed in 1 ml of Feulgen stain (Schiff's reagent, Fisher Scientific, U.K.) for 60 minutes.

pER8-*AtWEE1* seedlings were grown for 14 days under the conditions described above on M&S/hygromycin supplemented with 5 μM oestradiol (to induce *AtWEE1* expression) at 22°C. Wild type seedlings were grown on M&S medium for the entire duration of the experiment. Every two days (15, 17 and 19) of growth, the length of the primary root was measured by removing the samples from the medium, fixing and staining them as described above.

6.2.5. Analysis of lateral root development and primordia size

To analyse root development, a dissecting microscope was used. The primary root was taken as the root to have been the first root to emerge from the seedling. This was also the longest root. Lateral roots were categorized as all of the branch roots not classed as the primary.

In addition to the lateral root development analysis, I estimated average primordium volume. This was achieved by selecting randomly, five primordia from each plant. The primordia were then photographed using a Fujix Digital Camera attached to an Olympus BH-2 microscope for image analysis, using the program SigmaScan Pro (Version 5). The height and width of each primordium was measured and used to estimate primordia volume (assuming that a primordium was a cone) using the equation:

$$V = (\pi r^2 h) / 3$$

Where V = volume, r = radius (half of the width) and h = height (McKibbin *et al.*, 1998). Wild type primordium volume was compared with BIN HYG TX-*AtWEE1* primordium volume after 15, 17 and 19 days of growth, respectively.

6.3. Results

DNA extractions and PCR analyses were performed in order to ensure that the BIN HYG TX-*AtWEE1* and pER8-*AtWEE1* constructs were successfully incorporated into *Arabidopsis* plants. Only three plants transformed with BIN HYG TX-*AtWEE1* construct (line: 30, 58 and 61 see table 6.1) were found positive out of 16 analysed (Fig 6.2.a), whereas 4 plants (line: 5, 6, 36 and 40 see table 6.1) containing pER8-*AtWEE1* were positive out of 23 different plants analysed (Fig. 6.2.b). Lines 5, 6 and 40 were chosen for further studies because they showed the strongest product signals.

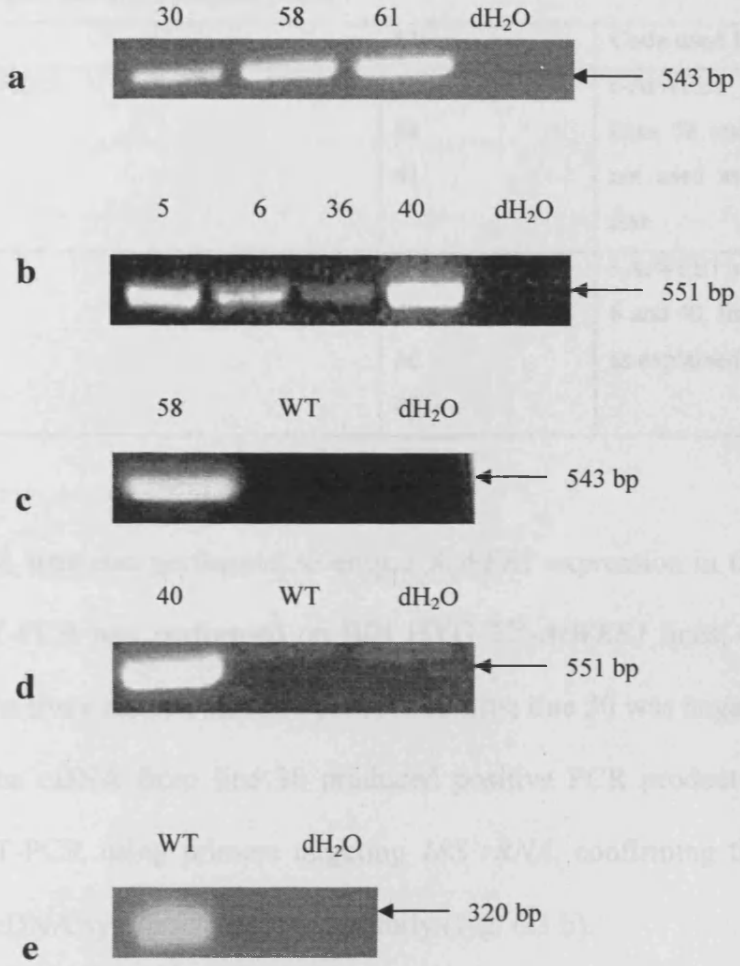


Fig. 6.2: PCR was performed on DNA extracted from *Arabidopsis* transgenic plants. **a)** BIN HYG TX *–AtWEE1*. The primers (BHTXF and P39) used were directed against the BIN HYG TX *–AtWEE1* construct. The numbers above the gel correspond to different transgenic lines; **b)** pER8-*AtWEE1*. The primers (35STRS and P61) used were directed against pER8-*AtWEE1* construct. Each number above the gel corresponds to a different transgenic line. Wild type (WT) cDNA was also used as negative control using **c)** primers BHTXF and P39, **d)** primers 35STRS and P61; **e)** the quality of wild type cDNA was tested using PUV2 and PUV4 primers.

Table 6.1: *Arabidopsis thaliana* transgenic plants

Plasmids:	**Lines**	**Code used in this thesis**
BIN HYG TX -*AtWEE1*	30 58 61	c-AtWEE1 referred only to lines 58 and 61, line 30 was not used as explained in the text
pER8-*AtWEE1*	5 6 36 40	i-AtWEE1 referred to lines 5, 6 and 40, line 36 was not used as explained in the text.

RT-PCR was also performed to ensure *AtWEE1* expression in the transgenic lines. When RT-PCR was performed on BIN HYG TX-*AtWEE1* lines, only lines 58 and 61 out of the three tested exhibited positive results; line 30 was negative (Fig. 6.3 a). However, the cDNA from line 30 produced positive PCR product bands when subjected to RT-PCR using primers targeting *18S rRNA*, confirming that the RNA extraction and cDNA synthesis were successfully (Fig. 6.3 b).

RT-PCR was also performed on induced and non-induced pER8-*AtWEE1* cell lines. In order to induce pER8-*AtWEE1*, seeds from *Arabidopsis* plants lines 5, 6 and 40, were sown on MS medium supplemented with 30 mg/l of hygromycin and 5 μM oestradiol (Zuo *et al.*, 2000). Induced seedlings were incubated in a growth room for two weeks as specified above. Seeds from the same *Arabidopsis* inducible lines (5, 6 and 40) were grown on MS medium supplemented with 30 mg/l hygromycin, but to prevent promoter induction oestradiol was not added to the culture medium. Seeds were incubated under the same conditions described above for the induced seeds. When the seedlings reached the stage of 3-4 leaves 1 – 1.5 cm in length, samples were collected for total RNA extraction and cDNA synthesis. Figure 6.3 c shows expression of *AtWEE1* in induced *Arabidopsis* plants compared to the negative mock

reactions, whereas figure 6.3 d shows expression of *AtWEE1* in the same non-induced lines, presumably because of leakiness in the inducible system. This could be because oestradiol is a very common estrogen hormone in nature and present in many different plastic materials including containers for distilled water and Petri dishes (Raloff, 1993, 1999).

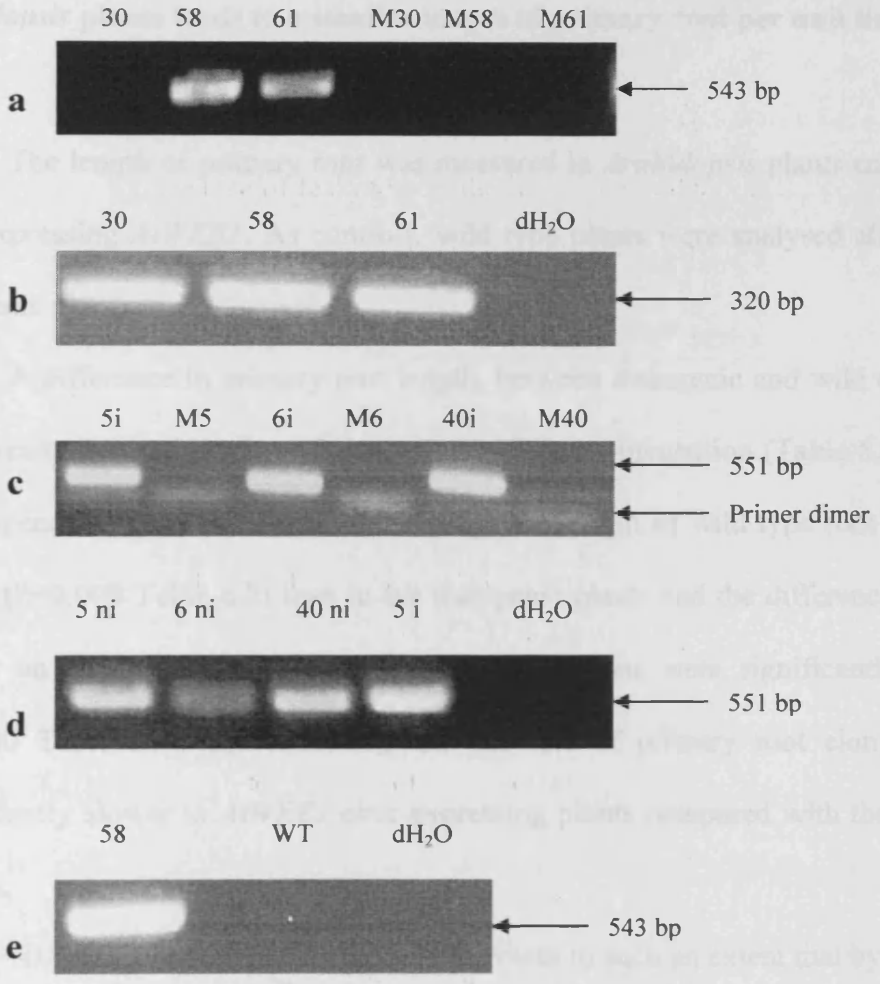


Fig. 6.3: RT-PCR was performed on *Arabidopsis* transgenic plants. The primers used BHTXF and P39 were directed against BIN HYG TX-*AtWEE1* cDNA. **a)** BIN HYG TX-*AtWEE1* RT-PCR. Positive bands were detected only for lines 58 and 61, but not for line 30. M30, M58 and M61 represent the respective mock reactions for each line (mocks contain all cDNA synthesis reagents except the reverse transcriptase). **b)** BIN HYG TX-*AtWEE1* RT-PCR. When the expression of *18S rRNA* was

investigated, positive bands were detected for all three lines, demonstrating that cDNA synthesis was successful. **c)** pER8-*AtWEE1* RT-PCR (using primers 35STRS and P61), *AtWEE1* expression in the induced lines (5 i, 6 i and 40 i) and corresponding mock reactions (M5, M6 and M40) used as negative controls. **d)** RT-PCR on non-induced transgenic plants (lines: 5, 6, 40) which showed a leakiness in *AtWEE1* expression, cDNA from line 5 induced was used as positive control. **e)** RT-PCR was also performed on *Arabidopsis* wild type (WT) cDNA (total RNA was extracted from leaf). The negative control in all cases was distilled water.

6.3.1. Constitutive and inducible over-expression of *AtWEE1* in transgenic *Arabidopsis* plants leads to a smaller length of primary root per unit time

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The length of primary root was measured in *Arabidopsis* plants constitutively over-expressing *AtWEE1*. As controls, wild type plants were analysed alongside the transgenic plants.

A difference in primary root length, between transgenic and wild type plants, was already noticeable after 15 days of growth, post-incubation (Table 6.2, Fig. 6.4; see Appendix VI for raw data); at this stage the length of wild type root was 3-fold longer (P=0.000 Table 6.2) than in the transgenic plants and the difference was even greater on days 17 and 19 (in both cases the means were significantly different P=0.000 Table 6.2), demonstrating that the rate of primary root elongation was significantly slower in *AtWEE1* over-expressing plants compared with the wild type control.

AtWEE1 over expression inhibited growth to such an extent that by day 19 the primary root of the transformed plants rarely exceeded 1.5 cm, whereas by this time, wild type primary roots were 4.5 cm in length on the same day. Hence wild type roots were at least 3-fold longer that *AtWEE1* over-expressing plants.

Table 6.2: The mean ± standard error (in parenthesis) of primary root length of WT and c-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test ($n \geq 15$).

Day	WT	c-AtWEE1	P
15	2.967 (±0.21)	0.947 (±0.15)	= 0.000
17	3.51 (±0.33)	1.080 (±0.082)	= 0.000
19	4.45 (±0.42)	1.47 (±0.28)	= 0.000

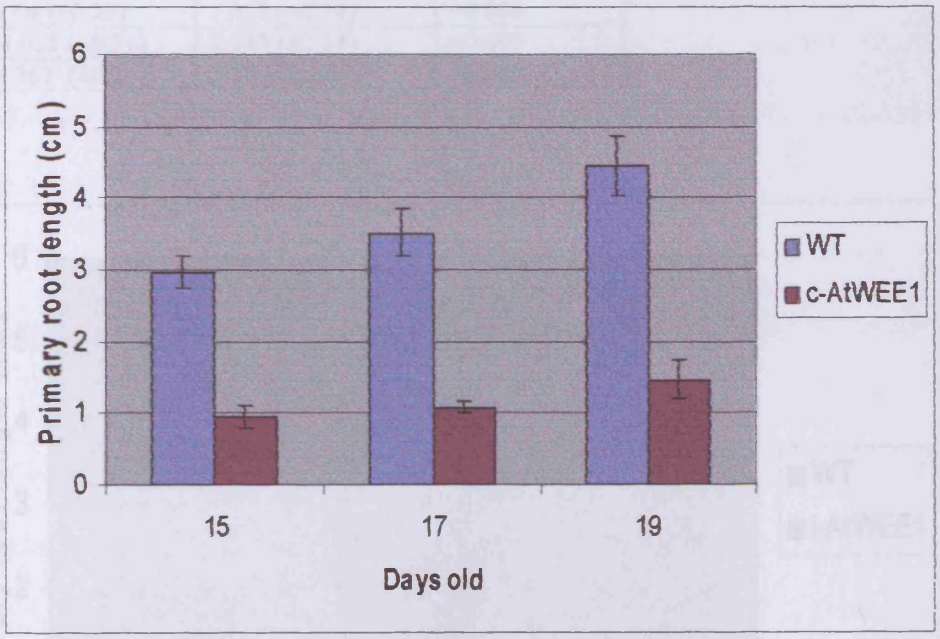


Fig. 6.4: Comparison of mean primary root length (±SE) between WT and c-AtWEE1 on days 15, 17 and 19. ($n \geq 15$).

Arabidopsis plants were also transformed with an inducible promoter to ensure the expression of *AtWEE1* gene in a controllable fashion. The choice of non-induced plants as control was not made because of the leakiness of *AtWEE1* expression in non-induced transgenic *Arabidopsis* plants, noted in the RT-PCR results; instead wild type plants were preferred as controls. *Arabidopsis* plants carrying pER8-*AtWEE1* were induced in order to investigate the development of primary roots compared to wild type on days 15, 17 and 19 post-incubation (Table 6.3, Fig. 6.5; see Appendix VI for raw data). On days 15 and 17 the difference in primary root length between wild type and transgenic plants is ~1.6-fold (P=0.000, Table 6.3, Fig. 6.5), whereas on day 19

the difference is ~1.4-fold (P=0.000, Table 6.3, Fig. 6.5).

Table 6.3: The mean ± standard error (in parenthesis) of primary root length of WT and i-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test. ($n \geq 21$).

Day	WT	i-AtWEE1	P
15	3.04 (±0.25)	1.857 (±0.14)	=0.000
17	4.022 (±0.21)	2.343 (±0.14)	=0.000
19	4.767 (±0.21)	3.23 (±0.12)	=0.000

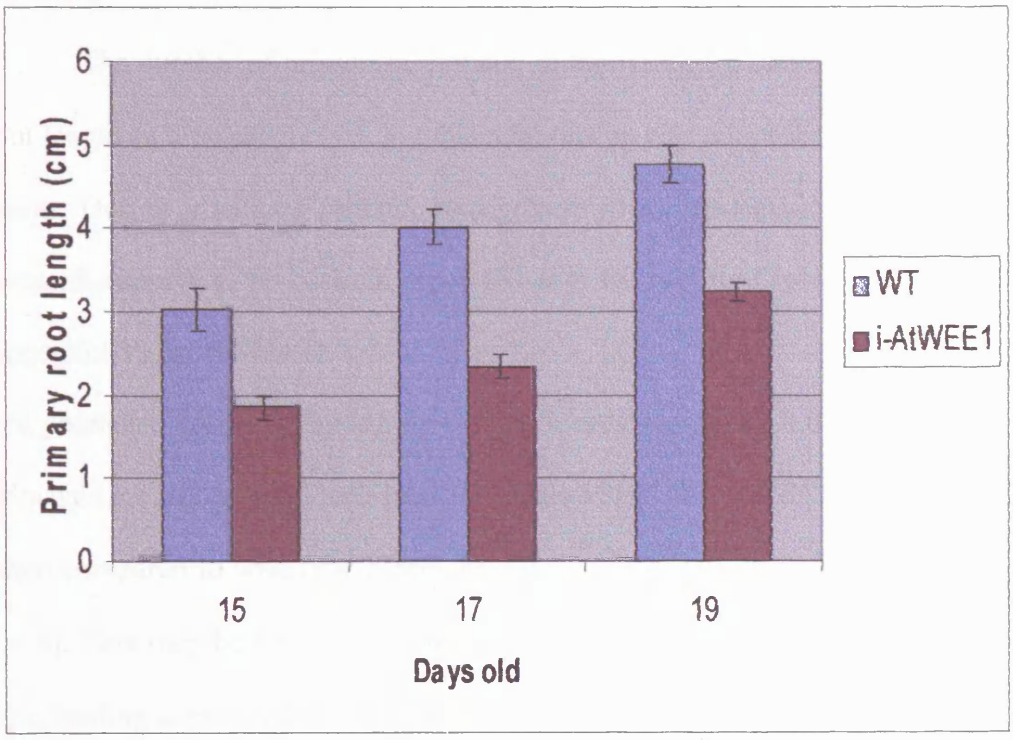


Fig. 6.5: Mean primary root length (±SE) of pER8-*AtWEE1* (i-AtWEE1) transgenic plants compared to wild type (WT) on days 15, 17 and 19 post-germination. ($n \geq 21$).

6.3.2. BIN HYG TX-*AtWEE1* plants initiate primordia at a slower rate per day than wild type

The number of primordia measured on the primary root of transgenic plants was compared to the number obtained from the analysis of wild type plants (Table 6.4). A significant decrease in number of primordia was observed in *AtWEE1* transgenic plants compared with wild type (Table 6.4, Fig. 6.6 a; see Appendix VI for raw data).

The number of primordia initiated in the wild type increases linearly per unit root length as time progresses, but this relationship was not observed in the transgenic plants. Due to a lack of primary root growth in the transgenic *Arabidopsis* plants, primordia appear to be initiated much closer to the root tip (Table 6.5, Fig. 6.6 b; see Appendix VI for raw data).

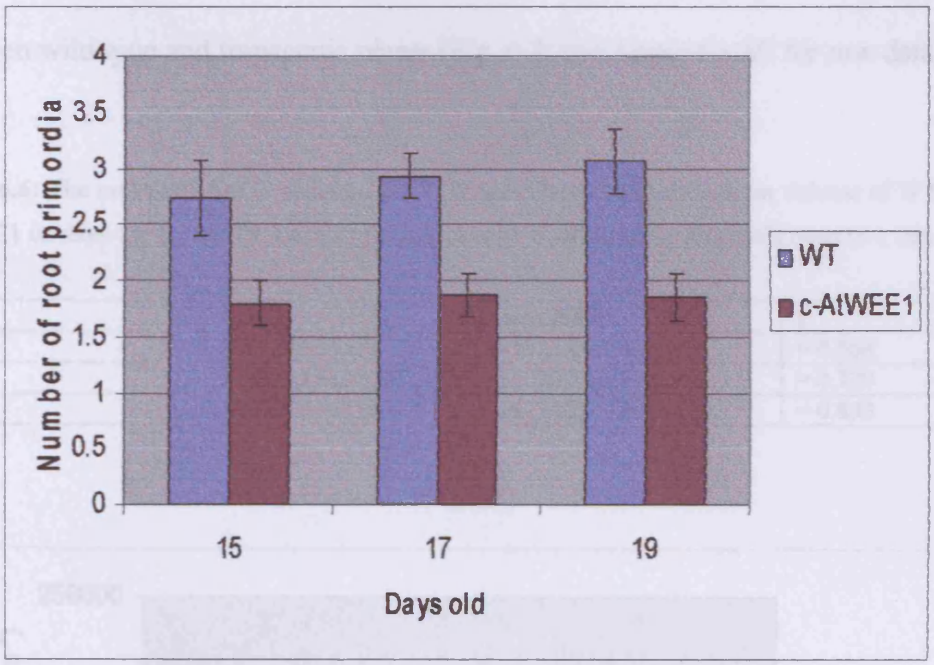
The youngest initiated primordium is significantly closer ($P \leq 0.002$, Table 6.5) to the primary root tip after 17 and 19 days of growth in the *AtWEE1* transformed plants when compared to wild type plants, by 1.5- and 3.5-fold respectively (Table 6.5, Fig 6.6 b). This may be because the transgenic primary roots grow slower than the wild type, leading to primordia formation nearer the root tip.

Table 6.4: The mean number (± standard error (in parenthesis)) of root primordia of WT and c-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test ($n \geq 15$).

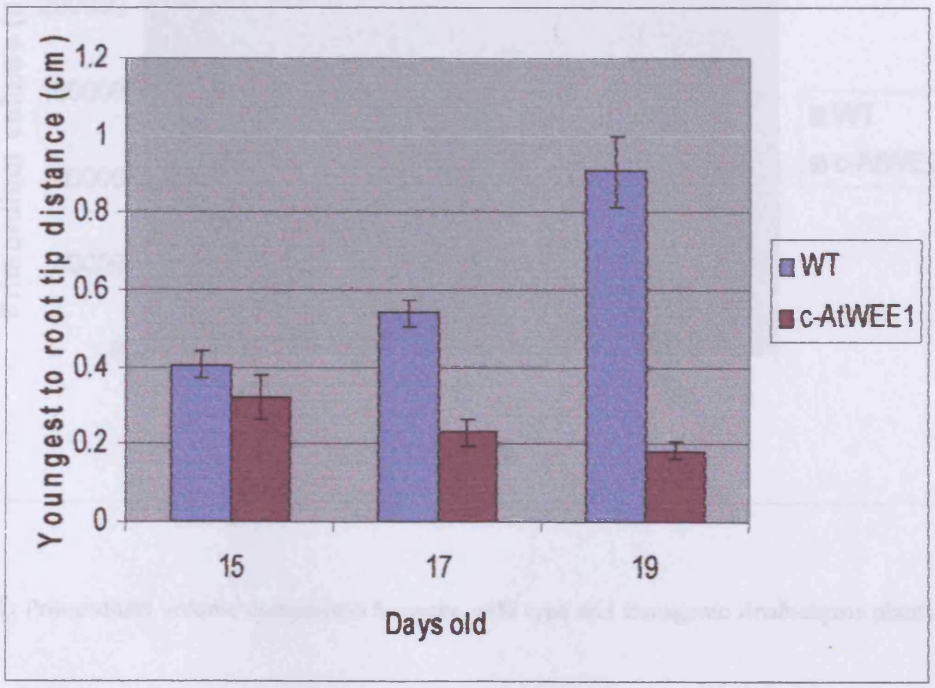
Day	WT	c-AtWEE1	P
15	2.73 (±0.33)	1.80 (±0.20)	= 0.024
17	2.933 (±0.21)	1.867 (±0.19)	= 0.001
19	3.07 (±0.28)	1.857 (±0.21)	= 0.002

Table 6.5: The mean distance (± standard error (in parenthesis)) of youngest primordia to root tip in WT and c-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test (n≥15).

Day	WT	c-AtWEE1	P
15	0.407 (±0.034)	0.321 (±0.058)	= 0.218
17	0.540 (±0.038)	0.232 (±0.034)	= 0.000
19	0.907 (±0.091)	0.185 (±0.023)	= 0.000



a



b

Fig. 6.6: The effects of constitutive *AtWEE1* (c-AtWEE1) expression in *Arabidopsis* plants compared to the wild type (WT) on **a)** the number of primordia initiated from the primary root ($n \geq 15$) and **b)** the distance between the youngest primordia to the tip of primary root ($n \geq 15$) (± SE).

However, the mean volume of the primordium was not affected by the over expression of *AtWEE1* (Table 6.6, Fig. 6.7). The average primordium volume measured on day 15, 17 and 19 was not significantly different ($P > 0.1$, Table 6.6) between wild type and transgenic plants (Fig. 6.7; see Appendix VI for raw data).

Table 6.6: The mean number (± standard error (in parenthesis)) of primordium volume of WT and c-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test ($n \geq 20$).

Day	WT	c-AtWEE1	P
15	88850 (±9558)	81825 (±7352)	= 0.564
17	127658 (±10960)	120202 (±18326)	= 0.729
19	191015 (±19196)	187342 (±19042)	= 0.893

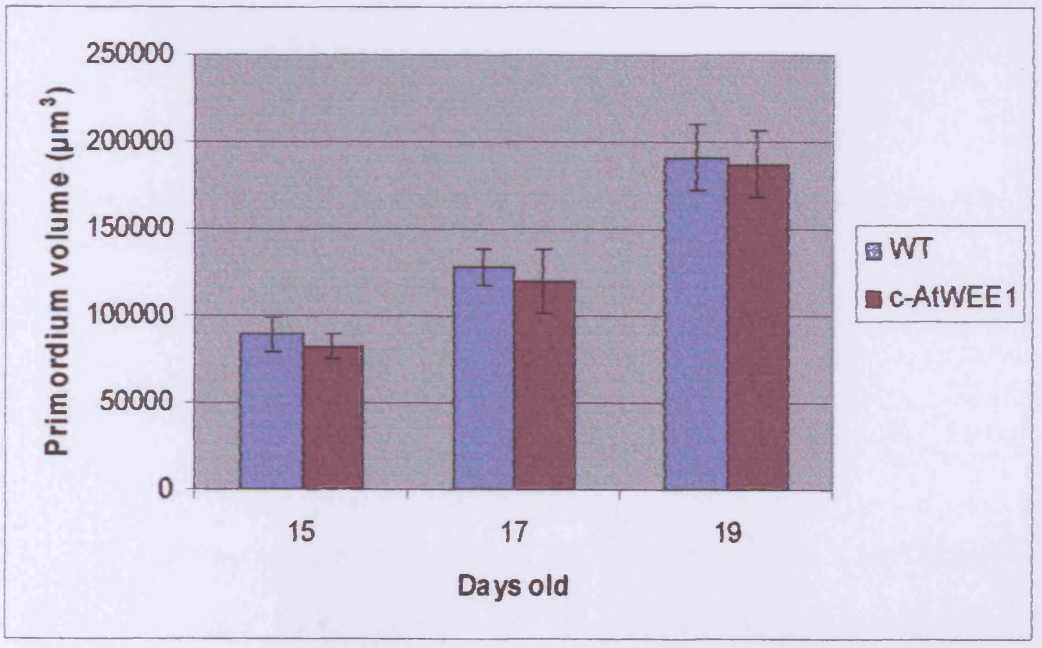


Fig. 6.7: Primordium volume comparison between wild type and transgenic *Arabidopsis* plants (± SE) ($n \geq 20$).

6.3.3. *AtWEE1* over-expression results in a reduction in the number of lateral roots

The number of lateral roots initiated from the primary roots on days 15, 17 and 19 after germination was investigated in both wild type and constitutively expressing *AtWEE1* plants (Table 6.7, Fig. 6.8; see Appendix VI for raw data). The number of lateral roots detected in wild type plants for each day of investigation was 2-fold of that in the transgenic plants (Fig. 6.8). This result was easily predictable because the number of primary root primordia was higher in the wild type than *AtWEE1* over-expressing plants.

Table 6.7: The mean number (± standard error (in parenthesis)) of lateral roots of WT and c-AtWEE1 on days 15, 17 and 19. Levels of significance are indicated by Student's t-test ($n \geq 14$).

Day	WT	c-AtWEE1	P
15	12.07 (±0.95)	5.86 (±0.67)	= 0.000
17	12.13 (±0.87)	7.07 (±0.74)	= 0.000
19	15.87 (±0.74)	7.71 (±0.76)	= 0.000

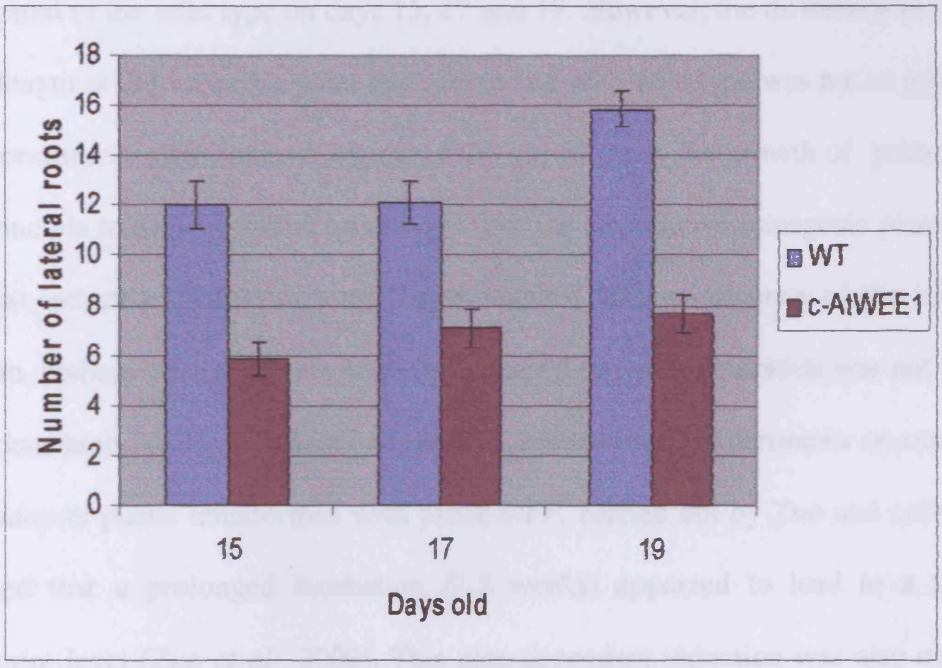


Fig. 6.8: The correlation between age in days and the mean (± SE) of lateral roots initiated on both wild type and transgenic plants ($n \geq 14$).

6.4. Discussion

The experiments reported in this chapter involved the transformation of *Arabidopsis thaliana* plants with vectors carrying *AtWEE1*. Subsequently, root phenotypes were characterized and compared with wild type *Arabidopsis thaliana* plants. In this study, particular emphasis was placed on the effects of *AtWEE1* over-expression on root growth and development over a five-day period. The investigation

of primary root growth in both constitutive and inducible transgenic lines showed a significantly shorter primary root length than the wild type. The wild type primary root appeared to elongate over the course of five days from 3 cm up to 4.5 cm in length, compared with the primary roots of the transgenic lines that did not grow more than 1 cm in length. In the inducible lines, the primary root length was shorter compared to the wild type on days 15, 17 and 19. However, the difference in primary root length in the inducible plant lines compared with wild type was not as great as in the constitutive plant lines. A hypothesis to explain why the growth of primary root in inducible transgenic plants was longer than the constitutive transgenic plants could be that inducible plants were no longer under a strong influence of the inducible system, perhaps oestradiol was partially degraded so its concentration was not enough to induce high levels of *AtWEE1* expression. Interestingly, experiments on transgenic *Arabidopsis* plants transformed with pER8-*GFP,* carried out by Zuo and colleagues, showed that a prolonged incubation (2-3 weeks) appeared to lead to a reduced transcript level (Zuo *et al.*, 2000). This time-dependent reduction was also observed with all other previously reported inducible systems (Aoyama and Chua, 1997; Martinez *et al.*, 1999). For this reason and since expression of *AtWEE1* was also found in non-induced plants, transgenic *Arabidopsis* plants carrying the inducible system pER8-*AtWEE1* were not used in further experiments.

In the experiments with transformed TBY-2 cells the non-induced cultures provided the ideal controls. Here the use of non-induced plants could not be used due to the leakiness of the inducible system. Unfortunately, empty vector lines were not available, so wild type was used as the control. Further experiments using an empty vector or a neutral transgenic line such as pER8-GUS would be important to confirm the results presented here.

The most plausible reason to explain the phenomena of shorter primary root length in the transgenic lines is that the over expression of *AtWEE1* leads the meristematic cells, responsible for root growth, to stop in G2 phase and not engage further in division. The over expression of *AtWEE1* is expected to increase the protein kinase level which subsequently phosphorylates CDKB-types. This would result in an inactivation of the CDK/cyclin complex, which normally leads cells to go into mitosis, causing a delayed primary root growth, since its rate of growth is in part dependent on the rate of meristematic cell production (Beemster and Baskin, 1998; Beemster *et al.*, 2002). This hypothesis is supported by many studies. The maize homologue to *WEE1* (*ZmWEE1*) is capable of phosphorylating and subsequently inactivating plant CDKs leading to an inhibition of mitosis (Sun *et al.*, 1999). Conversely, increased cyclin expression, which is required for CDK activity, increases the rate of mitosis in the primary root resulting in a faster rate of root elongation (Doerner *et al.*, 1996). Also, factors that lengthen the cell cycle reduce elongation. The SUC1/CSK1 *Arabidopsis* homologue *CKS1At,* significantly reduces primary root growth compared to wild type when over expressed in transgenic *Arabidopsis* plants by lengthening the duration of the cell cycle (De Veylder *et al.*, 2001b).

The over expression of *AtWEE1* clearly had significant effects on the rate of lateral root formation from the primary root. In particular, it resulted in a reduced number of lateral roots initiated per unit time in comparison with the wild type. The number of lateral roots and root primordia present on the wild type increased from day 15 to day 19, whereas they did not in the transgenic plants. These results imply that the transgenic plants are initiating primordia but a much reduced frequency that eventually falls to zero. Himanen and colleagues (2002) have demonstrated that

pericycle cell cycle arrest led to a reduction in lateral root primordium initiation. *AtWEE1* over expression in pericycle cells may delay their passage into division leading to a shorter root and a subsequent reduction in the rate of primordium.

AtWEE1 over-expression did not have an effect on the average primordium volume of transgenic plants, presumably because the smaller number of cells due to a reduced number of divisions is compensated by an increase in volume of cells stopped in G2. This implies that there were fewer large cells in primordia of transgenic plants compared with wild type. This hypothesis is based on the fact that the over-expression of *AtWEE1* in fission yeast cells leads to a long cell phenotype which probably reflects a delay in mitosis (Sorrell *et al.*, 2002). Also, very recently, epidermal cells in the primary root meristem of *Arabidopsis* plants transformed with BIN HYG TX-*AtWEE1* were found to be larger than corresponding cells in the wild type (Cardiff cell cycle laboratory, unpublished data). Those experiments carried out with and without hygromycin excluded any effects of this selective agent (Cardiff cell cycle laboratory, unpublished data).

Transgenic *Arabidopsis* plants over expressing *AtWEE1* initiated approximately half as many lateral root primordia than the wild type. In addition, the c-AtWEE1 lines formed primordia significantly closer to the root tip than wild type plants. Lateral roots are initiated from pericycle cells thought to have arrested in the G2 phase of the cell cycle after leaving the primary root meristem (Blakel and Evans, 1979). Thus lateral root initiation could be controlled via cells re-entering, and passing through, the G2-mitosis checkpoint (Casimiro *et al.*, 2003). Since AtWEE1 kinase is associated with blocking the cell in G2 it may play a role in determining which pericycle cells go on to form lateral root primordia and which do not. However, it should also be noted that a recent work on *Arabidopsis* has suggested that many

pericycle cells arrest in G1 phase, whilst only those nearest the developing lateral root are found to be in G2 (Beeckman *et al.*, 2001).

Cyclins like CYCD are well known to stimulate cell cycle progression. CYCD4;1 is generally expressed during lateral root formation, suggesting that the expression of cell cycle genes is a key step in controlling lateral root formation (De Veylder *et al.*, 1999). Cell cycle phosphoregulators have also been shown to have an influence on the rate and patterning of lateral root initiation. *Spcdc25* expression led to an increase in the frequency of lateral roots in tobacco (McKibbin *et al.*, 1998). Since positive cell cycle regulators are capable of influencing where and when root development takes place, a negative cell cycle regulator gene *AtWEE1*, also plays a role in the control of lateral root development.

6.5. Summary

The over expression of *AtWEE1* in transgenic *Arabidopsis thaliana* has significant effects on root growth and development, resulting in an inhibition of primary root growth and in a reduction in the number of primordia initiated. Transgenic plants also initiated primordia near the primary root tip possibly as result of a short length of the primary root.

AtWEE1 might exert its influence on root growth and development by inactivating CDKs which results in an inhibition of the onset of mitosis in dividing cells. This modulates root development by inhibiting mitotic progression and subsequently increasing cell size, confirming that *AtWEE1* is a cell cycle regulator.

Chapter 7: General Discussion

My aim was to gain a better understanding of *WEE1* function as regulator of the plant cell cycle, through the study of *AtWEE1* expression in the tobacco BY-2 cells and in the development of *Arabidopsis thaliana* roots. It became important to identify mechanisms in the higher plant that are either homologous or distinct from other eukaryotic systems such as yeast and animal. Different approaches based on molecular and microscopic techniques were employed to carry out this study.

Based on the experimental evidence that *AtWEE1* expression in *Schizosaccharomyces pombe* results in a long cell phenotype, expression of *AtWEE1* in tobacco cells should result in a large cell size phenotype and in an extended G2 phase. I tested this hypothesis by expressing *AtWEE1* both under constitutive and inducible promoters in tobacco BY-2 cells. Unexpectedly, the experimental evidence negates the hypothesis of increased cell size and extended G2 phase. In fact the exact opposite was found. TBY-2 cell size phenotype was smaller and G2 phase was shortened.

In an attempt to explain why the data found during my experimental work were so different from results previously published (Sorrell *et al.*, 2002), I investigated *NtWEE1* expression in TBY-2 cells transformed with *AtWEE1* under the control of an inducible promoter. Hence the expression of *NtWEE1* could be studied in the presence or absence of *AtWEE1* transcripts. My hypothesis was that expression of a foreign *WEE1* gene in tobacco cells inhibits or affects the normal expression of the endogenous *WEE1*. My experimental data do not support an inhibitory mechanism but instead indicate that induction of *AtWEE1* transcripts alters the timing of *NtWEE1* expression. Note that loss

of *WEE1* function in fission yeast is not lethal but causes cells to enter into mitosis prematurely at reduced cell size (Nurse and Thuriaux, 1980). The same result was found in the budding yeast when deletion of *Swe1*, the homologue of *wee1* in *S. cerevisiae*, led cells to divide at reduced cell size (Harvey and Kellogg, 2003). So too did loss of function of *WEE1* in transgenic *Xenopus* and *Drosophila* (Walter *et al.*, 2000).

Expression of *NtWEE1* peaks in S-phase, this is also confirmed by an independent study in TBY-2 cells (Gonzalez *et al.*, 2004). When *NtWEE1* expression peaked in S-phase the mitotic index peaked at 9 hours, whereas when the timing of *NtWEE1* expression was delayed, the mitotic index peak occurred at 4-5 hours and a smaller mitotic cell size was detected. In effect, these cells suffered loss of function of WEE1. In other words, the alteration in the timing of native *WEE1* expression resulted in a reduced cell size at division. From the observation of my data one question was then formulated: why is it that AtWEE1 does not replace NtWEE1 kinase activity? If it did, then perturbation of cell size would not be predicted. Two hypotheses were formulated: 1) perhaps *AtWEE1* is transcribed but not translated effectively, so that, the normal inhibition of tobacco CDK activity is removed; 2) alternatively, the AtWEE1 kinase may have insufficient affinity with tobacco CDKs to regulate them negatively. In support of this hypothesis is the extent of variation between the regulatory (N-terminal) components of AtWEE1 and NtWEE1 proteins, which is just 38% compared with 70% homology between catalytic domains (C-terminal) (Cardiff cell cycle laboratory, unpublished data). Resolving this issue will require a highly sensitive WEE1 kinase assay, able to distinguish NtWEE1 from AtWEE1 kinase activity.

In animals, 14-3-3 proteins play an important role in regulating WEE1 kinase activity (Honda *et al.*, 1997; Wang *et al.*, 2000). In *Xenopus* WEE1 (XWEE1), phosphorylation of a serine residue (Ser-549) that resides in a region that contains the RXXS sequence from the consensus motif is required for interaction with 14-3-3 proteins (Lee *et al.*, 2001). In humans, WEE1 binds to 14-3-3 proteins, and phosphorylation of WEE1 on Ser-642, inside the RXXS region is essential for the binding of 14-3-3 proteins to WEE1 (Wang *et al.*, 2000). In AtWEE1, there are two serines (Ser 450 and Ser 485) that reside in regions containing the consensus motif sequences RXXS for binding to 14-3-3 proteins. However, they are not in the same relative positions as those in human and *Xenopus* WEE1, because the predicted plant WEE1 proteins are over 50 amino acids shorter that the animal ones. Interestingly, only one of the potential serine residues (Ser-485) is conserved between *Arabidopsis* and tobacco (Cardiff cell cycle laboratory, unpublished data). Thus, despite a high overall homology between the predicted ORFs in *Arabidopsis* and tobacco, there may be significant divergence in specific regulatory amino acid residues.

In the TBY-2 cell lines, expression of *AtWEE1* induces a small mitotic cell size and in synchronized cells these data are in accord with a shortened G2 phase. The cell size data for weekly sub-cultured wild type TBY-2 cells also show a drop in mitotic cell area during exponential phase but an increase at stationary phase. Interestingly, during normal batch culture of TBY-2 cell lines expressing *AtWEE1*, cell size remained constantly small throughout batch culture. In fact, transformed TBY-2 cells were constantly dividing at smaller cell size. Following synchronization experiments, comparisons between controls and TBY-2 transformed cells revealed differences in

mitotic cell size; the control mitotic cells were always bigger than TBY-2 cells expressing *AtWEE1*. A smaller cell size and a shorter G2 phase were also observed when TBY-2 cells were transformed with a different cell cycle regulator *Cdc25* gene from *Schizosaccharomyces pombe* (*SpCdc25*) (Orchard *et al.*, 2005). Notably, in both *AtWEE1* and *SpCdc25* transformed TBY-2 cells, the shorter G2 phase is compensated by a longer G1 phase. As such, cell cycle length in TBY-2 cells remained remarkably stable regardless of the presence of a foreign cell cycle gene.

As outlined in the Introduction, transition of a plant cell from G2 to M is regulated by two CDKs. Whilst CDKA activity peaks at G1/S and G2/M, *CDKB1* expression is cell cycle regulated and kinase activity is restricted to late G2. In an attempt to explain why transformed TBY-2 cells were undergoing mitosis prematurely, the kinase activity of CDKA and CDKB1 were assayed to examine whether either or both enzymes were precociously activated in cells expressing *AtWEE1*.

In transformed TBY-2 cells, the kinase activity of both CDKA and CDKB1 were investigated in the presence or absence of *AtWEE1* expression. In non-induced cells, CDKA activity was high at both G1/S and G2/M in accordance with published data (Joubes *et al.*, 2000). In non-induced cells, CDKB1 activity peaked only at G2/M in complete agreement with published reports of its activity in wild type cells (Mironov *et al.*, 1999; Sorrell *et al.*, 2001). However, in *AtWEE1* expressing cells, CDKB1 activity was precociously high in early S-phase whereas CDKA activity peaked only at G1/S. Expression of *Spcdc25* in TBY-2 cells also generated a very early and persistent CDKB1 activity which also led to a shortened G2 and a small mitotic cell size (Orchard *et al.*, 2005). Note that in both cases, there was a differential effect on CDKB1 compared with

CDKA activity. From this it can be concluded that CDKB1 kinase activity drives cells into mitosis whereas CDKA may be linked more to the development of mitotic competence. Note that CDKB1 activity, but not CDKA activity, is restricted to proliferative regions of the plant (Magyar *et al.*, 1997; Mironv *et al.*, 1999; Umeda *et al.*, 1999: Joubes *et al.*, 2000; Sorrell *et al.*, 2001).

In fission yeast and animal cells, Wee1/Mik1/Myt1 competes with Cdc25 for the tyrosine 15 residue of Cdc2 (CDK) (Rhind and Russell, 2000). Recently a small CDC25 protein that lacks a regulatory domain has been cloned and is expressed at low levels in all *Arabidopsis* tissues (Sorrell *et al.*, 2005). In higher plants, *AtCdc25* seems to be the obvious counter player to *AtWEE1*. In plants, there is no evidence of a Mik1 or Myt1 so, as far as we know, WEE1 kinase is the only putative negative regulator of B-type CDKs. Normal TBY-2 cell lines exhibit a transcriptional peak of *NtWEE1* at S/G2 that presumably leads to NtWEE1 kinase activity that represses CDKB1 activity until the cell reaches the optimum conditions for division. However, in TBY-2 cell lines expressing *AtWEE1,* the timing of *NtWEE1* expression is shifted. This may result in the loss of the negative regulator of NtCDKB1, which results in a premature cell division. Notably, in TBY-2 cell lines when the timing of *NtWEE1* expression was perturbed, CDKB1 activity was high one hour following the induction of *AtWEE1* transcripts and represents the key difference in CDK activity between treatments. Since *AtWEE1* transcript began to be detected within 15 minutes of supplying DEX to the medium, it must start to perturb native *NtWEE1* regulation of CDKB1 very rapidly. In non-induced *AtWEE1*-transformed TBY-2 cells, NtCDKB1 activity peaks in mid G2 just as it does in wild type TBY-2 cells (Porceddu *et al.*, 2001; Sorrell *et al.*, 2001). However, NtCDKA peaks in early S-phase

regardless of the presence of *AtWEE1* transcript. Indeed, NtCDKA activity is much more constitutive, as found previously for wild type TBY-2 cells (Porceddu *et al.*, 2001; Sorrell *et al.*, 2001), and this could be part of a mechanism that regulates G1/S much like one of its suggested roles in *Arabidopsis* (Joubes *et al.*, 2000). It was previously demonstrated that CDK activity is dependent on native Cdc25 activity (Zhang *et al.*, 2005). Interestingly, in TBY-2 cells expressing *SpCdc25,* CDKB1 kinase activity was persistently high from early S-phase. However, CDKA activity was relatively constant. Collectively these results can show that premature and sustained CDKB1 activity is a key feature of cells that divide prematurely at a small size.

In plants, meristems are responsible for the formation of the entire postembryonic plant body. In *Arabidopsis* a small number of stem cells at the tip of the root generate all the cell types of the root through divisions followed by cell differentiation and regulated cell expansion (Scheres *et al.*, 2002). The primary root meristem of *Arabidopsis* is composed of three layers of initials, L1, L2 and L3 (Steeves and Sussex, 1989; Dolan *et al.*, 1993). L1 (the outermost layer) generates the root cap and epidermis, L2 produces the cortex, and the innermost layer L3 produces the vascular tissue (Scheres *et al.*, 2002). To gain a better understanding of *AtWEE1* function during primary root development, transgenic *Arabidopsis* plants were produced that over-expressed *Arabidopsis WEE1*.

The effect of *AtWEE1* over expression was studied on *Arabidopsis* plant roots. Lateral root primordia arise from the pericycle, the outermost layer of the vascular stele (McCully, 1975). Typically, primordia are initiated in a cluster which is then spatially and temporally separated from the next cluster (Mallory 1970). In the development of a lateral root, two distinct stages were identified: 1) the formation of a primordium, 2) the

subsequent formation of a meristem that is capable of emerging as a lateral root (Laskowski *et al.*, 1995). In *Arabidopsis* plants over-expressing *AtWEE1*, primary root elongation was severely inhibited and there was a lower frequency of lateral root primordia detected. Hence, *AtWEE1* over-expression induces a negative regulation of primary root growth and lateral root formation. It can be hypothesised in its negative setting, *WEE1* is a negative regulator of the G2/M transition in plants and when over-expressed causes yet more negative regulation reflected in slow root growth and fewer lateral root primordia.

The expression of *AtWEE1* did not completely inhibit lateral root development, but was associated with fewer lateral root primordia forming per unit length of primary root tissue. Interestingly, this result is the exact converse in transgenic tobacco plants where induction of *SpCdc25* expression resulted in more lateral root primordia forming per unit length of primary root (McKibbin *et al.*, 1998). The authors suggested that *SpCdc25* expression directly altered the control of cell division in the pericycle giving rise to the effects on cell size and subsequent development of lateral root primordia (McKibbin *et al.*, 1998). In the same way, the effect of *AtWEE1* expression on *Arabidopsis* root development can be explained by the fact that *AtWEE1* altered the control of cell division, delaying cells from entering into mitosis, which resulted in shorter primary roots and in fewer primordia. The molecular model to explain the experimental results is that over expression of *AtWEE1* maintained the native CDK in a phosphorylated inactive state that resulted in a delayed cell division.

The data reported here, show clearly that over-expression of *AtWEE1* in the native genetic background of *Arabidopsis* resulted in a predictable slow-growing phenotype.

Also, very recently, epidermal cells in the primary meristem of *AtWEE1* over-expressed *Arabidopsis* plants were found to be larger than corresponding cells in the wild type (Cardiff cell cycle laboratory, unpublished data). Again, this is a predictable negative effect of *WEE1* expression. Hence, it can be concluded that *AtWEE1* expression in tobacco cells is a transcriptional effect of the transgene on native *NtWEE1* and not, in itself, a typical *AtWEE1* cell cycle effect. Also, to my knowledge, this is the first demonstration of transcriptional regulation of mitotic cell size in higher plants.

The table below summarizes the effect of *AtWEE1* and *Spcdc25* expression on the cell size of fission yeast, *Arabidopsis* roots, tobacco roots and tobacco BY-2 cells:

Organism	AtWEE1 (cell size)	Spcdc25 (cell size)
Fission yeast	large	small
Arabidopsis roots	large	small
Tobacco roots		small
TBY-2 cells	small	small

Further Work

Nicotiana tabacum WEE1 (*NtWEE1*) was recently sequenced in our laboratory and a pTA7002 inducible vector was used to make a construct carrying *NtWEE1* gene.

pTA7002-*NtWEE1* plasmid was employed to transform tobacco BY-2 cells. Microscopic analysis on the newly established TBY-2 cell line over-expressing *NtWEE1* should be carried out to investigate the cell phenotype. Synchronization experiments can also give new information about the effect of *NtWEE1* on the tobacco cell cycle. It would be of particular interest to transform *Arabidopsis thaliana* cells with *NtWEE1* to investigate cell phenotype and cell cycle compared with the tobacco cell line over-expressing *NtWEE1*. *Arabidopsis thaliana* plants have also recently been transformed with *NtWEE1* to investigate its action on primary root development and primordium formation. These results will provide an important comparison to the effects of *AtWEE1* over expression in *Arabidopsis* plants and *AtWEE1* expression in TBY-2 cells.

Antibodies able to discriminate between AtWEE1 and NtWEE1 kinases, should be made to prove whether *AtWEE1* is translated in transgenic TBY-2 cells. Immunoprecipitation could also be employed to study AtWEE1 and NtWEE1 protein abundance during the cell cycle of transgenic tobacco cells, and this could be compared with NtWEE1 protein abundance of wild type TBY-2 cells.

A WEE1 kinase assay that uses as substrate CDK proteins, which contain tyrosine 15 residues that can be phosphorylated by WEE1, should be developed. The antibody should specifically detect only the phosphorylated form of the tyrosine 15 residue on the CDK. To investigate whether AtWEE1 is active in transgenic tobacco cells, an AtWEE1 kinase assay could be performed. An NtWEE1 kinase assay could also be performed on wild type and transgenic TBY-2 cells.

Gene silencing is, without doubt, one of the best approaches to study the function of a target gene in a living organism. To gain a better understanding of *WEE1* activity

during the plant cell cycle, the recently developed RNA interference (RNAi) technique is currently being employed in the Cardiff cell cycle laboratory to silence the expression of *NtWEE1* in wild type tobacco BY-2 cells. This system was successfully used in a variety of organisms and cell types (e.g., worms, fruit flies and plants). It is based on the utilization of long double-stranded RNAs (dsRNAs) to silence the expression of a target gene (as described in the mechanism of RNA interference (RNAi), Ambion's online appendix) the principal steps are: 1) the long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway; 2) the dsRNAs are processed into 20-25 nucleotide (nt) small interfering RNAs (siRNAs) by an RNase III-like enzyme called Dicer (initiation step); 3) the siRNAs are then assembled into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the RISCs to complementary RNA molecules, where they cleave and destroy the cognate RNA (effector step). Cleavage of cognate RNA takes place near the middle of the region bound by the siRNA strand (www.ambion.com/techlib/append/RNAi_mechanism.html) (Tang *et al.*, 2003).

Cell phenotype and the cell cycle can be studied using wild type tobacco BY-2 cells that have been subjected to RNAi treatment and are lacking NtWEE1 kinase. CDKA and CDKB1 kinase activity should be also investigated in order to determine the effect of silencing NtWEE1 on these two very important regulators of entry into mitosis.

The yeast two-hybrid assay, employed to find interactions between AtWEE1 and other proteins, gave putative positive interactions (see Appendix VII for more details). However, these results must be further tested to check that they are not falsely positive.

This can be done by transforming *Arabidopsis thaliana* plants with plasmids carrying the gene encoding for the putative positive proteins fused to non-fluorescent domains of the yellow fluorescent protein. The two non-fluorescent domains become fluorescent when brought together by association of AtWEE1 with the interacting protein (Hu *et al.*, 2000, Walter *et al.*, 2004).

The experiments in which *Arabidopsis thaliana* plants are transformed with *AtWEE1* using an inducible promoter (induced by oestradiol) and a constitutive promoter are described in chapter 6. RT-PCR analysis relative to non-induced transgenic *Arabidopsis* plants showed leakiness of *AtWEE1* expression. This could be due to the fact that oestradiol is a very common estrogen hormone in nature and present in many different plastic materials including containers for distilled water and Petri dishes. This inconvenience can be avoided by using glass, not plastic, Petri dishes for seed germination and seedling growth, and storing the distilled water required for all aspects of the technique in a glass container. RT-PCR could be then repeated to check if under these conditions *AtWEE1* is still expressed in non-induced transgenic *Arabidopsis* plants. Eliminating *AtWEE1* expression in non-induced plants presents two advantages: 1) the effect of *AtWEE1* expression can be efficiently studied only when it is required (induced); 2) non-induced transgenic plants can be used as the control.

It would also be instructive to measure the duration of the cell cycle in transgenic *Arabidopsis* root meristem cells using flow cytometric analysis to investigate at which stage of the cell cycle the majority of root meristem cells are delayed. These results can be then compared with wild type.

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Appendix I: Sequences of *AtWEE1* ORF

BIN HYG TX-*AtWEE1* clone 1S

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1s contig     1   ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG
athwee1 ORF   1   ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG

1s contig     51  GACAATCAAAACCAGGGCATCGAAGAAGATTCGGAAGATGGAAGGGACAT
athwee1 ORF   51  GACAATCAAAACCAGGGCATCGAAGAAGATTCGGAAGATGGAAGGGACAT

1s contig     101 TGGAGCGTCACTCTCTGCTTCAATTCGGTCAATTGTCGAAGATTTCTTTC
athwee1 ORF   101 TGGAGCGTCACTCTCTGCTTCAATTCGGTCAATTGTCGAAGATTTCTTTC

1s contig     151 GAAAATCGTCCGTCGTCGAATGTTGCTTCATCGGCGTTTCAGGGTCTCCT
athwee1 ORF   151 GAAAATCGTCCGTCGTCGAATGTTGCTTCATCGGCGTTTCAGGGTCTCCT

1s contig     201 GGATTCGGATTCTTCGGAACTCCGAAATCAGTTGGGTTCCGCTGATTCAG
athwee1 ORF   201 GGATTCGGATTCTTCGGAACTCCGAAATCAGTTGGGTTCCGCTGATTCAG

1s contig     251 ATGCCAATTGCGGAGAGAAGGACTTTATTCTTAGCCAAGACTTCTTCTGC
athwee1 ORF   251 ATGCCAATTGCGGAGAGAAGGACTTTATTCTTAGCCAAGACTTCTTCTGC

1s contig     301 ACACCTGACTATATAACCCCGGACAATCAGAACTTGATGAGCGGCTTAGA
athwee1 ORF   301 ACACCTGACTATATAACCCCGGACAATCAGAACTTGATGAGCGGCTTAGA

1s contig     351 TATCAGCAAGGATCATTCTCCATGTCCTAGGTCTCCTGTTAAACTAAATA
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1s contig     451 AATTCTACCTGGTCTTCAAAACATAGAGTAGATGAACAAGAGAATGATGA
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athwee1 ORF   501 TATTGACACAGATGAGGTGATGGGGGATAAACTCCAAGCTAATCAAACAG

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athwee1 ORF   551 AGAGGACTGGATACGTTTCACAGGCTGCAGTTGCTCTGCGGTGTCGGGCT

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athwee1 ORF   601 ATGCCACCCCCTTGCCTCAAGAATCCGTATGTGTTGAATCAGTCTGAGAC

1s contig     651 TGCTACTGACCCTTTTGGACATCAGAGATCAAAATGTGCAAGTTTTCTCC
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1s contig     701 CTGTAAGTACAAGTGGGGATGGCTTGTCAAGATATCTCACGGACTTTCAT
athwee1 ORF   701 CTGTAAGTACAAGTGGGGATGGCTTGTCAAGATATCTCACGGACTTTCAT

1s contig     751 GAAATCCGGCAAATTGGTGCTGGACATTTCAGTCGGGTATTTAAGGTGTT
athwee1 ORF   751 GAAATCCGGCAAATTGGTGCTGGACATTTCAGTCGGGTATTTAAGGTGTT

1s contig     801 GAAGAGAATGGACGGTTGCCTATATGCTGTGAAACACAGCACAAGAAAGT
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1s contig     851 TGTATCTAGATTCAGAGAGACGTAAAGCTATGATGGAAGTGCAAGCTCTT
athwee1 ORF   851 TGTATCTAGATTCAGAGAGACGTAAAGCTATGATGGAAGTGCAAGCTCTT

1s contig     901 GCTGCTCTTGGGTTTCATGAAAATATAGTAGGATATTACTCCTCGTGGTT
athwee1 ORF   901 GCTGCTCTTGGGTTTCATGAAAATATAGTAGGATATTACTCCTCGTGGTT

1s contig     951 TGAAAATGAGCAATTATACATTCAACTGGAACTCTGCGATCACAGCTTGT
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1s contig     1001 CAGCTTTGCCTAAGAAATCTTCTCTTAAAGTCTCAGAAAGAGAGATCTTG
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1s contig     1051 GTGATTATGCATCAGATAGCAAAGGCATTACATTTTGTGCATGAGAAAGG
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1s contig    1151 TTTGCAAGCTTGGTGACTTTGGTTGTGCCACACGATTGGACAAAAGCTTA
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1s contig    1351 CTCAATATCAAAGAAGGAAAACTTCCTCTCCTTCCTGGCCATTCGTTGCA
athwee1 ORF  1351 CTCAATATCAAAGAAGGAAAACTTCCTCTCCTTCCTGGCCATTCGTTGCA

1s contig    1401 GTTACAACAACTTCTTAAGACAATGATGGATCGTGATCCGAAGCGTCGGC
athwee1 ORF  1401 GTTACAACAACTTCTTAAGACAATGATGGATCGTGATCCGAAGCGTCGGC

1s contig    1451 CTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGATAGGATTCGAGGT
athwee1 ORF  1451 CTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGATAGGATTCGAGGT

1s contig    1501 TGA
athwee1 ORF  1501 TGA
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pTA7002-*AtWEE1* clone 1X

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athwee1 ORF  1    ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG
1x contig    1    ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG

athwee1 ORF  51   GACAATCAAAACCAGGGCATCGAAGAAGATTCGGAAGATGGAAGGGACAT
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athwee1 ORF  101  TGGAGCGTCACTCTCTGCTTCAATTCGGTCAATTGTCGAAGATTTCTTTC
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1x contig    401  ACAGTTAAAAGCAAAAGATGTCGCCAGGAGAGTTTCACAGGAAATCATTC

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1x contig    451  AAATTCTACCTGGTCTTCAAAACATAGAGTAGATCARSAAGACAAWGAWG

athwee1 ORF  500  ATATTGACACAGATGAGGTGATGGGGGATAAACTCCAAGCTAATCAAACA
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athwee1 ORF  1497  AGGTTGA
1x contig    1501  AGGTTGA
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pTA7002-*AtWEE1* clone 2X

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athwee1 ORF     1  ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG
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2x contig    1049 TGGTGATTATGCATCAGATAGCAAAGGCATTACAT-TTTGTGCATGAGAA

athwee1 ORF  1098 AGGAATAGCTCATTTAGATGTAAAACCTGACAATATTTACATTAAGAACG
2x contig    1098 AGGAATAGCTCATTTAGATGTAAAACCTGACAATATTTACATTAAGAACG

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2x contig    1148 GTGTTTGCAAGCTTGGTGACTTTGGTTGTGCCACACGATTGGACAAAAGC

athwee1 ORF  1198 TTACCAGTAGAAGAAGGAGATGCACGTTACATGCCTCAAGAAATTCTAAA
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athwee1 ORF  1248 TGAAGACTACGAACACCTTGATAAAGTCGATATCTTCTCTTTAGGTGTGA
2x contig    1248 TGAAGACTACGAACACCTTGATAAAGTCGATATCTTCTCTTTAGGTGTGA

athwee1 ORF  1298 CGGTTTATGAGCTGATTAAGGGATCTCCTCTTACAGAATCAAGAAACCAG
2x contig    1298 CGGTTTATGAGCTGATTAAGGGATCTCCTCTTACAGAATCAAGAAACCAG

athwee1 ORF  1348 TCGCTCAATATCAAAGA-AGGAAAACTTCCTCTCCTTCCTGGCCATTCGT
2x contig    1348 TCGCTCAATATCAAAGA-AGGAAAACTTCCTCTCCTTCCTGGCCATTCGT

athwee1 ORF  1397 TGCAGTTACAACAACTTCTTAAGACAATGATGGATCGTGATCCGAAGCGT
2x contig    1397 TGCAGTTACAACAACTTCTTAAGACAATGATGGATCGTGATCCGAAGCGT

athwee1 ORF  1447 CGGCCTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGATAGGATTCG
2x contig    1447 CGGCCTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGATAGGATTCG

athwee1 ORF  1497 AGGTTGA
2x contig    1497 AGGTTGA
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pTA7002-*AtWEE1* clone 3X

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3x contig     1    ATGTTCGAGAAGAACGGAAGAACACTGTTGGCGAAGAGGAAAACCCAAGG

athwee1 ORF   51   GACAATCAAAACCAGGGCATCGAAGAAGATTCGGAAGATGGAAGGGACAT
3x contig     51   GACAATCAAAACCAGGGCATCGAAGAAGATTCGGAAGATGGAAGGGACAT

athwee1 ORF   101  TGGAGCGTCACTCTCTGCTTCAATTCGGTCAATTGTCGAAGATTTCTTTC
3x contig     101  TGGAGCGTCACTCTCTGCTTCAATTCGGTCAATTGTCGAAGATTTCTTTC

athwee1 ORF   151  GAAAATCGTCCGTCGTCGAATGTTGCTTCATCGGCGTTTCAGGGTCTCCT
3x contig     151  GAAAATCGTCCGTCGTCGAATGTTGCTTCATCGGCGTTTCAGGGTCTCCT

athwee1 ORF   201  GGATTCGGATTCTTCGGAACTCCGAAATCAGTTGGGTTCCGCTGATTCAG
3x contig     201  GGATTCGGATTCYTCGGAACTCCGAAATCAGTTGGGTTCCGCTGATTCAG

athwee1 ORF   251  ATGCCAATTGCGGAGAGAAGGACTTTATTCTTAGCCAAGACTTCTTCTGC
3x contig     251  ATGCCAATTGCGGAGAGAAGGACTTTATTCTTAGCCAAGACTTCTTCTGC

athwee1 ORF   301  ACACCTGACTATATAACCCCGGACAATCAGAA-CTTGATGAGCGGCTTAG
3x contig     301  ACACCTGACTATATAACCCCGGACAATCAGAA-CTTGATGAGCGGCTTAG

athwee1 ORF   350  ATATCAGCAAGGATCATTCTCCATGTCCTAGGTCTCCTGTTAAACTAAAT
3x contig     350  ATATCAGCAAGGATCATTCTCCATGTCCTAGGTCTCCTGTTAAACTAAAT

athwee1 ORF   400  ACAGTTAAAAGCAAAAGATGTCGCCAGGAGAGTTTCACAGGAAATCATTC
3x contig     400  ACAGTTAAAAGCAAAAGATGTCGCCAGGAGAGTTTCACAGGAAATCATTC

athwee1 ORF   450  AAATTCTACCTGGTCTTCAAAACATAGAGTAGATGAACAAGAGAATGATG
3x contig     450  AAATTCTACCTGGTCTTCAAAACATAGAGTAGATGAACAAGAGAATGATG

athwee1 ORF   500  ATATTGACACAGATGAGGTGATGGGGGATAAACTCCAAGCTAATCAAACA
3x contig     500  ATATTGACACAGATGAGGTGATGGGGGATAAACTCCAAGCTAATCAAACA

athwee1 ORF   550  GAGAGGACTGGATACGTTTCACAGGCTGCAGTTGCTCTGCGGTGTCGGGC
3x contig     550  GAGAGGACTGGATACGTTTCACAGGCTGCAGTTGCTCTGCGGTGTCGGGC

athwee1 ORF   600  TATGCCACCCCCTTGCCTCAAGAATCCGTATGTGTTGAATCAGTCTGAGA
3x contig     600  TATGCCACCCCCTTGCCTCAAGAATCCGTATGTGTTGAATCAGTCTGAGA

athwee1 ORF   650  CTGCTACTGACCCTTTTGGACATCAGAGATCAAAATGTGCAAGTTTTCTC
3x contig     650  CTGCTACTGACCCTTTTGGACATCAGAGATCAAAATGTGCAAGTTTTCTC

athwee1 ORF   700  CCTGTAAGTACAAGTGGGGATGGCTTGTCAAGATATCTCACGGACTTTCA
3x contig     700  CCTGTAAGTACAAGTGGGGATGGCTTGTCAAGATATCTCACGGACTTTCA

athwee1 ORF   750  TGAAATCCGGCAAATTGGTGCTGGACATTTCAGTCGGGTATTTAAGGTGT
3x contig     750  TGAAATCCGGCAAATTGGTGCTGGACATTTCAGTCGGGTATTTAAGGKGT

athwee1 ORF   800  T-GAAGAGAATGGAC-GGTTGCCTATATGCTGTGAAAC-ACAGCACAAGA
3x contig     800  TTGAAGAGAATGGACCGGTTGCCTATATGCTGTGAAAXCACAGCACAAGA

athwee1 ORF   847  AAGTTGTATCTAGATTCAC-AGAGACGTAAAGCTATGATGGAAGTGCAAG
3x contig     850  AAGTTGTATYTAGATTCAGCAGAGACGTAAAGCTATGATGGAAGTGCAAG

athwee1 ORF   896  CTCTTGCTGCTCTTGGGTTT-CATGAAAATATAGTAGGATATTACTCC-T
3x contig     900  CTCTTGCTGCTCTTGGGTTTTCATGAAAATATAGTAGGATATTACTCCCT

athwee1 ORF   944  CGTGGTTTGAAAATGAGCAATTATACATTCAACTGGAACTCTGCGATCAC
3x contig     950  CGTGGTTTGAAAATGAGCAATTATACATTCAACTGGAACTCTGCGATCXX

athwee1 ORF   994  A--GCTTGTCAGCTTTGCCTAAGAAATCTT-CTCTTAAAGTCTCAGAAAG
3x contig     1000 ACAGCTTGTCAGCTTTGCCTAAGAAATCTT-CTCTTAAAGTCTCAGAAAG

athwee1 ORF   1041 AGAGATCTTGGTGATTATGCATCAGATAGCAAAGGCATTACAT-TTTGTG
3x contig     1049 AGAGATCTTGGTGATTATGCATCAGATAGCAAAGGCATTACAT-TTTGTG

athwee1 ORF   1090 CATGAGAAAGGAATAGCTCATTTAGATGTAAAACCTGACAATATTTACAT
3x contig     1098 CATGAGAAAGGAATAGCTCATTTAGATGTAAAACCTGACAATATTTACAT

athwee1 ORF   1140 TAAGAACGGTGTTTGCAAGCTTGGTGACTTTGGTTGTGCCACACGATTGG
3x contig     1148 TAAGAACGGTGTTTGCAAGCTTGGTGACTTTGGTTGTGCCACACGATTGG
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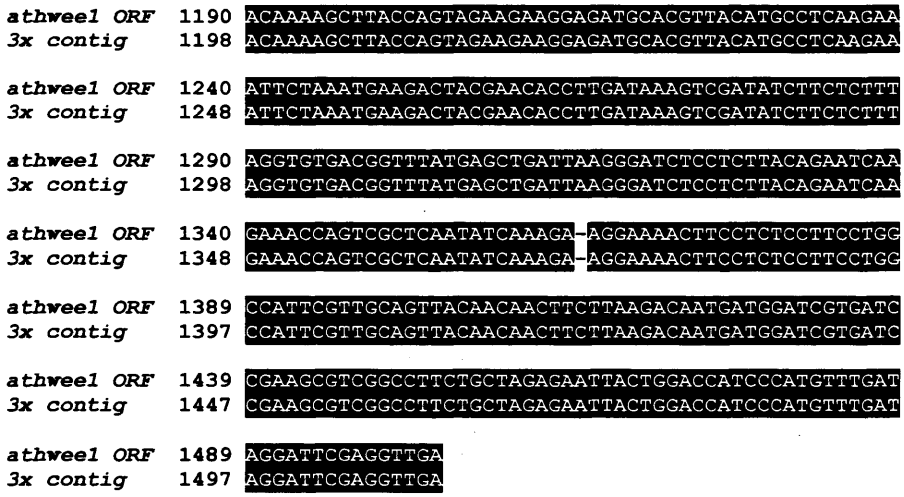
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3x contig 1198 ACAAAAGCTTACCAGTAGAAGAAGGAGATGCACGTTACATGCCTCAAGAA
athwee1 ORF 1240 ATTCTAAATGAAGACTACGAACACCTTGATAAAGTCGATATCTTCTCTTT
3x contig 1248 ATTCTAAATGAAGACTACGAACACCTTGATAAAGTCGATATCTTCTCTTT
athwee1 ORF 1290 AGGTGTGACGGTTTATGAGCTGATTAAGGGATCTCCTCTTACAGAATCAA
3x contig 1298 AGGTGTGACGGTTTATGAGCTGATTAAGGGATCTCCTCTTACAGAATCAA
athwee1 ORF 1340 GAAACCAGTCGCTCAATATCAAAGA-AGGAAAACTTCCTCTCCTTCCTGG
3x contig 1348 GAAACCAGTCGCTCAATATCAAAGA-AGGAAAACTTCCTCTCCTTCCTGG
athwee1 ORF 1389 CCATTCGTTGCAGTTACAACAACTTCTTAAGACAATGATGGATCGTGATC
3x contig 1397 CCATTCGTTGCAGTTACAACAACTTCTTAAGACAATGATGGATCGTGATC
athwee1 ORF 1439 CGAAGCGTCGGCCTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGAT
3x contig 1447 CGAAGCGTCGGCCTTCTGCTAGAGAATTACTGGACCATCCCATGTTTGAT
athwee1 ORF 1489 AGGATTCGAGGTTGA
3x contig 1497 AGGATTCGAGGTTGA

Appendix II: Tobacco BY-2 cell area raw data

II.I. Absorbance at 550 nm

WEE1-c-2	WEE1-c-10	WEE1-c-12	WT	EV-c-10
0.18	0.18	0.24	0.3	0.12
0.23	0.24	0.32	0.38	0.2
0.3	0.32	0.41	0.53	0.34
0.45	0.52	0.56	0.78	0.58
0.9	1.36	1.22	1.68	1.28
1.74	2.54	2.32	2.61	2.32
2.4	2.65	2.46	2.73	2.49

II.II. Mitotic cell area (μm^2) over a 6 day period

Wild type

day 1	day 2	day 3	day 4	day 5	day 6
2728.809	23271	1104.147	1627.609	2487.158	5310.475
3454.631	43357	1393.582	2093.58	2977.876	4610.181
3159.186	29448	916.0443	1749.683	3418.77	4006.447
2233.383	37656	1045.074	1713.432	4033.447	2919.304
2732.96	28219	2187.803	1624.127	2166.912	2419.246
1916.657	34153	3001.764	2407.508	3347.721	4145.916
3241.82	21813	2235.466	2056.799	2830.285	2459.58
4113.514	27418	1453.331	2219.969	2922.257	3685.662
2570.711	26646	1922.837	2084.347	2609.16	2763.49
2516.074	32882	1049.127	1924.583	2495.061	5221.254
2737.186	39532	1872.847	1956.597	3153.316	3658.344
1797.422	41535	1971.702	1890.148	4043.231	6001.461
4338.098	21098	1774.367	2842.98	3902.338	3782.753
4154.114	34135	1746.969	2386.847	4684.326	
3624.804	46274	1936.573	2502.866	2829.908	
3904.628	20409	1504.372	2204.605	3101.911	
3565.715	25538	1762.732	1671.807	2700.982	
3578.921	33015	1759.279	1596.882	2761.418	
4100.157	31166	1696.153	1924.432	3079.557	
3044.102	24788	1678.664	1718.578	3079.407	
4782.812	30506	1776.393	2363.991	3853.492	
5627.415	25973	2353.462	2055.21	3061.645	
3165.147	26283	1799.587	2022.061	3428.404	
3667.668	38904	2394.521	1753.619	4565.033	
2662.627	23658	1542.203	1718.578	3013.175	
3210.879	33907	2105.461	1625.565	3352.162	
3028.48	39469	1706.512	1273.04	3686.934	
3509.418	25930	1735.86	1937.601		
2151.805	30990	1241.659	2570.072		
2361.824	28682	1980.784	1619.586		
3197.371	23129	1293.526			
	33260	1451.304			
	31621	1493.789			
	20184	2200.563			
	19573	1735.035			
	19102	2106.512			
	21938	1939.276			
	24662	1903.321			
		2581.197			
		1995.872			
		2020.717			
		1577.482			
		1807.994			
		1648.865			
		1689.548			
		1659.974			

EV-c-10

day 1	day 2	day 3	day 4	day 5	day 6
4833.476	2291.097	1978.136	3115.138	1322.453	3173.991
4835.127	1836.656	2389.384	2569.084	1558.124	2872.782
4391.687	2032.341	1732.856	1722.459	2325.758	3043.708
3571.384	2347.996	1857.199	2248.233	1916.323	3767.003
4346.503	1504.969	2086.813	2349.331	2212.237	3133.123
5087.77	1917.489	1993.272	1759.463	2815.718	5069.611
2532.364	2689.391	1908.283	2288.036	3129.568	3948.617
5805.32	1899.425	2895.762	2060.716	3022.025	4598.603
5833.691	4052.112	1793.022	2369.838	2757.065	4542.757
2668.368	3254.997	2230.909	2250.427	3119.427	5031.15
1656.218	5643.56	4155.009	4138.231	2589.204	2852.837
8372.735	3683.547	2389.384	3626.533	3003.786	4889.878
3950.875	3816.914	2497.455	4631.238	2014.255	4728.209
4911.16	2208.683	2439.56	3310.449	2014.709	4219.645
5085.594	2997.595	2370.993	3949.806	3451.894	
4763.147	2955.598	2254.445	2372.563	3431.839	
5162.678	2221.628	2189.663	2572.868	2070.788	
4369.095	2320.525	3838.513	3794.829	3119.048	
4065.938	2021.879	2523.187	3370.987	2298.589	
5137.834	2478.051	2141.454	4162.522	3214.18	
	2632.868	2377.426	2119.892	2637.338	
	5798.151	1537.6	1948.645	3044.275	
	3053.591	2884.031	4507.059	2234.865	
	3852.514	2348.289	1793.062	2062.615	
	2087.81	2373.037	1781.711	4058.555	
	3194.409	1839.641	2029.917	3277.903	
	3411.168	2281.388	3373.862	2483.402	
	2372.607	2262.997	4479.893	3313.019	
	2777.525	2523.187	3876.177	2806.334	
	2840.52	2010.149	1833.093	2229.189	
	2208.608	2590.996	2040.965	2096.066	
	1863.525		1993.821	2154.265	
	2748.925		2389.135	2339.835	
	2760.967		2113.081	2917.509	
	2547.52		1842.779	2537.741	
			2117.168	2351.868	
				2345.738	
				1558.351	
				2316.752	
				2374.043	
				1845.183	
				1730.526	
				1535.344	
				3112.843	
				3189.281	

WEE1-c-10

day 1	day 2	day 3	day 4	day 5	day 6
5643.89	3443.532	2172.483	1470.318	4406.234	1911.44
3326.143	2491.298	1457.454	1875.848	1818.013	2149.86
1843.235	3560.416	3533.672	4299.792	1367.862	2188.88
1730.112	2363.801	1766.912	1820.077	1446.419	2397.035
1801.764	5560.243	1793.627	2310.435	1662.791	1859.74
2364.519	5184.527	1853.793	2433.176	2769.401	1824.872
2191.861	3576.071	1445.194	1905.284	2265.062	2403.526
2180.195	3266.136	1926.976	2563.712	2290.415	1466.524
2189.152	2386.155	2335.272	2129.729	1209.537	1686.831
1865.588	1623.735	2604.922	1425.596	1964.456	2028.65
2308.07	1938.186	2152.277	2054.586	1839.053	2910.632
2612.215	3282.618	1749.808	2312.1	3664.483	2450.32
2306.188	3100.255	2054.649	1303.006	1963.321	2124.35
2318.908	3404.846	2488.676	3100.609	2424.749	1280.935
1962.304	3053.215	1910.402	3580.675	1871.066	1520.714
2424.731	4210.392	1397.137	1170.731	2056.712	1850.608
	2776.095	1825.413	1925.867	1608.982	2665.796
	3286.381	2442.209	3596.188	1991.247	1997.328
	3412.749	1488.408	1333.578	1489.33	1527.582
	2117.991	2367.739	2568.479	1255.702	2475.226
	2462.547	7155.361	1916.257	1768.745	2447.603
	2204.619	1241.462	1467.443	2395.915	2166.162
	3959.011	2945.862	2671.394	3848.009	2079.594
	4806.78	2110.955	4309.251	1750.43	1892.496
		2131.086	1736.232	1912.615	3203.846
		2203.739	1859.502	1668.467	3084.9
		2326.115	1644.819	2172.277	2922.104
		1563.785	1673.348	1837.312	1987.819
		1956.491	1924.354	1491.676	2760.062
		2410.044	3444.843	2260.9	2493.717
		2113.301	3299.628	1873.109	1934.006
		1698.572	2159.771	2101.212	1108.402
		3117.203	1930.029	1971.495	
		1525.869	2079.407	1998.816	
		2926.715	1388.214	1535.571	
		2761.504	1460.33	1554.34	
		2045.492	3312.265	1617.837	
		2500.785	1740.848	1648.942	
		2915.665	1396.613		
			1109.284		
			1645.5		

II.III. Mitotic cell area (μm^2) following synchronization

Wild type

2938.952	2801.709	2799.265	3921.299	3311.524	3664.139
3486.39	3637.401	2866.189	3431.628	2212.048	2203.063
2857.107	3724.45	2095.968	3276.592	2856.629	2349.259
2770.659	3324.533	3100.209	3106.168	2644.086	3805.702
3675.291	3169.785	2155.697	3170.784	2811.7	2906.511
3320.573	3381.595	2109.984	2850.441	2859.066	3830.46
2607.429	3651	2721	3581.698	3557.116	
3620.295	2813.073	3097.047	2667.158	2368.521	
3458.215	2814.862	2760.46	2738.315	3872.147	
2655.658	3132.769	2572.002	3493.937	3226.495	
2894.932	3152.678	2370.749	3334.948	3029.915	
3412.646	2813.856	2234.689	2832.982	2579.836	
3318.273	2980.033	2192.066	3651.13	2302.108	
3555.319	3245.47	3198.32	3133.344	2728.331	
3498.465	2280.474	3031.28	3114.153	3706.043	
2502.354	1868.769	2344.155	3588.667	3410.712	
3159.931	2870.279	3153.325	3739.289	2870.782	
3567.538	3024.955	3829.983	3357.021	2837.647	
2677.364	3425.95	3533.627	2960.719	2542.094	
2815.222	3139.956	3378.304	3810.622	3257.976	

Constitutive lines

EV-c-10	EV-c-10 (-)Hyg	WEE1-c-2	WEE1-c-10	WEE1-c-10 (-)Hyg	WEE1-c-12	WEE1-c-12 (-)Hyg
3734.324	5442.708	1403.238	4110.473	1903.277	2301.642	3124.107
2040.267	2513.281	1723.695	1771.888	1331.985	2935.352	5040.923
2645.394	3486.143	2153.839	1900.333	1565.686	1615.287	2953.240
4435.438	2286.724	1852.335	1808.289	1517.366	2797.007	2703.227
3265.23	2247.798	1739.223	1623.339	1597.779	2760.397	2587.859
3794.874	2757.833	3535.840	2400.115	1218.976	2806.235	3663.969
5234.25	1635.214	2492.869	2707.049	1651.053	2989.358	2259.981
3331.129	2321.359	1491.991	2588.727	1699.157	3870.259	3096.395
3321.62	2914.292	2360.499	1248.485	2512.119	1928.057	2780.339
4289.153	1630.847	1778.119	4957.682	1888.128	6535.653	3644.541
2904.012	2492.425	1338.233	2666.555	2495.247	3348.950	3752.15
3662.63	3952.583	2017.130	2717.818	1781.652	2931.343	3774.743
2613.299	2826.274	1773.324	2350.575	1844.259	4231.136	7107.145
2427.935	3451.583	1292.638	1705.690	1919.575	2726.132	5194.772
3045.394	3890.014	1370.735	1437.455	2092.248	3143.966	3641.905
3007.652	3559.553	1760.536	1935.729	3549.591	3942.419	3448.522
3646.285	3471.837	2127.349	1799.386	2289.834	2622.884	2494.105
4750.743	3495.855	2191.289	2133.459	3852.218	2145.143	5033.092
4469.762	1985.703	1986.303	2717.603	1433.363	2757.523	3917.145
3265.527	1737.763	1911.326	1586.507	1341.606	1527.923	2486.047
4125.26	2211.507	2218.92	2742.014	1298.958	3624.052	2154.780
4094.502	3160.350	1997.949	2467.174	1750.779	1299.491	1960.191
2553.566	2027.490	1401.944	1731.107	1718.111	3493.876	1835.110
1817.979	2394.770	1751.021	2294.214	2074.801	2620.312	3376.982

1545.914	3895.285	1520.079	2448.937	1508.104	1739.033	2845.328
2791.753	3125.941	1388.623	3447.495	1433.363	3478.521	2179.630
3570.059	5063.984	1770.279	2216.385	1712.152	2540.663	2312.393
4874.889	5069.329	1547.634	1988.141	1679.054	2822.346	2267.361
2374.666	3643.430	2318.102	3277.623	3716.377	2289.237	2784.180
5251.932	3641.397	2343.829	8076.633	3257.017	6010.108	5434.543
3882.318	5050.355	1810.394	2150.547	3231.744	2190.300	7868.479
2957.355	5289.26	2233.839	2016.860	1750.995	1785.476	5016.299
2539.302	3134.599	3274.907	2470.907	1078.756	1915.173	1529.371
2410.03	2890.273	1927.311	2015.999	1665.771	2209.137	5215.255
3088.187	2122.360	1677.643	2397.458	1344.621	2380.604	2502.012
3136.924	3671.062	3390.530	3068.764	1680.418	1775.282	1901.228
4457.058	4765.296	3031.557	1628.149	3561.151	1929.804	2758.651
2898.96	3875.257	3513.689	1939.319	2672.155	3231.817	2195.294
3484.25	2648.206	2426.798	2868.162	2755.512	1502.742	1901.303
2359.435	2429.706	1973.210	1688.962	2371.683	2696.889	2298.989
3248.663	2477.216	1922.972	1931.063	3158.511	3607.671	1673.053
3744.577	3491.564	1240.573	2640.923	2867.229	1204.000	2097.699
1843.016	3534.104	1482.933	1794.647	1832.700	1135.323	2331.822
2547.994	4390.638	2776.104	1576.599	2384.750	2541.546	2424.598
3530.906	5264.941	1608.071	2320.994	1397.392	2014.455	1451.581
2629.792	2554.241	1975.418	2747.255	2184.005	2516.091	1510.168
3101.263	2322.865	2363.087	2188.743	3020.804	1922.862	3026.135
3309.435	3014.356	2131.231	1412.183	2596.050	1979.296	2162.536
2944.279	2282.734	2638.512	1871.901	2996.321	3008.395	2203.352
3637.147	3222.015	4765.7	1836.936	1347.780	2948.154	1854.840
4294.874	3197.469	5092.877	2083.776	1731.753	2670.463	1846.857
3983.581	4292.907	2852.123	3183.137	2078.032	2342.459	3182.920
4818.722	2638.343	5209.820	1789.119	2040.841	2099.330	3167.558
1944.056	6278.912	3708.618	1848.783	1451.169	2037.148	2166.979
1605.126	4144.731	2397.639	2550.746	2078.391	1918.233	2117.579
1803.789	2688.78	3023.2	1575.306	1581.337	1816.563	1656.486
3723.328	2811.517	2488.156	2566.111	1504.873	1847.392	1986.096
3723.328	3031.071	1639.255	2379.150	1787.539	2530.274	1599.405
3450.149	6251.204	2554.829	2574.726	1172.451	4081.985	2208.623
4815.973	5274.051	1752.249	2844.756	2133.316	1847.467	1764.925
2412.11	5275.030	1958.039	1906.508	2683.715	2215.482	2672.728
4959.435	3515.281	1875.192	1891.861	2121.397	2340.444	1637.660
3130.163	2095.555	2876.271	1880.374	1797.591	2745.708	1806.192
3626.226	3306.719	1896.378	2108.546	4503.348	2813.564	1588.636
3790.49	2439.41	1837.375	3151.618	2626.492	2918.519	1959.212
2685.587	3185.874	2609.428	1671.156	2149.686	2570.435	1845.652
3452.897	3095.221	3332.57	1950.017	1808.720	3018.697	2360.287
3568.945	2067.923	2489.979	2344.113	2015.568	1709.248	2507.961
4833.432	3733.330	3198.473	2300.460	3188.738	1990.172	2191.077
2622.66	5676.49	3382.848	1464.810	2459.420	2989.817	2428.213
2777.117	3393.532	2537.136	1256.454	2570.634	1968.750	2948.194
3706.166	3142.882	2504.787	1580.476	2293.783	2509.480	2954.294
3361.144	3352.573	3641.490	1330.477	1284.814	2164.212	2530.326
4382.095	2832.223	3219.052	1790.627	5283.714	1399.988	2013.206
3307.801	5049.753	2001.855	1891.143	7957.449	2288.493	2021.113
5960.327	2419.61	1838.970	1607.831	1891.574	1414.877	2064.338
4202.452	2256.833	2896.243	1951.812	2309.866	2461.393	2373.692
2982.764	3910.569	3301.747	1805.991	1937.093	2506.214	2297.634

3454.235	2040.215	2335.978	1843.039	2784.805	1454.760	4355.797
3107.281	3630.404	1938.22	2455.184	2666.196	1319.615	2451.783
4175.78	2788.025	2444.948	1618.887	1642.796	2138.839	1550.456
2844.428	2731.782	2068.201	2074.442	2237.996	2459.114	2502.991
2397.548	3129.555	2481.069	3313.952	2581.619	1832.010	1640.973
2895.914	2676.517	1801.747	1750.995	2507.811	2928.740	2079.023
2022.363	4246.226	2893.860	1377.863	1766.216	2522.243	2832.676
4024.22	3299.416	2015.608	1905.790	1606.969	2122.430	947.865
4663.596	3477.032	1276.114	2337.579	2458.702	2057.85	2814.151
4660.03	2445.743	1953.920	2127.644	1995.824	3431.33	2515.190
3383.21	2449.132	2441.358	1387.843	1644.304	2089.157	4579.228
4386.627	5297.166	2205.672	2177.256	2254.438	2756.448	3932.206
4476.969	3583.572	2003.861	1359.771	1480.247	2422.650	3224.639
2440.639	3635.675	7702.462	1638.344	1458.420	1283.075	2752.025
4774.591	2751.734	2910.533	1468.831	2438.239	1940.946	1915.084
2702.675	2436.031	4777.833	1726.009	1993.311	2000.808	2310.511
3284.101	2906.687	3657.908	1639.493	2147.388	2418.700	2670.017
3474.74	2554.994	5003.971	2908.153	1793.642	2650.626	1482.531
6383.061	4880.193	2376.033	2353.447	2047.949	2306.345	2065.468
6769.539	3872.019	2784.354	1590.527	1481.539	1552.225	2283.401
3623.254	2492.274	3436.242	2124.269	2700.946	2407.761	2274.440
3483.73	1686.639	2885.752	1834.567	1661.176	1991.996	1739.849
6467.311	7169.855	2481.524	1973.423	1324.159	3287.302	1670.794
3068.351		2988.362	2058.359	2067.190	3409.380	1597.974
4258.841		3250.345	1850.434	2576.449	2836.972	1558.439
6090.267		3158.799	1661.750	1683.505	1609.808	3343.471
		2604.141	1930.704	1689.680	2094.019	2841.487
		2476.825	1822.648	1882.025	4036.865	1959.288
		2413.015	2317.620	2520.878	1889.365	2177.145
		1730.511	1870.107	2105.530	1529.435	2511.801
		2363.605	1616.518	1857.757	1829.275	1728.026
		3170.924	1550.321	1873.696	1766.071	3216.883
		3384.860	3101.647	1949.084	1928.488	2001.383
		2886.661	2037.610	1888.989	1548.655	1787.818
		2805.042	2215.811	1964.089	2827.856	1641.500
		2601.185	2533.012	2270.880	1405.230	1697.603
		2165.204	2906.358	1815.828	2806.889	2343.268
		2376.943	2112.710	1472.492	1964.040	2047.244
		3598.873	1941.832	1720.337	2911.267	2396.509
		4001.585	1457.343	1295.871	3221.971	2037.756
		3453.293	1865.870	1958.058	2401.684	2241.456
		2644.912	1829.326	1838.587	2133.825	2054.925
		3627.215	2737.850	1649.688	1521.762	4520.490
		1684.738	3053.184	3135.033	1675.063	2119.085
		3509.903	2099.068	3561.797	2934.639	1500.604
		1975.822	1615.872	2327.959	3147.602	2517.299
		1927.245	1810.730	1883.245	2116.374	2662.562
		2748.281	1911.175	2043.497	2490.791	1512.879
		3618.046	1571.50	2666.771	2507.810	1496.011
		3495.428	1838.157	2467.748	2189.947	1990.313
		3571.818	1551.182	2118.238	1870.428	3265.304
		1500.206	1551.541	2153.060	3566.675	3737.015
		4521.989	1547.521	1395.310	2135.426	2632.214
		2941.907	1879.727	1708.778	2494.481	2504.045

2462.275	1512.699	2059.652	1712.136	2494.406
3153.646	1417.783	1827.674	2361.417	2651.794
2607.930	1140.573	2014.635	2126.616	2756.543
2298.28	2040.195	2198.292	3033.063	2079.701
2686.821	2195.492	2012.912	2821.155	2955.951
2685.532	2375.991	1969.115	2646.221	1625.234
3753.925	2031.363	2088.945	2932.682	4008.792
2787.309	2092.75	1520.166	2638.841	2502.463
2707.055	2139.562	2475.143	2351.401	2785.234
3696.557	2236.058	2117.377	2834.785	1641.425
2328.517	1629.657	2224.427	2279.334	2147.400
2436.129	1677.330	1810.443	3080.581	2260.056
2425.596	1563.173	1971.987	3729.259	1996.790
3049.595	2769.513	1436.307	2733.725	2079.776
3387.892	1541.203	1561.449	2441.240	3233.601
4078.278	2171.799	1712.296	2487.779	2589.290
	1769.231	5862.329	3163.341	3072.674
	3219.89	1347.565	3459.215	2930.121
	2330.615		1687.662	4347.062
			1708.974	5687.794
			1822.910	
			1925.626	
			3117.330	
			1811.238	
			1943.398	
			2084.445	
			2215.626	
			1807.548	
			2371.583	
			2864.229	
			2917.093	
			1772.531	
			3144.967	
			3101.666	
			2178.350	
			2480.248	
			2777.930	
			1723.056	
			2443.650	
			1800.620	
			2443.424	
			1988.431	
			2668.511	
			2483.788	
			2026.159	
			1947.088	
			3391.892	
			2589.215	
			2984.567	
			3116.049	
			3394.980	
			3103.323	
			2447.039	
			1844.222	

2906.701
2636.582
2027.213
1725.390

Inducible lines

EV-i-1	WEE1-i-1 (-)dex	WEE1-i-1 (+)dex	WEE1-i-6 (-)dex	WEE1-i-6 (+)dex
1615.854	2759.679	2495.094	2538.462	3238.2875
2594.865	1654.265	2796.774	5458.596	1986.730
1509.486	2753.481	2096.338	1857.061	2757.028
1545.158	3611.854	1848.997	6189.480	1482.204
1999.46	2976.884	2270.744	3308.180	1546.677
2004.72	2733.951	1977.928	2688.798	1380.053
2623.547	4897.288	2014.756	2569.688	1574.673
2558.977	2907.125	2077.095	2195.262	2364.261
2940.42	1980.650	1954.002	1796.927	1639.896
2478.768	2218.827	4776.073	3324.772	1701.892
1581.263	1912.116	1616.287	4294.165	1032.694
2788.506	3215.782	2861.276	3540.385	2402.690
2910.729	3577.695	2631.160	3592.478	1388.234
1925.808	3579.569	1900.238	3487.206	1359.637
2397.622	4979.803	1610.377	3107.636	1280.378
2035.348	1875.506	1703.202	3122.561	1638.020
1971.642	2912.890	1476.474	3061.123	1707.822
2035.853	2721.556	1444.043	2103.394	1127.264
2499.523	2163.552	1961.497	2103.032	1497.515
1717.539	2351.355	1497.734	2643.516	1894.336
1501.342	2595.369	2759.803	3528.721	1808.172
1776.272	2539.663	2188.730	2754.366	1565.366
2392.793	3185.226	2600.171	2507.236	2101.640
2680.623	2807.386	2427.710	3236.961	2333.113
1879.326	3727.880	3455.628	3287.025	1541.423
1850.716	3614.953	2092.518	2484.413	3654.697
2044.717	4244.374	1781.181	2705.171	4279.988
2490.298	3147.896	1765.470	2133.968	2198.087
2204.198	3607.098	2172.298	4186.285	3339.838
2030.015	2156.418	3225.079	4416.100	2231.562
2893.505	2004.215	1824.638	2628.229	1319.633
2441.51	2289.307	2382.379	3477.788	2641.292
1703.198	2833.114	2392.685	3279.127	2295.435
2639.69	2714.350	2821.206	2673.221	1496.239
2388.83	4519.520	6119.075	2384.649	2384.301
2769.336	2642.644	3345.939	2482.747	1742.573
1863.256	3289.433	2357.155	2826.020	1592.686
2180.056	3680.965	2133.021	3299.269	1945.299
2480.281	3283.812	1375.361	3502.131	3047.795
2382.993	4152.490	2309.877	2609.898	2308.269
1943.537	3488.262	2913.526	1879.376	1707.747
2170.687	2419.169	3399.990	1817.938	1737.319
2293.631	2406.702	3181.838	2411.166	2373.493
2246.14	2586.577	1892.022	2597.002	1740.021
2495.775	2292.261	2376.325	4463.555	2251.152
2240.807	2407.062	2267.645	3313.831	2453.953

2316.548	2471.056	3217.801	2745.961	2080.925
1802	3619.998	2946.245	3529.083	2361.484
1687.343	3552.400	2908.697	3446.851	2134.139
1627.457	2641.780	3034.746	2860.506	1643.574
1599.856	2758.021	1655.925	3484.598	2407.118
1842.573	2549.247	1974.109	2775.956	2817.824
1854.608	3847.365	2286.239	4568.536	2336.865
2302.207	3327.051	2972.983	2141.576	2495.008
2213.278	3449.418	2748.705	2564.254	2649.398
2205.567	3459.796	2194.063	3806.932	2601.738
2035.708	3989.910	2406.378	1764.034	2364.936
1855.761	2218.106	2692.707	3690.649	2193.208
2133.646	2034.195	2767.226	2681.190	2821.952
2927.952	2071.885	2961.740	2346.105	2698.185
2432.502	2866.408	1588.252	4076.015	2193.959
2195.982	2423.853	2099.220	2897.601	2625.230
2718.314	1700.963	1526.994	3575.741	3478.466
2808.107	2216.160	2200.261	3278.331	3430.655
1958.598	2890.982	1483.752	3978.569	1984.928
2077.507	2596.378	1620.107	2835.655	1974.946
1739.159	2549.103	1718.625	2096.222	2175.645
2398.703	2546.437	1947.876	1558.781	1959.034
2386.524	2004.215	3710.031	1850.541	2420.478
2533.105	2729.555	1709.400	2249.165	1989.807
2168.525	3014.142	1813.828	2187.220	2431.361
2472.426	3828.267	2224.476	2010.802	2673.791
2475.309	2890.118	2920.589	2056.808	3745.440
4070.264	3361.715	2613.143	2130.926	2433.763
2191.875	3613.007	2690.257	2729.587	2879.144
2179.263	3938.600	2813.278	2389.503	3834.756
2766.67	2895.955	2769.893	1850.468	3491.075
1650.734	2264.084	3563.875	1640.071	1824.984
2328.511	2936.312	1913.715	1907.632	1674.797
2187.046	2672.047	2967.073	1852.134	1896.212
2277.272	2733.159	2636.710	2568.022	1620.982
2432.79	2870.588	1458.528	2639.386	2785.174
2514.44	3357.391	1446.925	2183.308	2322.155
3754.401	2326.132	1793.144	1747.008	2004.368
2640.771	2374.416	1165.929	1891.330	1878.499
2566.688	2354.526	2875.618	1622.538	2017.427
1967.102	1655.058	3372.676	1870.247	2693.231
3134.492	1763.660	2877.275	1666.515	2549.874
2778.849	1701.684	2564.713	1891.620	2430.986
3978.669	1529.375	2669.933	1902.778	2915.096
2007.819	2104.386	2498.482	3017.870	3339.237
2630.394	2476.173	2973.199	2692.348	4921.341
2707.432	3035.834	2323.859	2495.933	3874.761
3135.069	2882.623	2370.776	2453.549	2751.850
2197.063	2179.911	2285.374	2170.049	1784.829
3629.295	617.313	2084.806	2148.821	1572.721
3415.26	2449.292	2562.623	2044.492	2165.438
2829.006	1998.882	3541.750	1952.696	1660.462
3101.054	2456.427	3515.084	2339.874	1778.750
2575.191	1986.920	3185.225	1527.844	1631.940

3545.915
3285.902
2357.625
2666.787
2709.594
2260.769
1946.708
3449.347
3832.448
3815.296
4041.726
2840.393
3622.953
1998.018
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2632.051
2793.046
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2952.311
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3467.075
3754.112
3367.552
3423.259
3350.185
4657.31
3516.224

2657.850
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1996.432
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2249.815
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3099.036
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2844.140
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2199.081
2633.060

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1826.151
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2694.220
2286.815
2404.071
2013.747
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2601.684
2163.650
2170.208
1984.198
2341.372
1948.596
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2114.211
1220.557
1623.998
1620.323
1786.586
1822.332
1297.238
1925.462
1516.544
1520.940
2084.879
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1552.506
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2536.390

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2962.445
1859.162
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3693.474
2994.97
2405.152
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2567.732
2586.787
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4045.441
4269.459
2926.944
2980.050
3836.637
3099.884
2639.821
3177.914
3097.638

2092.483
1755.557
1769.893
2823.978
2763.934
2563.309
2172.268

II.IV. Mitotic cell area (μm^2) following synchronization of EV-c-10 subcultured for 4 weeks without hygromycin

2616.374	2668.523	2308.57	3211.868	3115.946	2315.1	2949.773	3392.077
3434.634	2774.292	3973.836	1807.797	2226.609	3069.862	2630.71	2227.509
2875.843	2585.076	2973.866	1832.565	2292.508	1739.346	3053.875	2879.445
2388.955	4085.519	3145.068	2206.269	2221.355	1586.832	2782.698	5287.614
3044.718	3493.853	3737.785	1809.748	2079.875	1681.853	2835.312	5637.075
2553.627	2073.044	2457.106	2179.474	2528.334	1969.092	2113.95	3271.762
1071.798	3398.307	2219.178	2967.411	2174.22	1930.513	2766.486	1792.26
2684.825	1685.08	5710.404	3950.193	3535.809	2808.067	2712.296	2983.698
2730.759	1635.468	2250.101	4796.899	2943.018	3327.154	2656.004	2163.712
3243.016	2759.431	1501.794	5994.641	3142.516	2569.239	3041.416	2573.968
2411.622	2514.223	2240.119	3058.303	2377.471	2543.57	3894.877	2399.763
2040.695	3234.76	4170.482	2926.655	2140.144	2795.308	3195.055	2097.663
4144.588	3253.899	3722.173	3593.602	2736.989	2663.284	2018.028	3340.138
2926.355	1880.226	2753.952	2437.441	1912.95	1793.836	1841.647	2212.123
2096.087	1555.234	3813.291	3419.247	3409.64	2107.795	2649.849	2742.618
2247.775	2100.59	3797.079	2467.088	2665.536	2537.565	2164.538	
2943.093	2083.027	2064.938	2189.081	1650.93	2678.971	2519.327	
2116.502	1603.57	3909.137	1867.316	2889.503	4659.246	2872.465	
2341.069	1568.519	2672.891	1608.749	2556.254	2119.954	2315.1	
1824.759	1921.957	3527.178	2531.636	2502.064	2086.78	2080.325	

II.V. Mitotic cell area (μm^2) of EV-c-10 subcultured for 3 weeks without hygromycin

1 week	2 week	3 week
2196.059	1719.747	2226.083
2490.276	1383.615	2182.326
2404.972	2066.907	2325.608
2920.821	3696.469	1361.664
2250.892	2329.96	3057.928
1229.328	1775.494	1228.44
4449.395	1589.721	3290.526
2463.183	2259.208	2178.123
2393.761	2627.676	2066.515
3987.23	1820.736	1501.869
2505.296	1887.737	1455.935
2436.52	2323.881	1913.551
1855.993	1895.988	1918.354
3013.671	1794.287	1747.002
3247.664	3214.12	1806.296
3224.02	2182.701	1962.187
2405.341	2238.993	1434.844
1498.032	1598.541	3067.685
2635.765	1990.858	1579.552
3467.267	1861.687	1111.728
2456.256	1933.29	2331.237
2689.184	2499.587	3341.79
2234.987	2622.979	1169.897
2455.662	3011.619	1383.205
2231.508	1905.144	2468.814

3462.302	1970.443	2126.86
2369.98	4027.726	1397.992
1544.926	3840.386	1485.432
2345.432	1667.292	1787.081
2254.99	1386.433	1279.178
1845.098	1415.329	1643.199
1738.478	1029.467	1313.103
2298.09	1052.209	1777.474
	1287.509	1715.553
	2324.857	2267.214
	2114.55	2137.893
	1203.296	1486.783
	1405.197	1733.266
	1796.238	2807.392
	2031.914	1649.279
	2008.947	2676.269
	1290.962	2394.809
	1402.87	1691.235
	2425.132	1453.983
	1693.637	2202.216
	2329.51	2351.652
	1816.203	1895.912
	1643.95	851.4343
	1600.417	1676.299
	2243.196	
	1801.192	
	2105.168	
	1948.902	
	1683.729	
	2070.192	
	2692.406	
	2552.201	
	1998.364	
	2243.571	
	1668.718	
	2152.379	
	1934.641	
	2350.901	
	2405.017	
	1726.061	
	2291.532	
	1940.946	
	1906.42	
	1657.235	
	3352.523	
	3095.306	
	1949.502	
	3357.551	
	896.693	
	1290.737	
	1866.566	
	1330.216	
	1691.685	
	2413.723	

II.VI. Mitotic cell area (μm^2) of WEE1-i-1, WEE1-1-6, EV-i-1 and EV-i-3 (±) dexametasone at 0, 12, 24, 36 and 48 hour

WEE1-i-1 (+) dex

0 h	12 h	24 h	36 h	48 h
1390.031	1307.101	1909.789	1395.294	1281.538
721.2551	1776.485	1707.539	1870.617	955.7589
813.0567	1160.414	1189.962	2262.183	1247.329
1652.353	1293.868	1224.322	1490.704	2441.576
1096.506	1634.309	1363.791	1719.795	1859.64
623.3635	1282.29	2223.688	1855.805	1575.363
998.3891	1989.034	1892.12	1482.283	1592.731
1575.739	1138.911	1084.777	2071.513	1561.529
1066.582	1543.71	1220.111	1249.058	1482.509
922.6773	1810.393	1436.947	1567.995	1571.604
2421.201	1122.459	1592.957	1383.414	1698.216
2196.17	1298.372	1422.135	1552.732	962.4504
1268.381		1571.153	3162.38	1348.303
864.2581		975.4576	2642.848	2456.012
890.1971		1127.408	2099.858	2054.446
720.8792			1790.92	2258.499
944.1052			1832.056	1697.84
961.3979			1645.906	1999.56
1392.512				1559.574
1729.644				1161.692
1923.848				1850.693
1731.524				1692.202
1104.702				
657.573				
1008.614				
1549.424				

WEE1-i-1 (-) dex

0 h	12 h	24 h	36 h	48 h
1725.386	2558.959	1963.991	2575.606	2722.202
1957.112	2484.367	2073.169	2050.313	2724.861
1905.865	2068.21	1859.771	2514.698	2948.968
2689.664	1950.277	2427.011	2862	2760.224
2343.707	2187.235	1697.19	1843.815	2867.102
2665.708	1846.978	2240.063	2380.637	2015.453
2017.322	1307.051	2590.153	2099.116	3478.832
2280.745	2104.363	2289.657	2681.88	2329.404
2247.754	2001.869	2565.141	2457.342	2765.313
1840.581	1742.399	1086.897	2076.331	1972.629
2034.716	2224.754	2143.679	1857.256	2879.429
1794.796	1813.34	1940.2	2631.999	2387.048
1224.754	2121.182	2330.482	1614.318	1948.321
1985.941	1583.842	1694.818	2380.076	2941.637
1242.866	2468.555	2345.432	2403.436	2474.304
2048.588	1944.009	1471.501	2589.24	1854.812
1880.472	2405.808	1927.334	2463.164	3003.234
2699.202	2432.617	2277.941	2299.145	1994.537
1840.221	2073.96	1599.296	2429.023	3105.800
2265.651	1453.317	2783.44	2300.87	2690.936
1828.29	2045.353	1299.792	2091.282	2569.395
2647.452	1706.821	2338.964	1784.662	2083.662
1700.496	2198.807	2526.055	2247.898	2057.859
2293.898	1685.618	2412.923	2321.21	2284.769
	2568.821	1835.549	2563.214	2816.286
	1932.94		2568.389	2054.912
	2413.139		1648.027	3019.334
	2648.89		2229.929	3164.881
	2347.876		1798.318	2231.006
	2279.379		1768.202	2777.258
	2058.722		1802.199	2759.074
	2067.635		2296.507	2950.837
			2310.422	3316.829
			3145.26	2898.152
			2407.82	

WEE1-i-6 (+) dex

0 h	12 h	24 h	36 h	48 h
1671.45	1043.049	2654.201	1534.253	1530.252
1155.151	2990.205	1445.518	1793.852	1881.594
1116.506	2602.022	1518.222	1634.035	1649.346
1339.356	1049.741	1686.863	1209.36	2137.526
1428.601	1758.515	1595.889	1864.151	1595.513
1659.045	1804.078	1542.883	1475.899	1803.777
1741.899	1813.025	1185.3	1352.748	2012.116
1560.476	1195.375	1147.257	1179.511	1736.486
1487.321	1065.154	1610.926	1426.12	1924.525
1637.842	1942.945	1736.035	1497.621	1703.404
1307.627	1710.396	1649.421	1536.267	2249.627
1518.072	2430.073	1437.473	1574.072	2398.72
1861.82	948.5411	2012.417	1742.638	2171.885
1916.987	1467.322	2091.587	1660.038	1176.053
1754.098	1612.054	2328.046	1600.642	2853.743
2043.467	1292.515	2269.251	1464.765	2158.878
	961.9241	1487.095	1528.735	2530.145
	2078.956	1408.526	1317.101	2171.434
	1550.251	1128.46	1034.102	2302.257
	1365.295	1131.693	1350.784	5738.99
	929.5192	1910.24	1563.264	1947.908
	932.2258	1442.736	1545.986	2167.473
	1980.388	1774.079	1750.743	1935.902
	1735.374	1591.829	1764.133	2223.478
	1658.936	1496.569	15296.46	
	2234.154	1628.745	1698.305	
	1556.305	2090.986	1398.473	
	1856.362			

WEE1-i-6 (-) dex

0 h	12 h	24 h	36 h	48 h
2188.888	1498.465	1257.107	2666.727	2212.048
2413.714	2502.354	1770.659	2265.164	2856.629
2152.519	1159.931	1275.291	2903.054	2644.086
1784.59	1567.538	1320.573	3464.037	2811.7
1834.399	1558.122	1607.429	2899.963	2859.066
1670.093	2349.259	1620.295	2101.503	2557.116
1275.282	2305.702	1458.215	2632.226	3368.521
2346.946	2906.511	1905.857	2283.205	3072.147
1920.362	1830.46	1721.783	1701.73	2226.495
3633.221	1799.265	2631.515	2370.965	3029.915
1874.937	2012.808	2559.351	2484.737	2579.836
1878.171	1934.032	2047.74	1629.279	3208.67
1818.228	2061.828	1665.217	2328.127	2893.063
1343.492	2333.805	1811.987	1697.346	2665.145
1472.436	2009.143	1874.304	2443.337	1996.277
1402.429	2332.368	2047.884	2111.925	1926.989
1258.607	2280.474	1709.493	2370.462	2738.818
2240.063	1868.769	1816.659	2456.857	2866.189
1700.855	1870.279	1337.895	1419.546	3095.968
1386.832	2024.955	2517.16	1280.394	2100.209
2448.322	2425.95	2209.173	2229.435	2155.697
1254.654	2139.956	3533.627	2675.646	2109.984
1681.233	2311.524	2670.831	2599.099	2721
1234.816	1998.217	2302.108	1905.857	2097.047
1901.387	1675.639	2728.331	1768.718	2760.46
2093.797	1761.099	1706.043	1671.614	2572.002
2363.114	1815.222	2203.063	2678.018	2370.749
1951.7	1743.49	2166.838	2924.552	2234.689
1486.02	1878.185	2296.214	1944.526	3192.066
2151.888	2196.091	2313.824	1600.529	3198.32
1851.232	2516.945	2472.454	2237.923	3031.28
1987.22	4154.633	1135.924	3074.988	2344.155
2535.201		1716.536	2165.4	3153.325
2097.981		3378.304	1637.401	2767.568
1732.708		2360.831	2724.45	2938.848
1799.769		2152.032	2324.533	2784.387
2093.812		1803.865	3169.785	2943.161
1381.236			3381.595	3095.609
1912.829			3651	3175.679
1902.407			2813.073	2155.769
				2954.023

EV-i-1 (+) dex

0 h	12 h	24 h	36 h	48 h
1783.74	2396.768	1340.483	2772.679	2046.662
2463.972	2687.146	1896.01	1942.945	1998.002
1850.441	1807.531	2092.518	1845.985	1557.404
2513.351	2020.499	1558.482	2001.021	1453.974
2246.692	2980.184	1968.964	2124.288	2148.582
2808.257	1912.038	1847.135	2086.122	2228.723
1898.31	2333.374	2030.921	2356.015	2295.208
3234.265	2081.45	2802.435	1551.078	2475.832
1211.537	2316.34	1460.659	2231.382	2523.414
1429.177	2388.215	2317.849	2063.696	2951.865
1660.545	3341.288	2603.699	2540.736	2101.215
1527.647	2293.411	2861.805	3418.698	2335.315
1407.255	2564.814	3215.433	2832.192	2258.12
1492.787	1681.605	2355.656	2753.416	2552.451
1138.009	2007.274	2748.025	2008.783	2676.94
1212.256	2332.152	2013.527	2389.868	2867.626
1288.732	1957.392	2993.912	2406.903	3585.593
2377.65	2149.3	2050.04	2366.293	2445.213
2542.532	2316.699	2489.776	2257.833	1737.093
2537.271	2025.53	2435.941	3652.869	1749.456
2659.043	1959.98	1794.234	2107.612	3184.598
1862.229	2748.672	2733.722	3112.22	3380.891
2518.095	2220.314	2427.819	2288.667	3223.914
2099.49	1589.101	2331.074	2239.289	2617.786
2227.07	2993.337	2350.265	2357.021	2264.805
2149.228	2826.585	1716.752	1860.719	1924.76
2741.413	2172.516	2104.881	1810.622	2223.476
1826.291	1960.123	2770.522	2410.712	2396.912
1703.311	2095.681	2306.205	1813.281	1900.179
1635.101	2085.618	2270.411	2838.804	1820.469
2060.893	2716.903	1399.924		2207.52
2554.967	2502.498	2655.809		2195.588
2367.946	2376.212	2159.794		2189.623
2049.896	2856.486	2524.132		2335.171
3010.731		2107.828		2745.869
1773.318		2130.325		
1850.01		2597.805		
1705.324		2165.76		
2101				
2174.026				
2303.977				
2851.311				

EV-i-1 (-) dex

0 h	12 h	24 h	36 h	48 h
1761.315	2079.437	2084.468	2576.53	3670.335
1881.204	2699.725	1921.526	1942.442	1572.857
2133.56	2081.953	2263.583	2708.35	1765.556
2192.354	2443.128	2368.378	1969.97	2469.147
2341.006	3195.739	2752.266	2058.377	2574.373
1445.637	3009.437	2434.359	2232.101	2490.854
2743.928	3259.062	2733.075	2401.225	3037.109
1665.145	1529.803	2649.699	2347.534	2773.613
2456.497	2997.003	2117.747	2356.949	2125.941
2218.013	2475.329	2244.464	3115.813	1921.526
1448.009	2554.464	2095.839	3408.635	2417.037
2412.509	2727.253	2642.296	2199.613	2228.435
2640.068	1500.406	2549.289	1844.619	2229.37
1248.985	2131.331	2128.959	2009.789	2337.615
1878.257	2502.641	1968.978	2971.774	2561.508
2083.462	2354.434	2895.155	2616.708	2319.574
2319.071	2684.631	2394.476	1942.514	2062.762
2174.529	3259.709	2026.903	2083.606	1865.319
2217.798	2727.253	2366.868	2102.94	1865.535
2215.067	3089.076	2845.92	2744.503	2430.191
2651.352	3128.895	2709.715	1867.332	2497.97
1990.239	1984.345	2616.996	2198.679	
2479.138	2435.725	2314.119	2333.662	
3067.8	2518.382	2065.206	1948.551	
2551.373	1833.622	2537.645	2131.763	
1846.703	3169.72	2487.195		
1633.232	2335.674	2493.729		
2286.727	1489.193	2289.17		
2257.689	1882.066	2938.208		
2008.136	2215.857	2299.312		
2433.425	1824.35	2848.148		
2344.371	1978.739	2152.039		
2457.863	2565.892	3177.195		
1722.79	2544.617	2726.75		
1610.807	2140.603	3334.316		
1887.888	2679.312	2711.8		
1606.279	2167.269	2679.528		
3208.964	4330.585	2282.206		
2618.433	2698.934	2140.754		
2918.083	2727.469	2045.735		
2081.665		2702.463		
1888.463				
2031.568				
2891.633				

EV-i-3 (+) dex

0 h	12 h	24 h	36 h	48 h
1794.796	1307.051	2405.808	2565.141	2553.367
2427.011	1599.296	1700.496	1086.897	1583.267
1828.29	2783.44	2293.898	2143.679	1781.571
2279.379	1299.792	1932.94	1940.2	4418.817
1058.722	2048.588	1413.139	2330.482	2489.614
2067.635	1880.472	1648.89	1694.818	1860.49
2045.353	2699.202	2347.876	1625.386	1919.572
1706.821	1840.221	2121.182	2345.432	1846.69
2198.807	2001.869	1963.991	1471.501	2460.936
1685.618	1742.399	2073.169	1583.842	2423.776
2568.821	2224.754	1859.771	1468.555	1932.294
1976.497	2027.6	1765.974	1474.161	3136.85
2523.395	2039.819	1927.19	1627.902	3051.103
2147.488	2533.602	1475.958	2898.153	2190.829
1846.69	1484.655	1619.421	1502.192	2971.106
1627.543	2176.382	1420.973	2082.153	2614.893
2903.04	1975.994	1913.462	1446.417	2324.876
2613.096	1765.184	1863.15	1830.159	1719.974
2836.34	2366.779	2703.443	2300.007	2188.888
2417.236	1847.193	1415.439	2293.035	2413.714
2583.699	2524.258	2072.45	1722.202	2152.519
2361.748	1882.7	1974.053	1724.862	1784.59
1892.69	1579.099	1809.89	1948.969	1834.399
1992.453	1201.035	1785.884	2760.224	1670.093
2669.661	1135.341	2282.038	1867.103	1275.282
2500.18	1176.022	1681.88	1015.453	2346.946
3106.447	1591.317	2257.313	1478.833	
2104.003	1654.783	2389.391	1105.8	
1948.969	1735.571	1647.524	1690.937	
1813.124	1368.504	1927.909	2569.396	
1649.465	1379.429	2099.835	2083.663	
	1387.048	2505.189	2057.86	
	1948.322	2082.01	2284.77	
	1941.637	2099.116	2575.606	
	1474.305	2681.88	2050.313	
	1854.812	1457.342	2514.698	
	2003.234	1378.567	1862	
	1994.537	2248.688	1843.815	
	1568.389	2371.02	2380.637	
	1648.027	2287.213		
	2229.929	1415.295		
	1798.318	2202.113		
	1823.187	1588.227		
	1738.087	1768.993		
	2040.538			

EV-i-3 (-) dex

0 h	12 h	24 h	36 h	48 h
1944.009	1647.452	1813.34	2292.554	2294.76
1857.112	1104.363	1432.617	2420.283	1637.03
1805.865	1224.754	2073.96	2241.716	1771.868
2589.664	1185.941	1453.317	2666.571	1474.951
2343.707	1242.866	1697.19	1980.809	1365.917
2665.708	2558.959	2240.063	2325.739	2041.256
2017.322	1484.367	2590.153	1952.778	2998.203
2280.745	1068.21	2289.657	2147.344	2832.028
2247.754	1550.277	2265.651	2120.535	2603.393
1840.581	2187.235	1927.334	1540.574	2337.239
2034.716	1846.978	2277.941	1393.229	2335.298
2698.627	1175.735	2866.025	1801.409	3367.929
1886.078	1810.178	2245.813	3589.161	2457.126
2355.207	1087.544	2294.401	2440.811	2076.044
2976.497	1082.225	2616.689	1814.059	1900.884
1888.953	2445.303	1811.543	2303.529	2417.02
2170.776	1651.621	2224.323	1799.396	1924.819
2066.269	1782.721	1647.524	2103.428	2273.341
2511.033	1665.205	1472.436	2018.759	1943.65
3171.279	1909.222	1402.429	1937.612	1592.036
2248.76	2144.182	1258.607	1898.728	1389.132
2484.942	1732.48	2240.063	2482.225	1707.18
2254.726	1920.362	1700.855	2591.246	2764.752
2047.078	2076.331	1386.832	2338.964	2246.316
2899.734	1857.256	2448.322	2526.055	2575.936
3298.57	2631.999	1254.654	2412.923	1877.74
2724.574	1614.318	1681.233	1835.549	2403.436
1405.233	1380.076	1234.816	1777.259	2589.24
1704.665	1797.599	1901.387	2759.074	2463.164
1461.151	1587.436	2093.797		2299.145
2130.525	2192.554	2363.114		2429.023
1642.277	2240.998	1951.7		3145.26
	1733.415	1486.02		2407.82
	1308.488	1950.837		
	1294.904	2316.826		
	1971.537	1898.153		
	2455.761			
	1769.856			
	2191.835			
	2225.041			
	2046.503			
	1723.209			
	1869.978			
	1734.996			
	2300.87			
	2091.282			
	1784.662			
	2247.898			
	2321.21			
	2563.214			
	1965.356			

1838.496
1768.202
1802.199
2296.507
2310.422

Appendix III: Tobacco BY-2 mitotic index (MI %)

Wild type

Time (h)	MI (%)
0	0
1	0
2	0
3	0.66
4	1.6
5	2.6
6	13.1
7	28.1
8	34.7
9	15.2
10	7.7
11	3.6
12	4.3
13	1.3
14	1
15	2.3
16	2
17	3.8
18	4.1
19	7.1
20	8.8
21	15.4
22	20.1
23	13.4
24	5.8

Constitutive lines

Time (h)	EV-c-10 (+)hyg MI (%)	EV-c-10 (-)hyg MI (%)	EV-c-10 (-)hyg 4 week MI (%)	WEE1-c-2 (+)hyg MI (%)	WEE1-c-10 (+)hyg MI (%)	WEE1-c-10 (-)hyg MI (%)	WEE1-c-12 (+)hyg MI (%)	WEE1-c-12 (-)hyg MI (%)
0	0	0	0	1	0.33	1	0.94	0
1	0	0	0	1.75	2.2	1.7	1.8	6
2	0	0	0.33	5.3	10.3	2	1.3	8.6
3	0	0	0	20	17.4	14.6	1.9	12.2
4	0	0	0.33	20	22	31.6	8.7	21.2
5	0	0.66	5.2	13	10.3	18	13.8	12.6
6	0.33	1.66	6	6.4	9.3	12.4	10.2	8.8
7	1.99	7.95	15.7	6.1	9.4	9.3	8.6	9.8
8	14.33	24.43	23.3	3	6.4	6.5	9.4	7.4
9	18.99	27.66	29.7	3.5	4.3	5.5	10.8	6.2
10	15.66	17.33	15.5	4.4	2	2.3	8.6	4.3
11	10.96	7.33	5	3.7	2.3	2	4.6	6.6
12	7.8	4.65	2.3	2.6	2.3	2.3	2.5	5.3
13	7.3	1.33	2.6	6.8	2.9	3.3	4.3	7
14	4.8	2.6	6.3	11.35	6	5.8	3	5.4
15	2.56	3.3	1.2	15.4	11.7	9.9	2.4	6.8
16	2.17	0.92	1.3	15.6	11.8	15.5	3.6	6.6
17	1	2.6	3.2	14.3	14.4	14.9	5.2	7.8
18	2.3	2.3	5.5	7.6	11.5	10.7	9.8	14.7
19	2	2	5.3	7	10.3	11.4	5.2	7.3
20	8	7.3	11.4	6.2	8	9.2	4.7	8.3
21	1.97	12.3	6.7	5.6	6.5	8.5	4	4.8
22	2.8	5.3	3.3	3.1	5.6	6.7	3.6	4.7
23	1.33	3.5			4.2	5.7	3.3	3.5

Inducible lines

Time (h)	EV-i-1 (-)dex MI (%)	EV-i-1 (+)dex MI (%)	WEE1-i-1 (+)dex MI (%)	WEE1-i-1 (-) dex MI (%)	WEE1-i-6 (+) dex MI (%)
0	0	0	1.3	2.2	0.66
1	0	0.3	0.3	0.6	3.6
2	0.3	0.65	1.3	1.66	11.9
3	0.6	0.68	7	2.3	14
4	1.26	1.33	14.7	3.5	28.3
5	4.7	7.6	26.2	11.44	10.3
6	13.3	17.3	15.9	11.8	6.4
7	20.44	16.07	12.8	25.5	4
8	30.7	23.5	9.9	18.83	1.3
9	23.4	8.7	10.2	16.3	2.5
10	21.24	5.1	8	12	1.2
11	23.3	4.16	5.1	9.2	3
12	16.4	3	2.5	6.09	1.5
13	7.9	2.24	2.6	5.23	11.5
14	3.5	1.6	1.8	3.2	12.6
15	1.6	2.23	1.8	2.7	9.3
16	2.15	3.7	2.8	3.3	4.6
17	4.3	9.24	5.3	2.65	4.8
18	1.9	13.16	12.5	5	1.1
19	4.4	14	4.2	7.3	0.93
20	14.24	9.4	6.7	15.6	1
21	6.3	9.1	3.9	12.54	
22	9.65	5.23		8.33	
23	10.8	2.7		4	
24	7.23				

Appendix IV: *18S rRNA, H4, AtWEE1* and *NtWEE1* expression raw data

Wild type

Time (h)	18S rRNA 1	18S rRNA 2	18S rRNA 3	H4 1	H4 2	NtWEE1 1	NtWEE1 2	NtWEE1 3
0	1678.96	2500.36	2805.84	2519.95	2832.32	123.98345	99.87329	122.98264
1	316.063	19173.8	26880.9	14143.8	14098.5	3119.67	3469	3565.83
2	16628.9	9227.1	21715.4	5951.18	5297.09	3190.1	2974.95	3287.22
3	29762.8	10363.4	13932.7	5150.96	5314.65	5088.82	5276.13	4976.27
4	31001.7	10603.9	13952.6	5049.62	5087.25	6651.29	6531.62	6746.44
5	26638.5	8927.72	32126.8	392.403	262.624	2432.999	2369.44	2325.95
6	40526.3	21083.1	42430.2	638.129	201.759	529.819	571.115	537.855
7	14417.8	12632	17556.4	727.067	517.635	515.295	552.901	577.787
8	27049.7	25112.5	21523.4	974.275	858.236	1501.54	1574.5	1414.01
9	26831.8	2868.53	3466.41	421.222	422.618	615.002	575.911	599.057
10	21555.7	2685.29	25499.9	550.827	465.19	694.11	720.858	643.055
11	20280.3	36744.4	38743.5	427.655	190.474	722.979	734.446	786.066
12	27984.1	1284.77	1254.65	762.246	413.764	622.664	660.884	645.232
13	9545.08	840.591	9305.24	866.615	410.083	1575.2	1482.61	1579.25
14	984.009	10582.6	10726.7	575.967	246.416	860.998	886.396	893.399
Mix 25%	27145.5	36766.9	567.938	1848.42	2094.84	656.779	805.031	939.569
Mix 50%	2483.11	9364.79	13627.2	3187.67	3426.05	2019	2273.7	2349.57
Mix 100%	1440.65	36139.2	45205.3	7417.57	8334.92	4004.66	6459.02	4875.22

WEE1-i-1 non-induced

Time (h)	NtWEE1 1	NtWEE1 2	NtWEE1 3	NtWEE1 4	NtWEE1 5
0	495.469	12169.5	333.182	2648.08	13323.7
1	2688.3	6589	1627.3	9987.4	9057.5
2	5330	5078.6	2296.5	7045.6	7594
3	21321.19	15814.5	17308.7	12731.7	25901.9
4	35739.3	49037.1	43622.9	33166.3	49141.2
5	7259.12	8301.44	1532.87	6573.92	3182.95
6	13859.9	15916.6	825.609	25127.3	28241.7
7	31439.9	20528.9	20158.6	20241.4	23657.6
8	34497.7	12761.5	18581.6	25065.3	16251.4
9	37264.5	42651.8	29210.9	25690.9	43869.5
10	33081.7	42963.1	23256.3	19112.2	44673.1
11	27297.3	44971	36292.1	4720.7	47016.5
12	30503.6	14792.5	17388.2	24900.8	18495.6
13	36982.8	10068.6	23479	28323.7	13381.7
14	29699.9	42068.5	11248.6	31138.5	47182.5
Mix 25%	17745.8	38862.8	18048.8	31806.6	31159.6
Mix 50%	21270.1	41168.7	21087.7	31434.7	34231.6
Mix 100%	39134.3	49860.5	18808.2	33999.1	46264.8

Time (h)	H4 1	H4 2	H4 3	NtWEE1 1	NtWEE1 2	NtWEE1 3
0	459.023	403.41	341.103	579.999	599.5	583.873
1	4592.3	4963.08	4034.19	1113.44	1072.35	1123.05
2	4117.79	4652.56	4436.19	2894.02	3185.23	2998.12
3	11862.2	11659.5	10918.6	11221.8	11832	11150.2
4	8684.95	8869.32	8288.69	8683.93	9581.7	8300.45
5	1269.09	1994.96	1325	1115.35	1166.302	1012.07
6	2007.26	2593.14	2288.93	3275.82	3529.5	2790.85
7	2546.10	2633.36	2373.56	292.68	238.792	215.907
8	2374.24	2446.82	2611.08	1394.49	2247.16	1430.95
9	1540.54	1785.88	1366.95	1523.34	1648.26	1186.42
10	1820.02	1627.89	1726.38	491.986	490.781	420.791
11	1864.44	1948.3	1834.90	4905.1	4136.49	4331.91
12	1953.61	1856.86	1981.91	739.086	929.952	630.717
13	1601.55	1704.62	1995.13	723.85	898.951	564.839
14	2118.34	2423.02	2160.3	859.15	854.153	851.6
Mix 25%	815.084	977	1154.21	389.032	215.963	733.338
Mix 50%	1837.65	2146.55	2077.51	2193.7	2014.64	2833.62
Time (h)	3223.12	7229.29	3278.89	6437.23	4606.74	8224.3

WEE1-i-1 induced

Time (h)	18S rRNA 1	18S rRNA 2	18S rRNA 3	H4 1	H4 2	H4 3	NtWEE1 1	NtWEE1 2	NtWEE1 3
0	11803.9	22835.2	11187.4	983.092	973.127	979.382	278.984	235.359	288.865
1	11187.5	17571.8	15805.4	7487.04	7732.61	7539.94	1570.55	1340.39	832.673
2	10411.9	12083.3	6429.79	2865.54	2821.24	2961.02	590.213	670.411	274
3	5784.67	8101.52	7729.67	1804.21	1679.86	1235.62	1474.12	953.229	1522.93
4	27484.1	43924.6	24316.5	2863.07	2538.14	2152.61	8491.16	7573.75	7742.24
5	9334.71	13549.5	9611.84	1162.07	1871.3	1199.36	2523.09	3384.89	3823.87
6	7955.58	8679.52	7916.78	358.359	212.934	249.637	8227.3	8884.89	8309.6
7	7168.45	6098	5354.24	920.042	1027	684.375	3219.73	2610.2	2486.98
8	3574.86	4477.44	2714	390.894	485.573	500.441	1432.33	1446.79	1933.56
9	5848.82	8213.13	6597.89	593.158	454.379	445.071	1565.45	648.49	915.741
10	2528.4	3704.22	2447.69	209.564	501.111	307.434	741.371	564.717	151.201
11	16257.6	23567.4	15355.8	1073.97	879.143	1368.15	603.109	614.046	607.48
12	2496.26	3277.24	2774.32	611.102	495.428	727.056	654.986	387.182	892.84
Mix 25%	7658.93	4505.89	4799.01	1486.49	1183.66	1035.84	11861	17346.6	22675.8
Mix 50%	21515	14056	15188.9	2976.64	2247.85	2829.29	17572.8	24814.7	34549.9
Mix 100%	36511.2	27775.7	34514.6	4517.91	3688.21	4551.46	27087.3	35007.5	37649.5

Time (h)	AtWEE1 1	AtWEE1 2	AtWEE1 3
0	240.981	180.564	129.098
1	1249.19	1122.30	1481.81
2	1279.09	1347.47	1298.05
3	1950.38	2188.94	2219.59
4	3278.19	3266.96	3377.34
5	1236.64	1130.48	1371.76
6	440.263	259.323	1099.2
7	827.35	407.589	940.232
8	463.024	212.948	491.977
9	615.6	624.381	922.031
10	307.974	493.804	371.705
11	873.56	1176.80	1047.72
12	750.33	589.192	366.92
Mix 25%	542.081	413.654	262.301
Mix 50%	6670.1	6077.28	4754.64
Mix 100%	15675	13710.3	10341.1

Appendix V: CDKA and CDKB1 kinase activity raw data as percentage of maximum

WEE1-i-1 non induced

Time (h)	CDKA 1	CDKA 2	CDKA 3	CDKA 4	CDKA 5
1	100	100			
2	67.309014	65.5573503			
3	55.219807	57.4142102			
4	57.409191	57.7932567			
5	65.878511	57.4523634	90.932333	36.465628	60.05409
6	62.513976	54.8004999	75.599645	53.795134	80.38205
7	65.528723	70.8865126	100	62.109355	50.5921
8			81.641436	72.02204	99.33326
9			83.738376	100	100
10			75.529407	56.378313	92.77344
12			78.932017	60.916056	72.38456

Time (h)	CDKB1 1	CDKB1 2	CDKB1 3	CDKB1 4	CDKB1 5
1	69.0226538	34.27793961			
2	54.6464205	33.07220874			
3	55.1390765	40.1736447			
4	53.2202205	24.74461312			
5	60.4503629	65.28348997	91.31264962	100	94.0466846
6	68.5456619	70.0396686	100	99.19674386	100
7	100	100	84.0908945	90.37903514	85.6250143
8			62.65219149	79.23067636	66.5381303
9			69.4984821	66.02394678	46.6933646
10			50.92165307	58.35722693	51.3564902
12			34.02671254	52.35069913	55.4553006

WEE1-i-1 induced

Time (h)	CDKA 1	CDKA 2	CDKA 3
1	100	100	100
3	60.24659	94.36019	53.43055
4	64.81426	95.01102	63.25103
5	58.03517	97.53319	49.73508
6	45.45365	96.99092	56.70823
7	33.3446	95.98753	47.4936
9	42.85049	94.81163	38.94798

Time (h)	CDKB1 1	CDKB1 2	CDKB1 3
1	100	100	100
3	58.78956	63.15473	51.95804
4	68.41233	49.33648	34.51408
5	56.42295	60.84588	47.06691
6	54.99347	64.55488	44.09816
7	51.89385	53.14281	41.26649
9	73.26231	64.16574	48.82118

Appendix VI: *Arabidopsis* roots measurements raw data

Wild Type

Day	15	17	19
Primary root length (cm)			
	2.4	2.6	3.2
	2.2	2.8	5.7
	2.4	1.25	4.5
	5.2	2.9	3.5
	3.5	3.5	5.8
	2.8	3	6
	3	3.6	4.5
	3.1	4.7	3
	4.2	2.1	8
	2.2	5	2
	3.1	4	2.8
	2.3	5.4	3
	2.7	3	4.1
	2.3	6	4.6
	3.1	2.8	6
Last primordia to root tip distance (cm)			
	0.5	0.2	0.7
	0.3	0.5	1.2
	0.5	0.6	1.2
	0.6	0.6	0.4
	0.3	0.7	1
	0.5	0.6	1.1
	0.2	0.5	1.4
	0.3	0.4	0.6
	0.4	0.7	0.9
	0.4	0.6	1
	0.5	0.6	0.9
	0.3	0.5	0.4
	0.6	0.3	1.2
	0.5	0.7	1.3
	0.2	0.6	0.3

	15	17	19
Number of primordia			
	2	1	2
	2	2	4
	3	3	4
	4	3	3
	3	3	3
	6	4	2
	2	3	4
	3	3	5
	3	2	2
	2	3	1
	4	3	4
	1	4	4
	2	3	3
	3	3	3
	1	4	2
Number of lateral roots			
	11	10	16
	8	18	21
	10	7	15
	19	11	15
	11	14	17
	14	11	16
	12	12	18
	17	6	12
	8	14	9
	5	17	14
	16	11	19
	11	15	16
	12	9	15
	12	13	17
	15	14	18

Day 15			17			19		
Primordia radius	Primordia height	Primordia volume	Primordia radius	Primordia height	Primordia volume	Primordia radius	Primordia height	Primordia volume
41.75	27.25	49715	55.22	82.47	263207	60.67	56.56	217904
42.62	40.21	76448.6	50.14	62.72	165038	60.6	56.04	215403
44.77	47.89	100468	48.27	53.19	129716	59.36	47.59	175514
50.66	42.71	114728	52.57	65.31	188914	51.19	51.53	141331
47.82	54.68	130875	41.5	46.59	83984.1	45.85	44.47	97848.5
30.89	35.66	35614.4	40.71	51.69	89663.8	37.69	46.13	68587.4
30.5	37.21	36230	43.76	68.6	137495	68.19	21.57	104978
36.74	47.36	66911.1	42.37	36.79	69128.2	57.96	59.28	208436
52	42.96	121585	54.11	51.9	159049	45.32	45.37	97534.2
50.73	81.04	218292	43.11	55.55	108056	53.48	63.75	190841
42.61	45.7	86845.6	47.11	37.34	86737.9	58.79	66.45	240387
35	32.56	41747.3	49.14	22.43	56690.2	55.36	86.25	276668
39.78	45.89	76007.4	49.58	44.49	114468	56.19	95.84	316718
40.3	54	91793.6	51.22	46.78	128454	58.23	110	390422
35.72	48.34	64556.2	50	57.8	151243	49	99.81	250827
33.25	73	84472.3	38.98	80	127228	61.23	73.32	287713
42.12	54.32	100866	50	72.84	190598	53.98	53.98	164629
49	55	138218	42.7	58.43	111506	34.56	68.78	85984.1
35.67	62.54	83286.1	41.2	48.34	85883.4	48.32	70.32	171847
38.3	38	58343.1	39.21	65.93	106093	54.9	37	116723

Plants transformed with BIN HYG TX-*AtWEE1*

Day	15	17	19		15	17	19
	Primary root length (cm)				Number of primordia		
	0.8	0.6	0.7		3	1	2
	2.8	0.8	1.1		2	3	2
	1.4	0.9	0.6		1	2	2
	0.7	1	0.9		1	2	1
	0.6	1.4	1.2		1	2	
	1.1	1.7	1		2	2	1
	0.9	1.3	1.3		2	1	1
	0.7	1.1	0.9		2	3	1
	0.4	0.9	1.7		3	1	3
	1.2	0.6	1.9		1	1	2
	0.4	1.2	2		1	2	2
	0.4	1	0.9		2	2	3
	0.8	1.3	1		3	3	2
	1	0.9	5		2	2	3
	1	1.5	1.8		1	1	1
	Last primordia to root tip distance (cm)				Number of lateral roots		
	0.1	0.2	0.1		5	8	9
	0.3	0.3	0.1		12	6	10
	0.7	0.5	0.1		3	7	7
	0.2	0.5	0.2		8	14	6
	0.6	0.1	0.3		5	7	8
	0.1	0.2	0.1		5	5	6
	0.7	0.3	0.2		6	7	6
	0.2	0.1	0.2		4	7	9
	0.1	0.3	0.2		7	11	14
	0.3	0.2	0.1		3	4	11
	0.5	0.2	0.3		8	7	4
	0.3	0.3	0.1		8	3	7
	0.2	0.1	0.3		4	5	8
	0.2	0.4	0.3		4	8	3

Day 15			17			19		
Primordia radius	Primordia height	Primordia volume	Primordia radius	Primordia height	Primordia volume	Primordia radius	Primordia height	Primordia volume
39.98	41.54	69496.1	50.05	44.28	116098	64.52	74.93	326477
47.44	60.21	141829	37.62	52.74	78124.3	56.17	45.71	150948
36.71	55	77578.2	43.51	50.04	99152.5	45.29	41.81	89762.1
41.09	52.42	92635.6	40.89	61.31	107294	42.88	46.71	89893.4
30.69	46.47	45811.5	30.61	44.19	43337	68.18	75.48	367244
39.94	55.29	92314.8	35.09	54.2	69851.3	41.62	55.57	100752
48.8	55.15	137465	41.81	42.48	77723.7	44.41	63.65	131392
45.37	62.99	135712	33.65	49.97	59222.7	52.69	46.91	136311
38.3	49.17	75492.9	33.37	62.75	73136.6	51.02	79.51	216626
44.53	47.84	99289.9	63.03	47.15	196058	67.89	44.77	215977
33.33	55.39	64403.6	59.77	55.04	205804	79.62	30.77	204165
29.87	42.98	40137	45.63	25.95	56551.8	44.79	50.91	106899
24.39	35.42	22053.7	34.43	43.01	53364.4	54.54	37.82	117750
42.1	45.98	85298.5	61.29	98.61	387711	63.5	52.57	221868
35.34	55.87	73033.2	53.66	33.06	99635.2	55.94	51.39	168319
41	50.32	88535.4	55.71	49.31	160181	53.14	73.32	216708
33.65	47.92	56793.1	49.32	55.34	140894	62.11	88.99	359313
40.35	69.21	117941	55.55	68.31	220628	47.09	98.43	228451
30.85	48.29	48103.4	39.43	51.98	84585.9	44.32	90	185034
32.1	67.3	72582.8	37.78	50	74696.9	48.55	45.78	112944

Plants transformed with pER8-*AtWEE1*

Day	15	17	19
Primary root length (cm)			
	1.2	1.5	2.8
	2	2.4	3
	1.6	2.2	3.6
	0.8	1.4	4
	1.4	1.9	3.5
	2.8	3.2	2.6
	2.4	3.6	2.9
	1.9	2.6	3.5
	0.8	1.4	3.9
	2.3	1.8	2.6
	1.4	2.4	4
	1.6	3	4.2
	2.5	2.2	2.3
	3	3.2	2.5
	2	2.4	28
	2.7	2.5	3.5
	1.4	1.9	3.8
	2.4	2.1	3.7
	1.1	2.4	3.1
	1.3	3.2	2.9
	2.4	1.9	2.8

Appendix VII: The yeast two hybrid technique was employed to investigate possible interaction between AtWEE1 and proteins that regulate its activity

VII.I. Introduction

One technique used to study protein-protein interactions is the "yeast two hybrid" system. The first two-hybrid system was described by Fields and Song in 1989. It is based on the fact that proteins are composed of modules, or domains, which are units within the same polypeptide (protein) chain. The presence of these individual domains allows the same protein to perform different functions. The yeast two-hybrid technique uses GAL4 protein which is a transcriptional activator with two separate domains: 1) DNA-binding domain (BD) that is capable of binding to DNA, and 2) activation domain (AD) that is capable of activating transcription of the DNA. Both of these domains came from the same gene *GAL4* of *Saccharomyces cerevisiae* and are required for transcription. Transcriptional activators (TA), like the GAL4 protein, use their binding domain to interact (bind) to the promoter, a region situated upstream from the gene (coding region of the DNA) (Fig.VII.I). Once the TA has bound to the promoter, it is then able to activate transcription via its activation domain, which interacts with RNA polymerase. Hence, the activity of a TA requires both a DNA binding domain and an

activation domain. If either of these domains is absent, then transcription of the gene will fail.

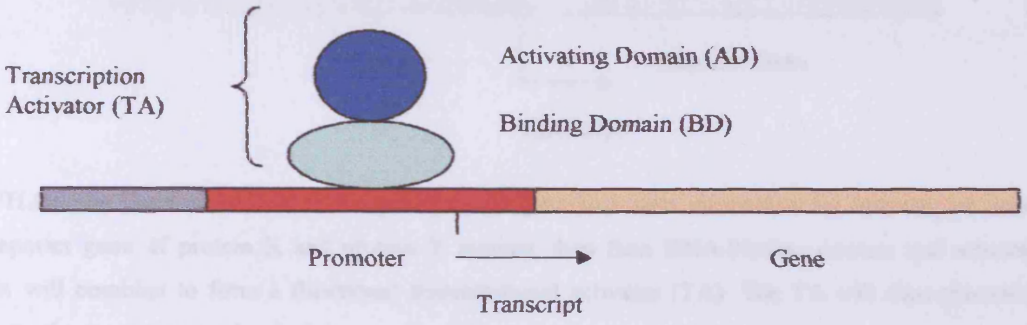


Fig. VII.I: Normal transcription requires both the DNA-binding domain (BD) and the activation domain (AD) of a transcriptional activator (TA).

The binding domain and the activating domain do not necessarily have to be on the same protein. In fact, a protein with a DNA binding domain can activate transcription when bound to another protein containing an activation domain. This principle is used in the yeast two-hybrid technique, where the *GAL4* gene is divided in two parts: 1) the DNA binding domain fused to the protein of interest (X), known as the bait and 2) the activation domain which is fused to a potential binding partner (Y). If protein X interacts with protein Y, the binding of these two will form an intact and functional transcriptional activator. This newly formed transcriptional activator will then go on to transcribe a reporter gene, which is a gene whose protein product can be easily detected or can guarantee the survival of yeast cells growing on a medium lacking one or more amino acids (Fig. VII.II).

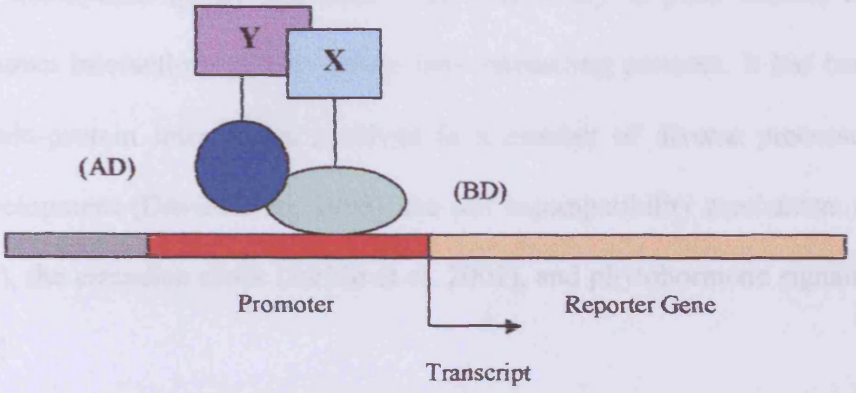


Fig. VII.II: The yeast two-hybrid technique measures protein-protein interactions by detecting properties of a reporter gene. If protein X and protein Y interact, then their DNA-binding domain and activating domain will combine to form a functional transcriptional activator (TA). The TA will then proceed to transcribe the reporter gene that is downstream of its promoter.

For the two-hybrid analysis of protein interaction used in this work, the bait protein AtWEE1 was fused to the GAL4 binding domain (GAL4 BD), while the potential binding partner was fused to the GAL4 activation domain (GAL4 AD). For a screen to find unknown interacting proteins, the GAL4 AD was fused to an *Arabidopsis* seedling root cDNA library. The two plasmids carrying these fusion proteins also contain selection genes, or genes encoding proteins that are required for cell survival on a particular medium. Yeast two-hybrid assays typically use selection genes encoding proteins capable of synthesizing amino acids such as histidine, leucine and tryptophan.

In the original system, only one reporter gene (*LacZ*) was used (Fields and Song, 1989). However, as the technology developed, yeast strains containing a number of reporter genes were generated, such as *HIS3* in the case of the GAL4 system, and used as a reporter gene in conjunction with *LacZ* to provide a more stringent assay for protein-protein interactions.

The yeast two-hybrid system has been used extensively in plant science research to analyze known interactions and to isolate new interacting partners. It has been used to study protein-protein interactions involved in a number of diverse processes such as flower development (Davies et al. 1996), the self incompatibility mechanism (Mazzurco et al. 2001), the circadian clock (Jarillo et al. 2001), and phytohormone signaling (Oullet et al. 2001).

In non-plant systems the yeast two-hybrid screen has been used successfully to find proteins interacting with WEE1. In mouse and human the yeast two-hybrid system was employed to isolate proteins that may interact with WEE1. Interestingly, in both mouse and human the results obtained through the yeast two-hybrid technique showed interaction between WEE1 and a member of the 14-3-3 family. In the case of mouse, WEE1 interacted with 14-3-3 ζ (Honda *et al.*, 1997), whereas in the case of the human gene, WEE1 interacted with 14-3-3 β (Wang *et al.*, 2000).

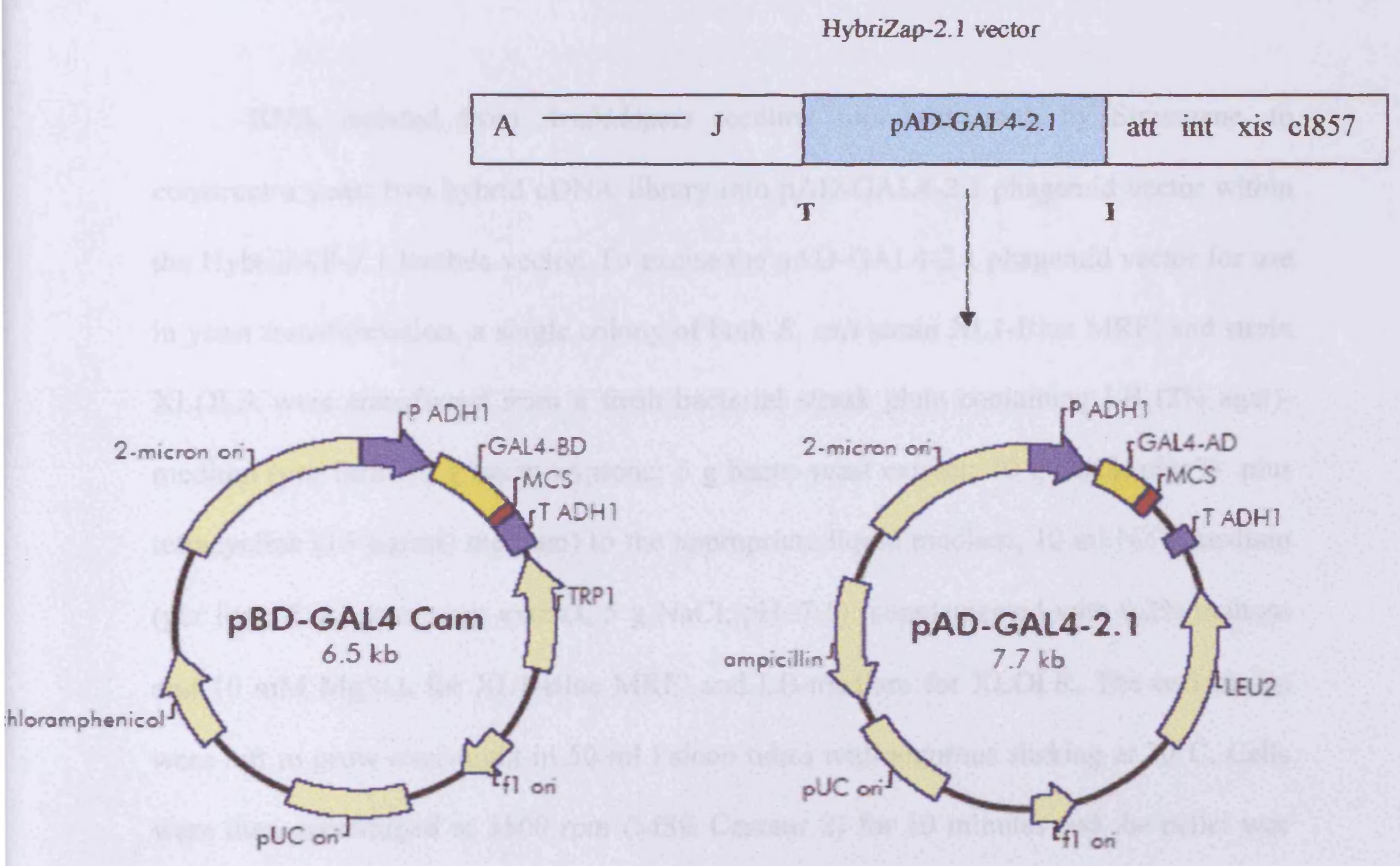
The aims of the work reported in this chapter were to examine whether AtWEE1 protein interacts with members of the 14-3-3 family and other proteins. The study of proteins interacting with AtWEE1 could lead to the discovery of protein regulators of AtWEE1 kinase activity.

VII.II. Materials and Methods

VII.II.I. pBD-GAL4 Cam and pAD-GAL4-2.1 plasmids

In the yeast two-hybrid system, used in this work two different plasmids were constructed:

1. pBD-GAL4 Cam (Fig. VII.III a): encoding the protein of interest fused in frame with the DNA-binding domain of the transcription factor GAL4
2. pAD-GAL4-2.1 excised from the HybridZap-2.1 vector (Fig. VII.III.b): encoding the potential binding protein fused in frame with the GAL4 transcription activation domain.



a b

Fig. VII.III: a) DNA encoding the bait protein is inserted into the region of insertion (MCS) of pBD-GAL4 Cam vector close to a segment of GAL4 BD; **b)** The HybriZap-2.1 contains lambda genes *A* through *J* in the left arm and *att*, *int*, *xis* and *cl857* in the right arm. The f1 initiator (I) and terminator (T) allow efficient in vivo excision of the pAD-GAL4-2.1 phagemid vector from the HybriZap-2.1 vector. DNA inserts are ligated into the HybriZap-2.1 vector to generate the primary lambda library. This primary lambda library is amplified and converted by in vivo mass excision to a pAD-GAL4-2.1 vector library.
Both pBD-GAL4 Cam and pAD-GAL4-2.1 vectors contain the pUC origin for replication in *E.coli*, an f1 origin for production of single stranded DNA in *E.coli*, and 2 micron origin for replication in yeast. The pBD-GAL4 Cam vector contains the chloramphenicol resistance gene and promoter for selection with chloramphenicol in *E.coli*. The pAD-GAL-2.1 vector contains the ampicillin resistance β-lactamase gene for selection with ampicillin in *E.coli*. For selection in yeast the pAD-GAL4-2.1 vector contains the *LEU2* gene and the pBD-GAL4 Cam vector contains the *TRP1* gene. Hybrid proteins are expressed in yeast from the ADH1 promoter and terminated by the ADH1 terminator.
(Extracted from http://www.stratagene.com/products/showProduct.aspx?pid=256).

VII.II.II. Mass library in vivo excision

RNA isolated from *Arabidopsis* seedling root was used, by Stratagene, to construct a yeast two hybrid cDNA library into pAD-GAL4-2.1 phagemid vector within the HybriZAP-2.1 lambda vector. To excise the pAD-GAL4-2.1 phagemid vector for use in yeast transformation, a single colony of both *E. coli* strain XL1-Blue MRF' and strain XLOLR were transferred from a fresh bacterial streak plate containing LB (2% agar)-medium (per litre: 10 g bacto-tryptone; 5 g bacto-yeast extract; 10 g NaCl; pH=7) plus tetracycline (15 μg/ml) medium) to the appropriate liquid medium, 10 ml NZY-medium (per litre: 5 g bacto-yeast extract; 5 g NaCl; pH=7.5) supplemented with 0.2% maltose and 10 mM $MgSO_4$ for XL1-Blue MRF' and LB-medium for XLOLR. The two strains were left to grow over-night in 50 ml Falcon tubes with vigorous shaking at 30°C. Cells were then centrifuged at 3500 rpm (MSE Centaur 2) for 10 minutes and the pellet was resuspended in 10 mM $MgSO_4$ at an OD_{600} of 1.0. The cell suspension (0.25 ml) was

added to 50 ml of NZY broth supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ in a 250 ml flask. Cell cultures were incubated at 37°C with shaking until an OD_{600} of 0.3-0.4 was reached and then centrifuged at 3500 rpm (MSE Centaur 2) for 10 minutes. XL1-Blue MRF' cells were resuspended in 10 mM $MgSO_4$ (4 ml) to an OD_{600} of 5.0 (assuming an OD_{600} of 5.0 is equal to a final cell concentration of $4x10^9$ cells). XLOLR cells were resuspended in 10 mM $MgSO_4$ (10 ml) to an OD_{600} of 1.0.

To mass excise an entire library, a portion of the amplified library stock was combined with the XL1-Blue MRF' cells at a multiplicity of infection (MOI) of 1:10 lambda phage to cell ratio in a 250 ml flask. ExAssist helper phage (Stratagene) at a 10:1 helper phage to cells ratio was added to ensure that every cell was coinfected with lambda phage and helper phage. The cells were incubated for 15 minutes at 37°C before adding 20 ml of LB broth and the culture was incubated with gentle agitation for a further 3 hours at 37°C. Cultures were incubated for 20 minutes at 70°C to lyse the lambda phage particles and the XL1-Blue MRF' cells. The cells were then centrifuged at 3500 rpm (MSE Centaur 2) for 10 minutes and the supernatant was transferred to a fresh tube. The titer of Amp^r colonies per milliliter (or colony forming units per milliliter (cfu/ml)) was determined. Each Amp^r colony represents a single excised phagemid. The supernatant (containing the excised phagemid particles) was diluted in different volumes of TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH=7.5) and mixed with 200 µl of XLOLR cells (OD_{600} of 1.0). The cells were then incubated at 37°C for 15 minutes and spread onto an LB agar plate containing 50 µg/ml of ampicillin. The plate was incubated over-night at 37°C. The titer of excised phagemid (in cfu/ml) was determined as follows:

$$\frac{\text{Number of colonies (cfu) x dilution factor}}{\text{Volume of phagemid plated (µl)}} \times 1000\ \mu l/ml$$

VII.II.III. Amplification of the excised phagemid library

A culture of XLOLR cells was transferred from a fresh bacterial streak plate (LB-tetracycline medium) to LB medium and incubated over-night with vigorous shaking at 30°C in a 50 ml Falcon tube. Cells were then centrifuged at 3500 rpm (MSE Centaur 2) for 10 minutes; the pellet was resuspended in 10 mM $MgSO_4$ at an OD_{600} of 1.0.

To amplify the mass excised library, XLOLR cells were combined with a portion of the excision supernatant in a 2 l flask at minimum cells to phagemid ratio of 10:1, assuming an OD_{600} of 1.0 equals a cell concentration of $8x10^8$ cells/ml. The phagemid and the cells were incubated at 37°C for 15 minutes, and then 500 ml of LB broth containing 50 µg/ml of ampicillin was added. The cells were incubated with shaking at 37°C for 3 hours to an OD_{600} of 0.4. The cells were then centrifuged at 3500 rpm (MSE Centaur 2) for 10 minutes.

VI.II.IV. Isolation of DNA plasmid library

The plasmid DNA was extracted from XLOLR pelleted cells using a QIAfilter Plasmid Maxi Protocol (QIAGEN). The bacterial cells were harvested by centrifugation at 3500 rpm (MSE Centaur 2) for 10 minutes. The kit is designed for preparation of up 500 µg of high- or low-copy plasmid DNA. The kit provides lysis buffers to disrupt the bacterial cell wall and membrane and ribonuclease (RNase A) to remove the RNA

molecules. Cell debris and proteins are separated from the plasmid DNA by centrifugation. The supernatant containing the plasmid DNA is applied to the QIAGEN-tip 500 column. This column contains a resin able to bind the plasmid DNA, but not other elements that contaminate the DNA, which are washed through. Elution DNA buffer is then applied to the column to remove the plasmid DNA from the resin. The DNA is then precipitated by adding isopropanol to the eluted DNA and centrifuged. The pellet was washed with 70% ethanol and centrifuged at 15000 g for 10 min (Beckman Coulter J-E centrifuge, rotor JA-14). The ethanol was then removed and the pellet was air-dried for 30 min, and redissolved in 300 µl distilled water.

VII.II.V. Insert preparation and ligation

The bait used in the two hybrid screen was constructed by PCR amplifying the ORF of the *Arabidopsis thaliana WEE1* (*AtWEE1*), using forward primer (5'-CATGGAGAATTCATGTTCGAGAAGAACG-3') and reverse primer (5'-TAAGCGGTCGACTCAACCTCGAATCCTATATC-3'). One µl of cDNA was amplified with 0.625 U of Qiagen Taq polymerase, Qiagen buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 µg of each of the primers. Thermocycle conditions were: 35 cycles of 95°C (1 min), 55°C (1 min) and 72°C (1 min) in a PTC100 thermocycler (see chapter 2 sections 2.4.1, 2.4.2 and 2.4.3 for details about RNA extraction, DNase treatment of cDNA and cDNA synthesis respectively; also see section 2.4.4 for details about cDNA amplification, except that a hot start step of 15 min at 95°C was used with this Taq polymerase). The primers introduced *EcoR*I (5'-

CATGGAGAATTCATGTTCGAGAAGAACG-3') and *Sal*I (5'-TAAGCGGTCGACTCAACCTCGAATCCTATATC-3') restriction sites (see chapter 2 section 2.1.1 for more details about restriction and ligation reactions). The product was then cut with *EcoR*I and *Sal*I restriction enzymes, purified using the "QIAquick Purification Kit Protocol" (QIAGEN), and cloned into the *EcoR*I and *Sal*I sites of Gal4 BD (binding domain) plasmid vector pBD-GAL4Cam (Stratagene) giving pBD-GAL4Cam-*AtWEE1*.

VII.II.VI. Transformation into yeast YRG-2 cells

To transform YRG-2 cells with pBD-GAL4Cam-*AtWEE1*, 50 ml of YPD broth (per litre: 10 g yeast extract; 20 g bacteriological peptone; 20 g glucose; 0.1 g adenine; 0.1 g uracil) were inoculated with YRG-2 cells harvested from a plate (YPD-medium) using a flamed loop. The cells were incubated at 200 rpm over-night at 30°C to a cell density of 1-5 x 10^7 ml^{-1} (OD_{600} 1-2). Cells were then harvested by centrifugation at 8000 rpm (Beckman Coulter J-E centrifuge, rotor JA-14) for 1 second; the pellet was resuspended in 20 ml TE buffer and spun down at 8000 rpm for 1 second. This was repeated and the pellet was then resuspended in 10 ml LiAc (0.1 M) and incubated at 200 rpm for 1 hour at 30°C. Competent YRG-2 cells (150 µl) were transferred into two Eppendorf tubes and 5 µl of pBD-GAL4Cam-*AtWEE1* (1 or 2 µg) were added to each tube. As a control 5 µl TE buffer were also added to a different Eppendorf tube containing YRG-2 competent cells. PEG4000 (350µl) was added to each tube of competent cells. The transformation mixtures were then incubated at 30°C for 1 hour

without shaking. The cells were heated at 42°C for 5 minutes and quickly cooled down on ice for 3 minutes. The transformed cells (200 µl) were then plated on minimal medium lacking tryptophan and incubated at 30°C for 3-4 days. Single colonies were then patched onto minimal medium lacking histidine and containing 0, 1, 5, 10, 25, 30, 35, 40, 45 and 50 mM 3-AT (3-amino-1, 2, 4-triazole, Sigma). 3-AT is an inhibitor of the *HIS3* gene (*HIS3*-encoded IGP-dehydratase, an enzyme required for histidine biosynthesis) product and reduces the background due to basal *HIS3* expression.

VII.II.VII. Double transformation into YRG-2 cells

For the two hybrid screen the pAD-GAL4-2.1 library was transformed into YRG-2 cells containing pBD-GAL4Cam-*AtWEE1*. YRG-2 cells carrying pBD-GAL4Cam-*AtWEE1* were inoculated in 10 ml minimal medium lacking tryptophan and incubated at 30°C on a shaker at 200 rpm over-night. The titre of the over-night culture was determined by measuring the OD_{600} of a 1 in 10 dilution into water, considering an OD_{600} of 0.1 to correspond to approximately $1x10^6$ cells/ml. The volume of the over-night culture containing $2.5x10^8$ cells was then calculated and added to prewarmed (30°C) YPD medium, to give a final volume of 50 ml in a 250 ml flask. The diluted culture was then incubated at 30°C on a shaker at 200 rpm until the cells reached approximately a concentration $2x10^7$ cells/ml. The cells were harvested in a sterile 50 ml disposable centrifuge tube (Falcon) by centrifugation at 3500 rpm (MSE Centaur 2) for 5 minutes. Cell pellets were resuspended in 25 ml of sterile water to wash the cells before pelletting them again by centrifugation as described above. The pellet was then resuspended in 900

µl of sterile water and transferred to a 1.5 ml microcentrifuge tube. Cells were centrifuged again at 13000 rpm (MiniSpin, Eppendorf) for 1 minute; the pellet was resuspended in 100 mM LiAc to a final volume of 1 ml and incubated at 30°C for 10 minutes. For each 1x transformation reaction aliquots of 100 µl LiAc cell suspension were transferred into a new 1.5 ml microcentrifuge tube. The cells were spun down at top speed (MiniSpin, Eppendorf) for 1 minute and the following reagents were added: 50% PEG (240 µl); 1 mM LiAc (36 µl); ss-DNA 2 mg/ml (50 µl); plasmid DNA from the mass excision and isolation of the plasmid library 1, 2 and 5 µg, sterile water to a final volume of 360 µl. The transformation mixtures were incubated at 30°C for 30 minutes, heat shocked at 42°C for 30 minutes and spun down at top speed (MiniSpin, Eppendorf) for 1 minute. Sterile water (400 µl) was added to resuspend the cell pellet and cells were plated onto minimal medium (per litre: 1.62 g yeast nitrogen base; 5 g ammonium sulfate; 20 g glucose; 20 g agar) lacking tryptophan and leucine (0.2 ml of appropriate "drop-out" solution were spread onto each Petri dishes immediately before use). Petri dishes containing the transformed cells were incubated at 30°C until colonies appeared.

VII.III. Results

VII.III.I. Preparation of yeast two-hybrid construct

The yeast two hybrid screen was performed to identify *Arabidopsis thaliana* proteins that bind *Arabidopsis thaliana* WEE1 kinase (AtWEE1). The *AtWEE1* open reading frame was amplified by PCR from the BIN HYG TX-*AtWEE1* plasmid and

ligated in frame with the binding domain of the pBD-GAL4 Cam plasmid. The construct was sequenced to confirm that no PCR errors had been generated and that the test protein was in frame with its fusion partner. AtWEE1 was then used as a bait to screen an *Arabidopsis thaliana* seedling root cDNA library in the GAL4 transcriptional activation domain vector pAD-GAL4-2.1.

VII.III.II. YRG-2 cells transformed with pBD-GAL4 Cam-*AtWEE1* plasmid

The Stratagene-2-hybrid yeast strain YRG-2 was transformed with *AtWEE1* in the pBD-GAL4 Cam vector. Following transformations, positive colonies growing on selective medium (minimal medium lacking tryptophan) were tested by PCR (Fig. VII.IV) to confirm *AtWEE1* presence inside the cells.

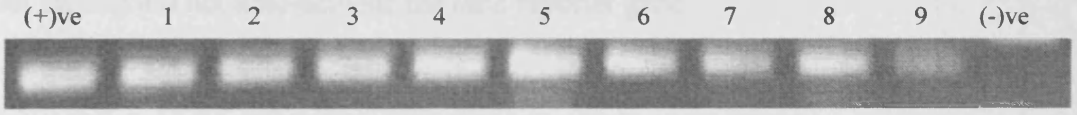


Fig. VII.IV: Indipendent colonies (from 1 to 9) of YRG-2 cells transformed with pBD-GAL4 Cam-*AtWEE1* were amplified by PCR using primers AtWEE1 (forward and reverse) to confirm *AtWEE1* presence. As a positive control ((+)ve) BIN HYG TX-*AtWEE1* plasmid was used, whereas sterile distilled water was used as the negative control ((-)ve). Product size is 229 bp.

VII.III.III. Testing the pBD-GAL4Cam-*AtWEE1* plasmid for auto-activation

Auto-activation of reporter gene expression can be a problem with some proteins fused to the DNA-binding domain. To test that the AtWEE1 bait protein itself was unable

to activate the transcription of the *HIS3* and *lacZ* reporter genes, the YRG-2 cells transformed with pBD-GAL4 Cam-*AtWEE1* were plated onto minimal medium lacking histidine with different concentrations of 3-AT. Yeast cells containing the AtWEE1 bait protein were able to grow on minimal medium lacking histidine up to a concentration of 35 mM 3-AT, but were unable to grow on minimal medium lacking histidine with 40 mM 3-AT.

These results show that the AtWEE1 bait protein could auto-activate transcription of the *HIS3* reporter gene, but the growth of yeast cells on histidine free minimal medium could be blocked by the addition of 40 mM 3-AT. The two-hybrid screen could therefore be performed on minimal medium lacking leucine, tryptophan and histidine with 40 mM 3-AT. Leucine and tryptophan were used to select yeast cells containing the two plasmids. The lacZ filter-lift assay was also performed showing that yeast cells containing pBD-GAL4 Cam-*AtWEE1* plasmid did not have lacZ activity. This suggests that the AtWEE1 bait protein did not auto-activate the *lacZ* reporter gene.

VII.III.IV. Transformation of YRG-2 cells containing AtWEE1 bait protein with pAD-GAL4-2.1 cDNA library

To transform YRG-2 cells with pAD-GAL4-2.1 cDNA library, yeast cells already transformed with *AtWEE1* were used in the exponential phase of growth. Cell growth was thus measured every 2 hours for a 24 hour period (Fig. VII.V).

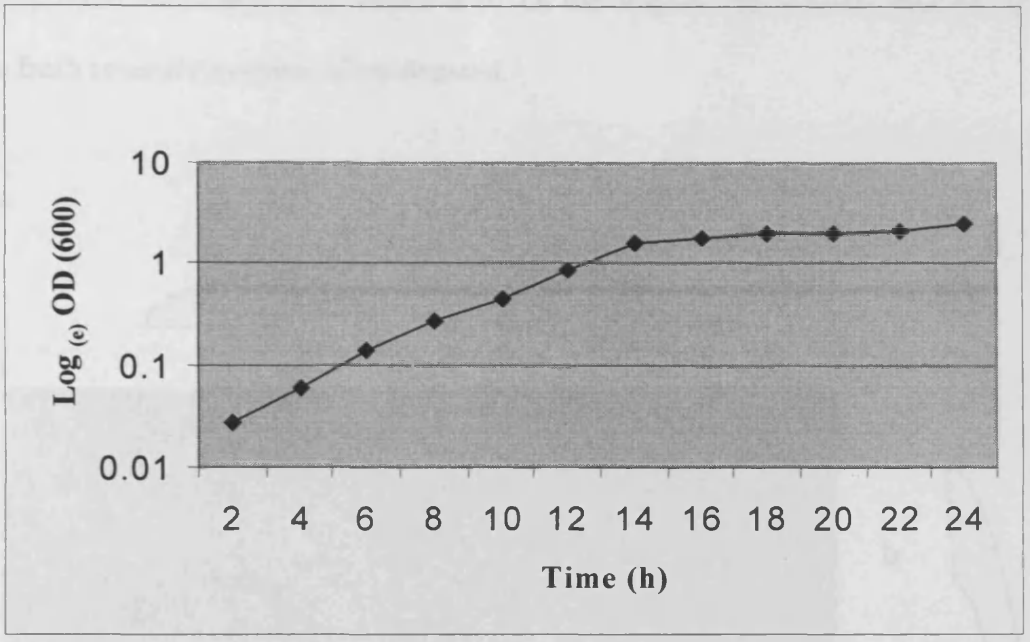


Fig. VII.V: Growth of YRG-2 cells transformed with pBD-GAL4 Cam-*AtWEE1*. YRG-2 cells containing the bait protein grow exponentially for 12 hours.

Aproximately $1x10^7$ YRG-2 cells transformed with both pBD-GAL4 Cam-*AtWEE1* and pAD-GAL4-2.1 cDNA library were selected on minimal medium lacking tryptophan, leucine and histidine with 40 mM 3-AT. After 4 days of incubation, the primary two-hybrid screening plates were covered with a background layer of yeast cell growth. However, after further incubation, larger distinct colonies appeared to grow out from the background. These fast-growing colonies are putatively positive for a protein-protein interaction. Only 700 colonies larger than the overall background of colonies became apparent out of $1x10^7$ transformants screened on the selective medium (minimal medium lacking histidine, leucine and tryptophan with 40mM 3-AT) (Fig. VII.VI a). The 700 colonies were harvested and re-selected onto a fresh selective medium for the selection of

protein-protein interaction (Fig. VII.IV b, c). Of the original 700 colonies only 442 grew on the fresh selective medium when retested.

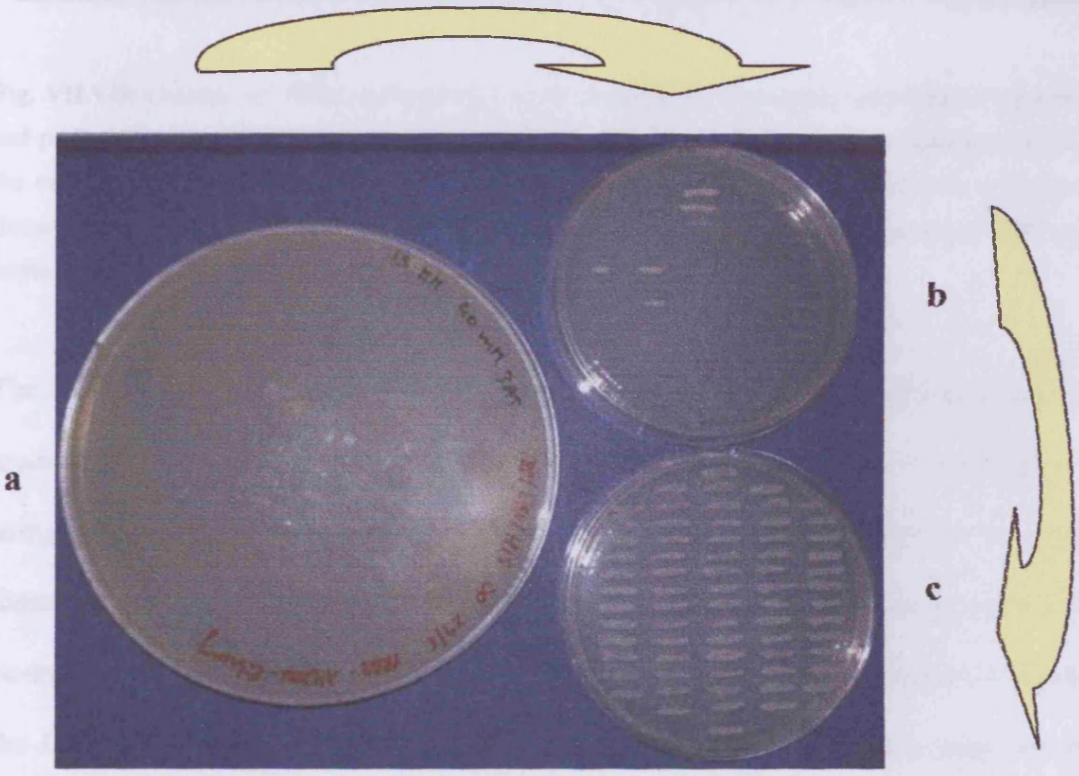


Fig. VII.VI: Example of the method used to screen $1x10^7$ transformants: **a)** large Petri dishes (13.5 cm in diameter) containing minimal medium lacking tryptophan, leucine and histidine with 40mM 3AT, were used to screen YRG-2 cells carrying both the bait protein and the activating domain. Large distinct colonies, which appeared to grow out from the background, were harvested and transferred to **b)** small Petri dishes (8.5 cm in diameter) containing the same selective medium. Positive colonies growing on the fresh selective medium were then transferred to **c)** new small Petri dishes containing the same selective minimal medium. These colonies were then used for the LacZ assay.

The presence of the pAD-GAL4-2.1 cDNA library was confirmed by PCR on randomly chosen colonies (Fig. VII.VII).

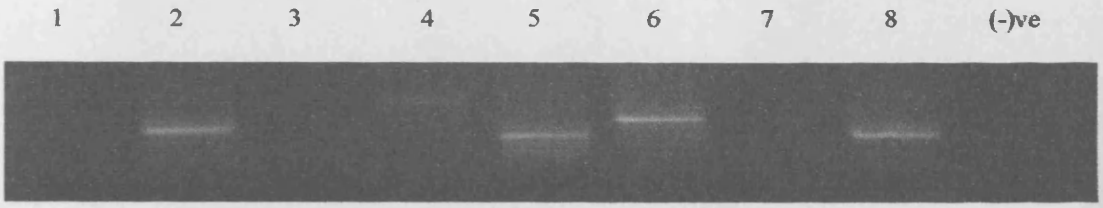


Fig. VII.VII: Colonies of YRG-2 cells (from 1 to 8), containing both plasmid pBD-GAL4 Cam-*AtWEE1* and pAD-GAL4-2.1 cDNA library, were randomly chosen to check presence of the activating domain in the cell. The different band size reflects the different inserts contained in the pAD-GAL4-2.1 plasmid. Some of the colonies appeared to give a negative result which may be due to a low quantity of YRG-2 cells in the PCR reaction. Distilled water was used as negative control ((-)ve).

The 442 colonies selected were replica-plated onto minimal medium lacking tryptophan, leucine and histidine with 40mM 3AT for the second library screen which was performed using the lacZ assay to reduce the number of false positives. Surprisingly, not one of these colonies gave a positive blue color signal. YRG-2 cells containing Spcdc25 as bait co-transformed with 14-3-3 κ, λ and ω respectively were chosen as positive controls in the LacZ assay. As previously demonstrated by Sorrell and colleagues using the two-hybrid screen, Spcdc25 protein can interact with three *Arabidopsis* 14-3-3 proteins: GF14 κ, λ, and ω (Sorrell *et al.*, 2003). The LacZ assay was repeated three times on the 442 colonies and the control, each time making sure that: 1) the LacZ buffer with X-gal were freshly prepared, 2) the colonies transferred on fresh plates were between 4-5 days old at the time of screening, 3) the colonies were transferred from the plate to the filter paper by visual inspection of both the filter paper and the colonies left on the plate.

VII.IV. Discussion

Eukaryotic cell cycle regulation is controlled by the activity of CDKs. WEE1, as a key regulator of CDK, plays a critical role in the G2/M transition of the cell cycle. Previous work indicates that protein phosphorylation may play an important role in the control of WEE1 activity in yeast (Russell and Nurse, 1987; Coleman *et al.*, 1993; Parker *et al.*, 1993; O'Connell *et al.*, 1997; Boddy *et al.*, 1998). However, little is known about how WEE1 activity is regulated in plants.

In an attempt to gain a better understanding of how WEE1 activity is regulated in plant cells during the cell cycle, the yeast two-hybrid screen approach was used to identify proteins that interact with WEE1. To investigate AtWEE1 interactions with other proteins, the pBD-GAL4-Cam *AtWEE1* plasmid was successfully transformed into the Stratagene two hybrid yeast strain YRG-2 as demonstrated by the growth on selective minimal medium lacking tryptophan and by PCR. Positive colonies carrying the binding domain were re-transformed using the pAD-GAL4-2.1 cDNA library. The presence of the activating domain inside YRG-2 cells already containing pBD-GAL4-Cam *AtWEE1* was confirmed by PCR and growth on selective minimal medium lacking histidine, leucine and tryptophan with 40mM 3-AT.

The yeast two hybrid system was used in previous work on mouse and human cells to identify proteins which bind to WEE1 kinase. In mouse cells, the yeast two-hybrid technique was performed. Using the carboxyl half of WEE1 kinase the 14-3-3 ζ protein was isolated. Recombinant 14-3-3 ζ binds to WEE1 kinase *in vitro*, but the functional significance of this protein-protein interaction was not addressed in the study (Honda *et*

al., 1997). 14-3-3 proteins are a family of acidic proteins of low molecular weight found in all eukaryotic cells. There are 7 known 14-3-3 isoforms in humans and 15 in plants (Rosenquist *et al.*, 2000, 2001). In human cells, the yeast two hybrid screen demonstrated that WEE1 activity in mammalian cells can be regulated by 14-3-3 β. The 14-3-3 β protein binds directly to WEE1 at the COOH-terminal RSVSLT motif. Phosphorylation of a serine residue within this motif is required for the binding. The interaction of WEE1 with 14-3-3 β protein may change WEE1 protein conformation and block the degradation motif contained in the NH_2-terminal domain (Wang *et al.*, 2000; Rothblum-Oviatt *et al.*, 2001).

In my study the yeast two hybrid technique was performed using the whole AtWEE1 ORF as the bait domain to screen an *Arabidopsis* root cDNA activation domain library. Interactions between *AtWEE1* and other proteins were not detected using the LacZ assay when glucose (2%) was used as the carbon source to prepare the minimal media. Interestingly, subsequently in the Cardiff cell cycle laboratory when Anne Lentz (unpublished data) changed the composition of the minimal medium adding galactose (2%) and raffinose (1%) instead of glucose (2%) as specified in the Clontech Yeast Protocol Handbook (published the 14th March 2001), 82 positive yeast patches were obtained from the LacZ assay. The 82 positive results were submitted for sequencing and 77 of those gave a good quality sequence. In the table below (Table VII.I) there is a list of proteins that were detected as interacting with AtWEE1 via the two-hybrid screen in more than one case.

Table VII.I: proteins interacting with AtWEE1 detected more than once using the yeast two-hybrid technique (Anne Lentz, Cardiff cell cycle laboratory, unpublished data)

Protein	Number of hits	Function	Reference
bZIP Transcription factor GBF5	2	This transcription factor regulates processes including pathogen defense, light and stress signaling, seed maturation and flower development. The *Arabidopsis* genome sequence contains 75 distinct members of the bZIP family.	Jakoby *et al.*, 2002
SCL6	3	Member of GRAS proteins. Although the *Arabidopsis* genome encodes at least 33 GRAS protein family members only a few GRAS proteins have been characterized so far. However, it is becoming clear that GRAS proteins exert important roles in very diverse processes such as signal transduction, meristem maintenance and development.	Bolle C., 2004
Chitinase AtCTL1	2	Chitinase-like proteins have been proposed to play roles in normal plant growth and development and lignin deposition.	Zhong *et al.*, 2002
Strictosidine synthase	3	Strictosidine synthase (STR1) is a central enzyme that participates in the biosynthesis of almost all plant monoterpenoid indole alkaloids.	Treimer and Zenk, 1979
Cdc48A	2	Cell cycle gene involved in the spindle pole body separation.	Feiler *et al.*, 1995
Brix domain protein	3	Role in ribosome biogenesis and rRNA binding.	Eisenhaber *et al.*, 2001
Copper chaperone ATX1	3	Bind copper ions and deliver them to specific cellular pathways.	Liu *et al.*, 2003
GSTs AtGSTF9 Phi class GST	2	Glutathione *S*-transferases (GSTs) appear to be ubiquitous in plants and have defined roles in herbicide detoxification. In contrast, little is known about their roles in normal plant physiology and during responses to biotic and abiotic stress.	Wagner *et al.*, 2002

To confirm that the putative positive interactions occur *in planta* and are not false positives further tests need to be done. The specificity of the interaction between AtWEE1 and the proteins listed in Table VII.I has to be verified *in vivo*. One method for doing this is to transform *Arabidopsis thaliana* plants with plasmids carrying the gene encoding for the above proteins fused to non-fluorescent domains of the yellow

fluorescent protein. The two non-fluorescent domains become fluorescent when brought together by association of AtWEE1 with the interacting protein (Hu *et al.*, 2000, Walter *et al.*, 2004).

VII.V. Summary

To gain a better understanding of WEE1 kinase regulation in plants, the yeast two hybrid screen was used to isolate proteins that may interact with AtWEE1. The construction of pBD-GAL4 Cam-*AtWEE1* plasmid and its transformation into yeast YRG-2 cells were successfully performed. Co-transformation of YRG-2 cells carrying AtWEE1 bait with pAD-GAL4-2.1 cDNA library from *Arabidopsis* root was also successful. The first screening on medium HIS- and 40 mM 3-AT gave 442 positive colonies, which were all negative when re-screened using the LacZ assay to identify blue (positive) colonies.

This work is now taken on by Anne Lentz; she found very exiting interactions between AtWEE1 and other proteins. These interactions need to be further tested to confirm that they are not false positive.