Functional analysis of B1-type cyclins in *Arabidopsis thaliana*

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CONTENTS

ZUSAMMENFASSUNG

Zykline spielen eine entscheidende Rolle bei den Durchtrittskontrollen des eukaryontischen Zellzyklus. Für jeden Zellzyklusübergang werden spezifische Zykline benötigt, die durch Untereinheitenbindung ihren katalytischen Partner aus der Familie der Zyklinabhängigen Kinasen aktivieren. Der Fokus dieser Arbeit liegt auf den Zyklinen des B1-Types, welche während der G2- und M-Phase exprimiert werden und dadurch den Eintritt in die Mitose regulieren. Zykline der B1-Familie werden in *Arabidopsis thaliana* durch die vier Mitglieder *CYCB1;1*, *CYCB1;2*, *CYCB1;3* and *CYCB1;4* vertreten, deren Mutanten in der zugrunde liegenden Arbeit untersucht werden. Morphologische Analysen des Wachstums ergeben lediglich geringfügige Unterschiede im einfach mutanten Hintergrund. Während die Anzahl der Rosettblätter in der *cycb1;2*-Mutante abnimmt, steigt sie für *cycb1;4* an. Die Anzahl der Seiten- und Nebentriebe wird nicht durch Mutation von B1-Zyklinen beeinträchtigt. Die Mutanten *cycb1;1* und *cycb1;2* zeigen ein verstärktes, das Fehlen von *CYCB1;4* hingegen ein gehemmtes Wachstum der Rosettblätter. Wachstumsanalysen der Wurzel erwiesen, dass es sich bei CYCB1;4 um den wichtigsten Vertreter der B1-Zykline handelt, dessen Aufgabe die Regulation des Wurzelwachstums ist. Demzufolge ist dieses in der *cycb1;4*-Mutante deutlich reduziert. Sämtliche Mutanten für die vier Zykline des B1-Types zeigen eine verlängerte Wachstumsphase und eine verspätete Blühinduktion. Die nur geringfügigen Effekte, die in den einfachen Mutanten beobachtet werden, lassen eine starke Redundanz der B1-Zykline untereinander vermuten. Entsprechend ist die *cycb1;1*$^{-/-}$*;cycb1;2*$^{-/-}$-Doppelmutante letal: die Embryonen sterben zwischen Herz- und Torpedostadium. Während die Expression des CYCB1;4 unter Kontrolle des *CYCB1;1*-Promoters die Doppelmutante *cycb1;1*$^{-/-}$*;cycb1;2*$^{-/-}$ nicht retten kann, wird die wildtypische Morphologie durch die Expression von CYCB1;1, CYCB1;2 sowie CYCB1;3 auch in der Doppelmutante wiederhergestellt. Die Doppelmutanten *cycb1;1*$^{-/-}$*;cycb1;4*$^{-/-}$ und *cycb1;2*$^{-/-}$*;cycb1;4*$^{-/-}$ führen zu keinem deutlich vom Wildtyp abweichenden Phänotypen. Doppelmutanten von *cycb1;3* mit anderen *zyklinen des b1-typs* werden in dieser Arbeit nicht beleuchtet. Untersuchungen an Zyklinen des B1-Types bestätigen sowohl eine starke Redundanz zwischen CYCB1;1, CYCB1;2 und CYCB1;3 aber auch, dass sich *CYCB1;4* unabhängig von den anderen Familienmitgliedern entwickelt hat.

In allen Eukaryonten, deren Zellteilung größtenteils auf dem Wechselspiel zwischen Zyklinabhängigen Kinasen und den Zyklinen beruht, ist der „Destruction box“-abhängige Abbau der Zykline des B-Typs durch den APC/C-Komplex reguliert. Während die Degradation von CYCB1;1 und CYCB1;2 in der Abstammungslinie der Spaltöffnungen von *Arabidopsis thaliana* ein Abbaumotiv erfordert, ist dieses in endoreplizierenden Blatthaaren nicht essentiell. In dieser Arbeit wird das neuartige Motiv der „Barbie box“ beschrieben, dass sich für den Abbau von Zyklinen in Blatthaaren verantwortlich zeigt. Die „Barbie box” ist ein für Pflanzen spezifisches Abbaumotiv, welches nur in einigen pflanzlichen Zyklinen des B-Types gefunden werden konnte. Die Abbaubox scheint nur eine untergeordnete Rolle im Zyklinabbau in Blatthaaren zu spielen. Anhaltspunkte für diese Annahme kommen von Markerlinien, deren Reporter GUS an eine Abbaubox fusioniert wurde. Darüber hinaus wurde ein RNAi-Konstrukt blatthaarspezifisch gegen APC11 – einer zentralen Komponente von APC/C – gerichtet und zeigte dabei keine Abweichung vom wildtypischen Blatthaarphänotyp.

ABSTRACT

Cyclins play a vital role in controlling progress through the eukaryotic cell cycle. Specific cyclins are required at each cell cycle transition to activate their partner cyclin-dependent kinase. The focus of this study were the B1-type cyclins that are expressed in G2/M phase and control entry into mitosis. B1-type cyclins are represented by four members in *Arabidopsis thaliana* and in this study mutant lines for all family members, *CYCB1;1*, *CYCB1;2*, *CYCB1;3* and *CYCB1;4*, were analyzed. Morphological analyses revealed only minor growth alterations of the single mutant plants. While the number of rosette leaves decreased in the *cycb1;2* mutant, *cycb1;4* increased the number of rosette leaf. Side and auxiliary shoots numbers did not affected by mutation in B1-type cyclins. *cycb1;1* and *cycb1;2* mutants increased the rosette leaf growth while knock out of *CYCB1;4* reduced the growth of rosette leaf. Root growth analysis revealed that CYCB1;4 is the main B1-type cyclins in root growth and root growth significantly decreased in *cycb1;4* mutant. All B1-type cyclin mutants prolonged the vegetative phase and flowering was delayed. The minor effects seen in the single mutants suggested a high level of redundancy among the B1-type cyclins. Consequently, a $cycb1;1^{-/-}$ $cycb1;2^{-/-}$ was lethal and embryos died in the middle of heart and torpedo stage. While the expression of CYCB1;4 under the *CYCB1;1* promotor could not rescue $cycb1;1^{-/-}$ $cycb1;2^{-/-}$ mutants, the expression of CYCB1;1, CYCB1;2 and CYCB1;3 could restore wild type morphology in the double mutant. The $cycb1;1^{-/-}$ $cycb1;4^{-/-}$ or $cycb1;2^{-/-}$ $cycb1;4^{-/-}$ double mutant did not induce any severe phenotype. Double mutants of *cycb1;3* with other *b1-type cyclins* were not analyzed. Analysis of B1-type cyclins shows that there is high level of redundancy between CYCB1;1, CYCB1;2 and CYCB1;3 but the*CYCB1;4* developed independent from other B1-type cyclins.

In dividing cells of all organisms, the destruction box dependent degradation of B-type cyclins is mediated by the APC/C complex. While the degradation of CYCB1;1 and CYCB1;2 in the stomata lineage of *Arabidopsis thaliana* is required a destruction box, it is interestingly not required in endoreplicating trichomes. In this study, a new motif was identified that mediates cyclin degradation in trichomes, the motif was designated Barbie box. The Barbie box is a plant specific degradation motif which was found only in some plant B-type cyclins. The Destruction box appeared to be not of primary importance for degradation of cyclins in trichomes. Evidence for this hypothesis came from the analysis of GUS marker lines that were fused to a destruction box. Moreover,

an RNAi construct directed trichome-specifically against ACP11, a central component of the APC/C, .resulted in no deviation from wild-type trichome phenotype.

Abbreviations and gene names

%	percent
°C	degree Celsius
3'	three prime end of a DNA fragment
35S	35S promotor from the Cauliflower Mosaic virus
5'	five prime end of a DNA fragment
ATP	adenosinetriphosphate
Bp	base pair
cDNA	complementary DNA
CDS	coding sequence
CAK	CDK ACTIVATING KINASE
CDK	CYCLIN DEPENDENT KINASE
CKI	CYCLIN DEPENDENT KINASE INHIBITOR
CKS1	CDC KINASE SUBUNIT 1
CLF	CURLY LEAF
CYC	CYCLIN
CYCB	CYCLIN B
E2F	ADENOVIRUS E2 PROMOTOR BINDING FACTOR
DP	DIMERIZATION PARTNER
APC/C	anaphase-promoting complex/cyclosome
CAK	CDK ACTIVATING KINASE
CaMV	Cauliflower Mosaic Virus
CCS52	CELL-CYCLE SWITCH 52
CDC6	CELL DIVISION CYCLE DEFECTIVE 6
CDC25	CELL DIVISION CYCLE DEFECTIVE 25
CDK	CYCLIN DEPENDENT KINASE
CLSM	confocal laser scanning microscopy
CPC	CAPRICE
CUL1	CULLIN 1
DEL	DP-E2F LIKE
EF1	ELONGATION FACTOR 1
E2F	ADENOVIRUS E2 PROMOTOR BINDING FACTOR
FZR	FIZZY-RELATED
FZY	FIZZY
GL2	GLABRA2
GL3	GLABRA3
ICK	INTERACTOR/INHIBITOR OF CDKs
KRP	KIP RELATED PROTEIN
Rb	RETINOBLASTOMA
RBX1	RING BOX PROTEIN1
Col	*Arabidopsis thaliana* Columbia accession
sim	siamese mutant
d.a.g.	days after germination
DAPI	4',6'-diamidino-2-phenylindole
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid

EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* [Lat.] for example
et al.	*et alii* / *et aliae* [Lat.] and others
F1, F2, F3	first, second, third... filial generation after a cross
FDA	fluorescein diacetate
Fig.	Figure
G1	Gap phase between M phase and S phase
G2	Gap phase between S phase and M phase
gene-/-	homozygous mutant of a gene
gene+/-	heterozygous mutant of a gene
YFP	Yellow fluorescent protein
GUS	*beta-glucuronidase*
i.e.	*id est* [Lat.] that is

aa	amino acid
CDS	coding sequence
Kb	kilo bp
N	number
NLS	nuclear localization signal/sequence
PCR	polymerase chain reaction
RNAi	RNA-interference
Rpm	rounds per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SCF	Skp1; Cdc53 (cullin); F-box protein
SD	standard deviation
SEM	scanning electron microscopy
SIM	SIAMESE
T-DNA	transferred DNA
TIS	trichome initiation site
UTR	untranslated region
WT	wild type

All gene and mutant names are written in italics. WT-genes are written in capital letters. Proteins are written in non-italic letters.

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1. Introduction

1.1. THE BASIC CELL CYCLE MACHINERY

The eukaryotic cell cycle is typically defined on the basis of chromosomal events. Early in the cell cycle, the DNA is replicated and chromosomes are duplicated in S-phase. The second major phase of the cell cycle is the M-phase, which is typically composed of two events: nuclear division (mitosis) and cell division (cytokinesis). Between M phase and S phase, there are two gap phases (G1 and G2). Gap phases provide additional time for cell growth, and serve as important regulatory transitions for entry into the next cell cycle phase.

G1 is a particularly important regulatory period because during this phase most cells become committed to continue the cell cycle. Unfavorable growth conditions or inhibitory signals from other cells may result in a longer time in the G1 phase or removal from G1 into a prolonged non-dividing state called G0.

Similar to G1, G2 is an intermediate phase, a time for the cell to ensure that it is ready to proceed into the next cell cycle step, which is controlled by the activity of cyclin–dependent-kinase/cyclin complexes. Between the end of DNA replication in S phase and the beginning of cell division in mitosis, G2 can be thought of as a safety checkpoint to ensure that all of the cell's DNA and intracellular components were properly duplicated. In addition to acting as a checkpoint along the cell cycle, G2 also represents the cell's final chance to grow before it is split into two independent cells during mitosis (Fig1-1)(Morgan, 2006).

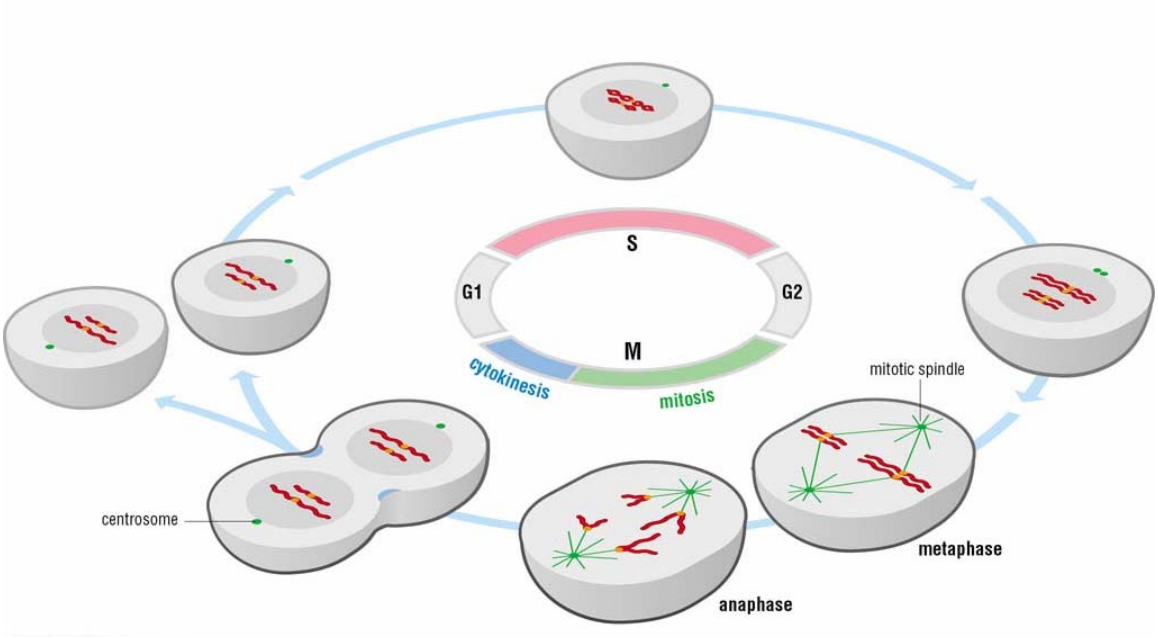


Fig. 1-1. The cell cycle:

Cell reproduction begins with duplication of the cell's compartments in S phase. These compartments are then divided equally between two daughter cells in M phase (taken from Morgen 2006).

In plant and animal cells a different cell cycle mode called endoreplication exists that leads to replication of DNA without any subsequent mitosis and cytokinesis (Fig2-1)

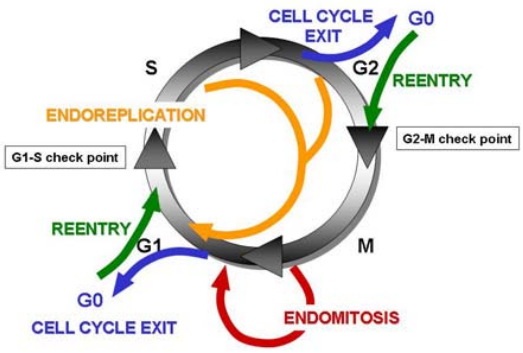


Fig. 2-1. Different cell cycle modes:

Simplified model of different cell cycle modes. The length of the individual phases (S, G2, M and G1) and the entry into an endoreplication cycle can vary (Taken form Jakoby and Schnittger 2004).

Endoreplication is frequently observed in some, but not all, plants. The level of ploidy varies between species and tissues (Sugimoto-Shirasu and Roberts, 2003, Inze, 2005 #1240).

The action of endoreplication in cell differentiation and cell growth can be observed in the development of *Drosophila melanogaster* nurse cells, *Medicago truncatula* nodule cells, and *Arabidopsis thaliana* leaf hairs (trichomes) (Edgar et al., 2001)(Kondorosi et al., 2000) (Hulskamp, 2000).

The physiological role of endoreplication is still under investigation. Endoreplication may safeguard against mutations that accumulate during a plants sessile lifetime. Unfavorable conditions that fall upon many plants can cause mutations within the plants genome (Larkins et al., 2001). Alternatively, endoreplication might be essential for an enhanced metabolic capacity, e.g. observed in plant endosperm tissue, (Kowles and Phillips, 1985). There is also little information to explain how a new round of DNA replication can occur simultaneously to the inhibition of mitosis. Questions also remain concerning how plant cells switch from a mitotic to an endoreplication cycle during their differentiation, as well as how endoreplication is terminated and how they manage to regulate starting another round of DNA replication while at the same time inhibiting mitosis (Larkins et al., 2001).

1.2. The cell cycle control

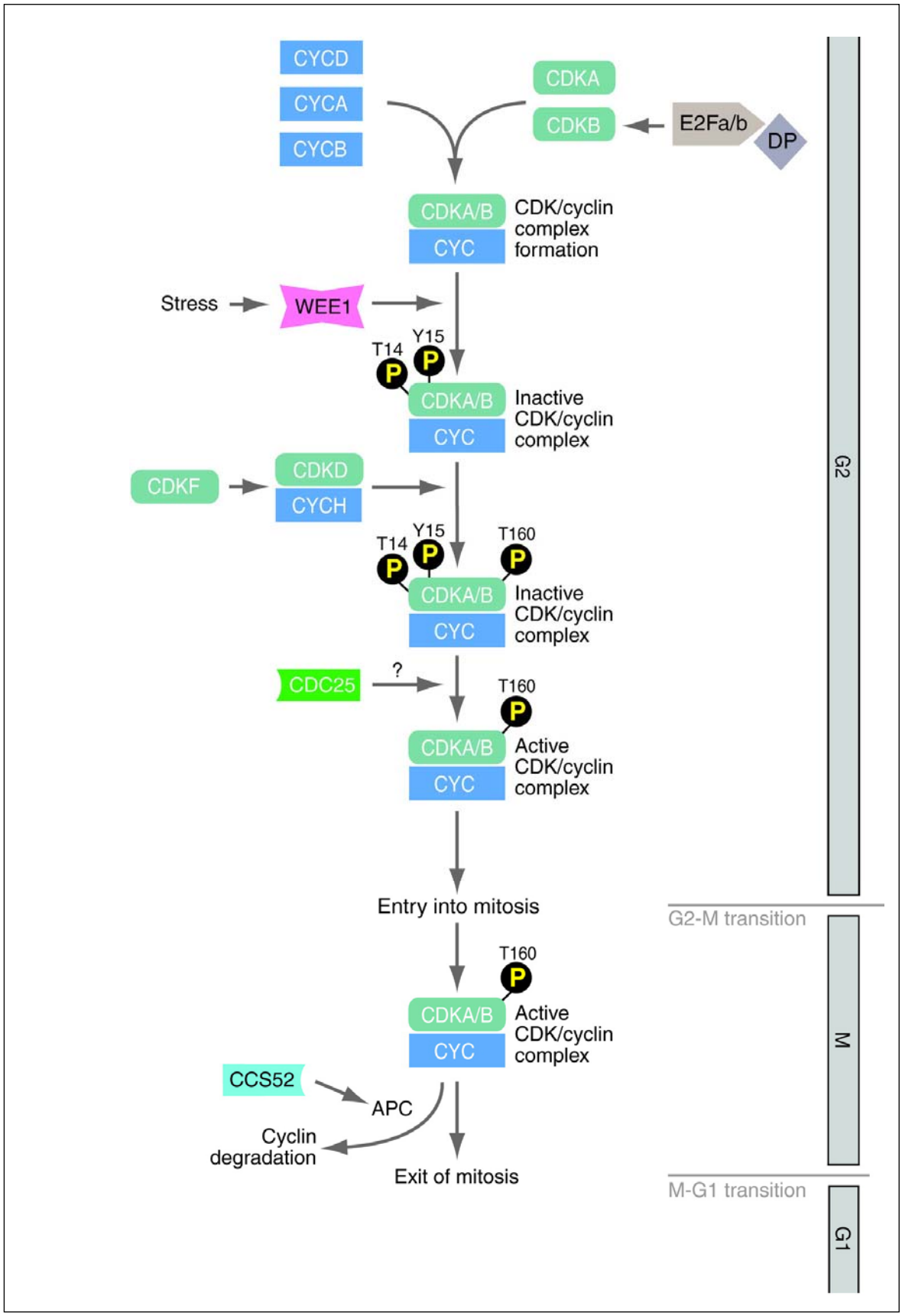
1.2.1. Cyclin-Dependent Kinases

All eukaryotic organisms possess at least one CDK with the PSTAIRE hallmark in their cyclin-binding domain, designated CDKA;1 in Arabidopsis. CDKA;1 protein levels remain constant throughout the cell cycle (Magyar et al., 1997; Porceddu et al., 1999; Sorrell et al., 2001). Overproduction of a dominant negative CDKA;1 of *Arabidopsis thaliana* in tobacco (*Nicotiana tabacum*) plants results in an overall reduction of cell division rates, thus yielding smaller plants. However, the G1/G2 ratio remains unaltered, corresponding with the observation that CDKA;1 regulates both checkpoints (Hemerly et al., 1995; Porceddu et al., 2001; Joubes et al., 2004). The requirement of CDKA;1 at least for entry into mitosis has been demonstrated by null mutants, whose primary defect appears to be a failure to progress through the second mitosis during male gametophytic development (Iwakawa et al., 2006; Nowack et al., 2006).

Plants possess a unique class of CDKs, the so called B-type CDKs that have not been described for any other organism (Hirayama et al., 1991; Joubes et al., 2000; Inze and

De Veylder, 2006). In B-type CDKs the PSTAIRE hallmark present in CDKA is replaced by either PPTALRE or PPTTLRE, reflecting the existence of two subgroups, CDKB1 and CDKB2 (Vandepoele et al., 2002). *Arabidopsis* harbors two CDKB1 (CDKB1;1 and CDKB1;2) and CDKB2 (CDKB2;1 and CDKB2;2) family members. The presence of both CDKB subgroups in Monocotyledonous and dicotyledonous species suggests that each of the CDKB subgroups have a unique role in cell cycle regulation. Timing of CDKB in cell cycle phase dependent transcription varies slightly. *CDKB1* transcripts accumulate during S, G2, and M phases, whereas *CDKB2* expression is specific to the G2 and M phases (Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001; Inze and De Veylder, 2006). The accumulation of CDKB proteins follows their transcription pattern, and their associated kinase activity reaches a maximum during mitosis. (Porceddu et al., 2001; Boudolf et al., 2004)

Fig. 3-1 (following): Representation of cyclin kinase activity during G2 to M phase transition in plants. CYCA, CYCB, and CYCD bind with CDKA or CDKB to form CDK/cyclin complexes. The E2F pathway controls transcription of some CDKBs. The Activation of specific CDK/cyclin complexes is controlled by CDKF, and CDKD associated with CYCH. Phosphorylation of T14 and Y15 by WEE1 inhibits the pathway. Dephosphorylation of T14 and Y15 is thought to be done by a CDC25-related kinase. Active CDK/CYC complexes trigger G2-to-M transition by phosphorylation of many substrates. Exit from mitosis requires degradation of the cyclin subunits and is initiated by the association of APC with the CCS52 protein (modified after Inze and De Veylder 2006).


CYCD
CYCA
CYCB
CDKA
CDKB
E2Fa/b
DP
CDKA/B
CYC
CDK/cyclin complex formation
Stress
WEE1
T14
Y15
Inactive CDK/cyclin complex
CDKF
CDKD
CYCH
T160
CDC25
?
Active CDK/cyclin complex
Entry into mitosis
CCS52
APC
Cyclin degradation
Exit of mitosis
G2
G2-M transition
M
M-G1 transition
G1

1.2.2. CDK/cyclin complex regulation

CDK activity is regulated by posttranslational modification and regulators. Phosphorylation of Thr160 (or the equivalent residue) of CDKs induces a conformational change allowing proper recognition of substrates and is performed by CDK-activating kinases (CAKs). *Arabidopsis* contains four CAK-encoding genes, divided into two functional classes (CDKD and CDKF) (Vandepoele et al., 2002)(Inze and De Veylder, 2006). CDKDs are functionally related to vertebrate CAKs, whereas CDKF is a plant specific CAK displaying unique enzyme characteristic.

Phosphorylation and dephosphorylation of specific CDK residues are essential in yeast and animals for a fully active CDK/cyclin complex.

WEE1 kinase phosphorylates CDKs at residues Thr14 and Tyr15, thereby inhibiting ATP fixation and substrate binding of the CDK (Fig3-1). In order to activate the CDK/cyclin complex the phospho groups at position 14 and 15 have to be removed by the CDC25 phosphatase (Fig3-1). In the *Arabidopsis* genome orthologs have been identified for most of the components involved in the phosphorylation and dephosphorylation of CDKs (Vandepoele et al., 2002). Recently a *CDC25*-like gene has been identified in *Arabidopsis*. The protein has been shown to stimulate kinase activity of *Arabidopsis* CDKs *in vitro* (Landrieu et al., 2004b; Landrieu et al., 2004a). The in vivo role of this CDC25-like protein, however, remains to be determined.

1.2.3. Cell cycle-dependent expression of cyclin genes

Cyclins were first identified in sea urchin eggs as proteins whose amounts increase during interphase and then abruptly decrease at each meiotic or mitotic division (Evans et al., 1983). Subsequently, cyclins were found in various organisms from yeasts to man and plants. Cyclins have a well-conserved amino acid sequence known as the cyclin box (Minshull et al., 1989). All proteins thus far designated as cyclins contain this structural motif, which has been shown to contain information necessary for binding to and activating of cyclin-dependent kinases (CDKs) (Lees and Harlow, 1993)(Stals et al., 2000).

Cyclins are classified into different groups on the basis of their structural similarity, functional period in the cell cycle and regulated expression. At least 10 classes of cyclins (designated cyclins A through I) have been reported thus far in animal cells. Several of these subclasses have multiple members. In *S. cerevisiae*, there are 22 cyclins that bind to one of five CDK enzymes (Andrews and Measday, 1998)Stals, 2000 #452}

Most cyclins show periodic expression during the cell cycle (Muller, 1995) (Stals et al., 2000). In animals, this group consists of cyclins A, B, E, and F. The first of these genes to be induced is that encoding cyclin E in late G1 {{Ohtani, 1995 #176}, followed by the genes encoding cyclins A and F, which are both activated around the G1/S transition and reach peak expression levels in late S-G2 phases (Desdouets et al., 1995). Finally, the gene encoding cyclin B is induced in S phase and is expressed at its maximum level in G2/M (Piaggio et al., 1995).

Cyclins in plants

Despite its small size the *Arabidopsis thaliana* genome contains at least 32 cyclins with a putative role in cell cycle progression. The nomenclature of plant cyclins is based on the functional similarities with mammalian counterparts. *Arabidopsis* gene annotation identified 10 A type, 11 B-type, 10 D-type, and 1 H-type cyclins {Vandepoele, 2002 #25; {Wang, 2004 #1191}. In a broad sense, it is thought that, D-type cyclins regulate the G1-to-S-transition, B-type cyclins assist in the G2-to M-transition as well as intra-M-phase control, and A-type cyclins control the S-to-M phase **(**Fig. 3-1 and Fig. 4-1). (Mironov et al., 1999; Potuschak and Doerner, 2001).

A number of deviations of this general functional assignment have been reported. For *Medicago sativa*, *CYCA2* has been shown to contribute to cell cycle kinase activity at S-phase entry as well as during the G2-to-M transition (Roudier et al., 2000; Inze and De Veylder, 2006). In contrast to animals, some preliminary evidence suggests that D-type cyclins may have an additional function at the G2-to-M transition that animal cyclins do not have. For example, ectopic expression of *CYCD3;1* in trichomes not only promotes S-phase entry but also induces mitosis (Schnittger et al., 2002b). Similarly, S-phase and mitotic entry were stimulated by induced overexpression of snapdragon (*Anthirrhinum majus*) CYCD1;1 and *tobacco* CYCD3;3 in tobacco Bright Yellow-2 (BY-2) cell suspensions (Nakagami et al., 2002; Koroleva et al., 2004).

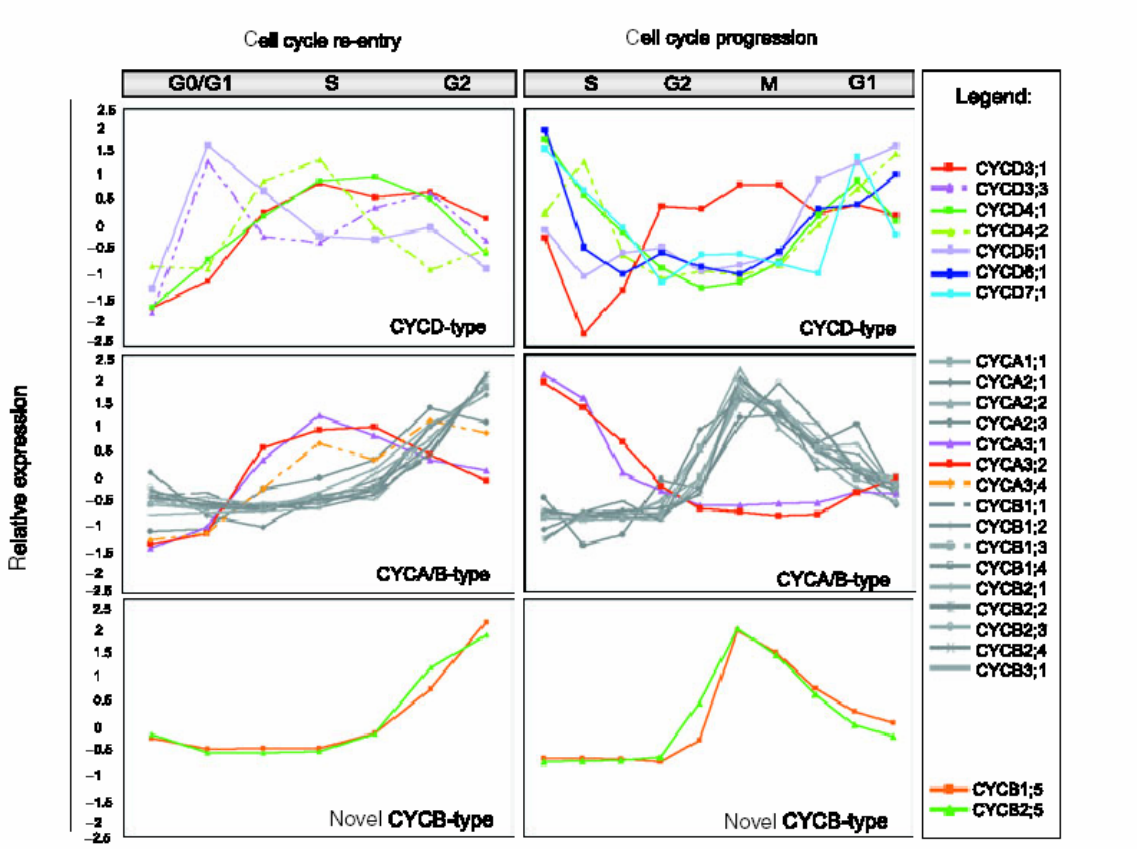


Fig4-1. Relative expression data of expressed cyclin genes were plotted against the different time points during cell cycle re-entry (left column) and further cell cycle progression (right column) as indicated. For clarity, CYCD, CYCA and B, and the novel CYCB1;5 and CYCB2;5 are shown in separate panels. (taken from Menges *et al.*2005)

D-type cyclins have a large sequence divergence and were originally identified by functional complementation of a yeast strain deficient for G1 cyclins (Dahl et al., 1995; Soni et al., 1995; Inze and De Veylder, 2006).

In *Arabidopsis*, the 10 CYCDs are classified into seven groups, designated CYCD1 to CYCD7, with the CYCD3 and CYCD4 groups consisting of three and two members, respectively (Vandepoele et al., 2002). The large number of cyclins might reflect the high developmental plasticity of sessile plants to respond to both intrinsic developmental signals and extrinsic environmental cues. Possibly, the complex cell cycle machinery is the trade-off for the tremendous plasticity and robustness of plant growth, which requires the presence of flexible regulatory networks (Inze and De Veylder, 2006). The large number of cyclins might possess a wide range of expression patterns and confer different substrate specificities. There is probably an extensive functional redundancy among D type cyclins, because the genome-wide insertional mutagenesis surveys have yet to report severe phenotypes for D-cyclin knockouts

(Campisi et al., 1999; Swaminathan et al., 2000). Just as for D-type cyclins, only marginal phenotypes have been reported for CYCA mutants. Knockouts for *CYCA2;3* display a slight increase in their DNA ploidy level (Imai et al., 2006). And only a relatively mild phenotype can be explained by the fact that A-type cyclins are part of a family of closely related genes; as such, multiple knockouts will presumably have to be combined before any severe phenotype is revealed (Yu et al., 2003; Inze and De Veylder, 2006). In contrast to their knockdown, overexpression of the A-type cyclin genes triggers an acute phenotype: *Arabidopsis* plants that overproduce the tobacco CYCA3;2 cyclin show ectopic cell division and delayed differentiation, correlated with an increase in expression of S phase–specific genes and CYCA3;2-associated CDK activity. In addition, overproduction of CYCA3;2 impairs shoot and root regeneration in tissue culture (Yu et al., 2003).

The potential of B-type cyclins to trigger the G2-to-M transition was originally shown by Colon-Carmona et. al, Criqui et al and Weingartner et al (Colon-Carmona et al., 1999)(Criqui et al., 2000; Weingartner et al., 2003). CYCB has 11 members which are divided into 3 subfamilies (CYCB1, CYCB2 and CYCB3. CYCB1 contains five members (CYCB1;1- CYCB1;5), CYCB2 contains 5 members CYCB2;1-CYCB2;5) and CYCB3 has one member (Menges et al., 2005) and (Vandepoele et al., 2002). The expression of B-type cyclins was also shown using Microarray analysis (Fig.4-1) (Menges et al., 2005)

Entry into mitosis can be accelerated by inducing cyclin B2 expression in cultured cells during G2 phase (Weingartner et al., 2003).

Factors that regulate mitotic B-type cyclin genes in higher plants

All B-type plant cyclin genes examined so far show cell cycle-regulated expression. Promoter activity of three B1 cyclin genes, *CYCB1;*1 from *Nicotiana sylvestris (Trehin et al., 1997)*, *CYCB1;1* (*CYM*) from *Catharanthus (Ach et al., 1997)* and *CYCB1;1* from *Arabidopsis* (Shaul et al., 1996); Planchais, 2002 #4} have been examined independently by different groups, and all of them were found to be cell cycle-regulated and are activated specifically in G2/M. The mechanism of promoter activation seems to be conserved among various plant species. Cell cycle-regulated promoter activation occurs in a heterologous system, namely tobacco BY2 cells. Deletion analysis of the *CYM* promoter revealed that 80 bp of the proximal region of

the promoter retained G2/M-specific promoter activity in BY2 cells. Further analysis of the 80 bp fragment by inducing mutations resulted in identification of a 9bp sequence that has been identified as a critical fragment for cell cycle-regulated promoter activation. (Ito et al., 2000). The full-length *CYM* promoter contains three other sequences similar to the 9 bp element. These 9 bp sequences, in a heterologous context, could direct G2/M-specific expression of a reporter gene called MSA (M-phase-specific activator), can function in an orientation-independent fashion. Multiple MSA elements are present in the promoter of B1 and B2 classes of cyclin genes from various plant species (Ito et al., 1998; Ito, 2000)

1.3. Regulation of the cell cycle by APC/C-type ubiquitin ligases

The degradation of cell cycle regulator proteins is necessary to maintain the periodic fluctuations in protein levels during the cell cycle, and serves as a means of cell cycle control. The highly regulated proteolysis of B-type cyclins involved in sister chromatid separation is required at the onset of anaphase. Proteins subjected to degradation are marked with ubiquitin tags and subsequently are targeted for degradation by the 26S proteasome (Hochstrasser, 1995). The ubiquitin/26S proteasome proteolytic pathway is highly conserved in eukaryotes and is involved in many other important cellular functions aside from cell cycle progression (Hochstrasser, 1995; Genschik et al., 1998; Nakayama and Nakayama, 2006).

Degradation via this pathway is a two-step process: the protein is first tagged by the covalent attachment of ubiquitin; subsequently, it is degraded by a multicatalytic protease complex called the 26S proteasome. Conjugation of ubiquitin to the protein involves a cascade of three enzymes: E1, E2, and E3. The E1 (ubiquitin-activating) enzyme forms a high-energy bond with ubiquitin, which is then transesterified to a ubiquitin-conjugating enzyme (E2). E3 ubiquitin ligase activity is then used for transfer of the ubiquitin to the target protein substrate (Genschik et al., 1998; Castro et al., 2005).Transfer of ubiquitin to the target protein substrate requires specificity and versatility, which are provided by the existence of 500–1,000 different E3 ligases. RING-finger-type E3s are thought to be the largest family and are further divided into subfamilies; the cullin-based E3 subfamily, is one of the largest single classes of E3. There are seven cullin-based E3s, including the SKP1–CUL1–F-box-protein (SCF) complex (Zachariae and Nasmyth, 1999) and the anaphase-promoting complex/cyclosome (APC/C) (Zachariae and Nasmyth, 1999; Buschhorn and Peters,

2006; Eloy et al., 2006) both of which are involved in the proteolysis of core components of the cell-cycle machinery. The SCF complex has three invariable components — RBX1 (RING-finger protein), CUL1 (scaffold protein), and SKP1 (adaptor protein) — along with one variable component the F-box protein. The F-box protein is responsible for substrate recognition and binds to SKP1 via its F-box motif. The APC/C is structurally similar to the SCF complex, and consists of invariable core components — APC11 (RBX1-related RING-finger protein), APC2 (CUL1-related scaffold protein) and at least 9 other Components (Fig5-1) (Nakayama and Nakayama, 2006)

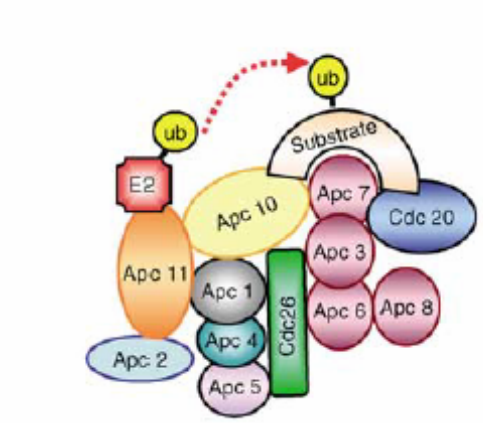


Fig. 5-1. APC contains a Cullin, and a Ring-H2 finger protein, designated here as Apc2 and Apc11 respectively. The cullin and Ring-H2 finger proteins are required to bind E2 and catalyze the ubiquitination of APC/C substrates. (Adapted from Castro et al. 2005)

APC regulates exit from mitosis and events in G1 (Fang et al., 1998{Peters, 2006 #25) #1423}}. APC/C activity needs activator. WD-containing proteins Cdc20/Fizzy and a related protein Cdh1/Fizzy-related can activate APC/C (Fig5-1) (Visintin et al., 1997; Fang et al., 1998). Each activator associates with APC/C in a cell cycle-dependent manner and is thought to target a distinct set of substrates. Cdc20-activated APC corresponds to the mitotic form of the APC. A destruction box composed of the sequence R-X-X-L-X-X-X-X-N has been found in all known Cdc20-APC substrates (Glotzer et al., 1991). In late mitosis Cdh1 activates the APC. Cdh1–APC recognizes both D-box and non-D-box-containing substrates (Fang et al., 1998) including the non-D-box (in vertebrates) substrate Cdc20 itself. The Cdc20 protein present fluctuates throughout the cell cycle (Weinstein, 1997; Prinz et al., 1998), Ubiquitination and degradation of non-D-box containing substrates is directed by the KEN box (Pfleger and Kirschner, 2000; Buschhorn and Peters, 2006).

1.4. Model systems to study the function of B1-type cyclins

Since many mutants in cell cycle regulators are either embryonic or gametophytic lethal, e.g. *apc2* (Capron et al., 2003b), or display no alteration from wild type plants due to redundancy, e.g. D-type cyclins (Swaminathan et al., 2000). The ubiquitously active 35S promotor from the *Cauliflower Mosaic Virus* (*CaMV*) has been employed for carrying out misexpression experiments. An advantage of ectopic overexpression a cell cycle regulator can be analyzed in a wide range of cell types. On the other hand ectopically expressing cell regulators can severely effect plant growth. For example plants that misexpress *Pro35S:E2Fa* together with *Pro35S:DPa* experience severe retardation in growth (De Veylder et al., 2002). In these lines, it is difficult to distinguish whether the observed phenotype is caused by the misexpression of the cell cycle regulator directly, or whether this phenotype reflects the misregulation of multiple genes challenged by the misexpression or whether it is an indirect effect, e.g. root development in *Pro35S:KRP1* misexpressing plants is severely affected.

Misexpression in specific cells, such as *Arabidopsis* leaf hairs (trichomes), have been proven to be suitable to study the function of cell cycle regulators in a developmental context, also largely avoiding general growth and fertility problems (Schnittger et al., 2002b; Schnittger et al., 2002a; Weinl et al., 2005).

Trichomes are single-celled leaf hairs, which are initiated with a controlled distance to each other in the basal part of young and developing leaves. Archetypical for many differentiating cells, incipient trichomes exit the mitotic program and switch to an endoreplication mode. Concurrent with outgrowth and initiation of branches, trichomes undergo approximately four rounds of endoreplication resulting in mature three-branched trichomes with a DNA content of approximately 32C (Marks, 1997; Hulskamp et al., 1999).

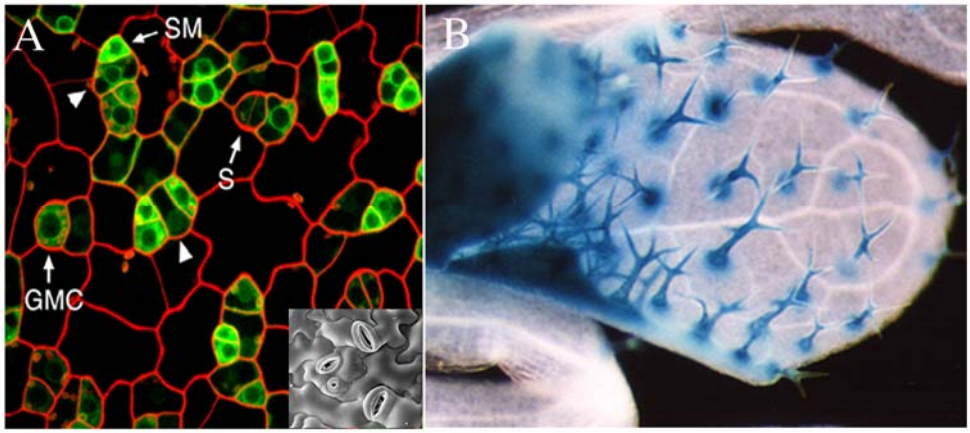


Fig. 6-1. (A) Confocal scanning micrograph of leaf epidermal cell from plants expressing *ProTMM:TMM:GFP*; to visualize cell walls the leaf was stained with propidium iodide; (Taken form Nadeau and Sack, 2002a). right down, a Scanning electron micrograph of stomata from a plant expressing *ProTMM:TMM*. GMC: guard mother cell, SM: satellite merisetemoid.
(B). Expression pattern of pro*GLABRA2:GUS* in rosette leaves.

Promotors such as CAPRICE, GLABRA2 or TRIPTYCHON can be used to study the role of cell cycle regulators in an endoreplicating context. These three genes play important roles in trichome development and are expressed from very early stages until late stages of trichome development (Fig6B-1) (Szymanski et al., 1998; Schellmann et al., 2002). Thus, expression of cell cycle regulators under control of the *GLABRA2* promotor provided a tool to analyze their function in an endoreplicating context.

To analyze the function of cell cycle regulators in dividing epidermal cells during post-embryonic development, the promotor of the *TOO MANY MOUTH* gene (*TMM*) has been used. TMM is involved in the control of stomata distribution and has been found to randomize the plane and alter the number of asymmetric divisions in stomata neighboring cells (Geisler et al., 2000). *TMM* expression occurs during early leaf development in cells of the stomatal lineage.

Expression has also been detected in guard cells, guard mother cells and some of their neighboring cells, and meristemoids (Fig5A-1) (Nadeau and Sack, 2002b). Expression could be detected in meristemoids, guard mother cells and some of their neighboring cells, but also in guard cells (Fig5-1) ((Nadeau and Sack, 2002b)

1.5. Aim of this work

In this work, I studied the function of B1-type cyclins, the key regulators of CDKs in G2/M phases in *Arabidopsis thaliana*. The analysis focused on three aspects: First, loss of function analysis of B1-type cyclins which was done by analyzing knock out lines. Second, the function of CYCB1;2 in endoreplicating and dividing cells was analyzed by performing cell type specific misexpression experiments in the stomata lineage as dividing cells and trichomes as endoreplicating cells.

The third aspect involved analyses of the APC/C function in trichomes. Silencing of APC11 using RNAi provided an interesting tool to analyze the function of APC/C in trichomes.

2. Results

2.1. Studying CYCB1 function: loss of function approach

Plant cell cycle regulators constitute much larger families than animal regulators. For example in Drosophila there are two B and one A type cyclins whereas in Arabidopsis 11 B-type cyclins and 10 A type cyclins have been identified (Pines, 1995)(Wang et al., 2004). The specific function of the different members is not understood. To analyze whether the different B1-type cyclins (Fig.1A and B) have specific roles in development or whether these genes have a solely redundant function, I analyzed CYCB1 knock out lines.

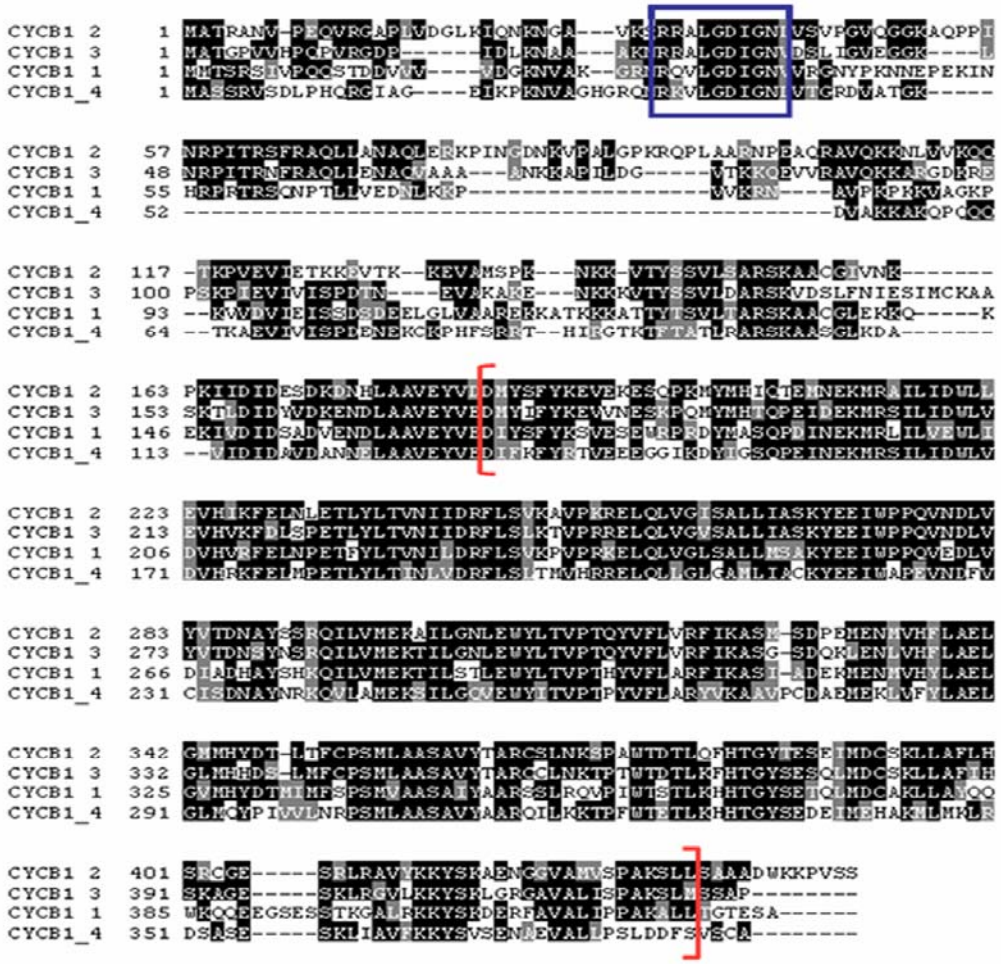
2.1.1 Characterization of B1-type cyclins

A data base search was used to screen four collections of insertion mutants:, the GABI-Kat collection, the SIGNAL collection hosted at the SALK Institute, the transposone insertion library from RIKEN and the Koncz collection at the Max Planck institute of Plant Breeding was screened manually. T-DNA lines of *cycb1;1* (At4g37490), *cycb1;2* (At5g06150) from the Koncz T-DNA collection, *cycb1;3* (At3g11520) from SALK, GABI and RIKEN collections, and *cycb1;4* (At2g26760) from Koncz and GABI collections was analyzed, (Fig2-2).

To remove potential extra unlinked T-DNAs from the respective insertion mutants, T-DNA lines were crossed to wild type plants. Segregation analysis of F2 generations on MS media containing antibiotics proved the presence of one T-DNA per genome (table 2-1) Using a PCR genotyping assay, I was able to follow the transmission of the T-DNA insertions in the self-progeny of F1 plants.

Fig. 1-2 (A). Amino acid sequence alignment of *Arabidopsis thaliana* B1-type cyclins. The sequences are presented using the single-letter code. The parenthesis indicate cyclin box and sequences of Destruction box are indicated by a rectangular.
(B). Phylogenetic tree of *Arabidopsis thaliana* B1-type cyclins. The tree was constructed using Vector NTI software. The GenBank accession numbers of the cyclin sequences and their distance are indicated.

A


CYCB1 2
CYCB1 3
CYCB1 1
CYCB1_4

B

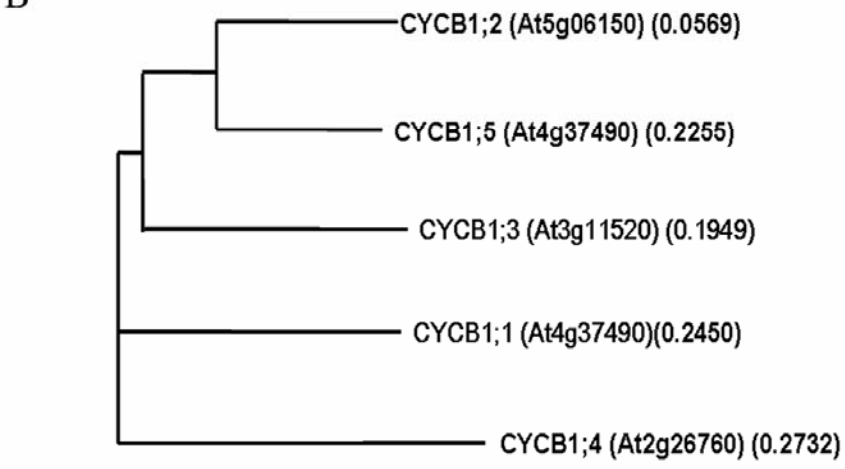

CYCB1;2 (At5g06150) (0.0569)
CYCB1;5 (At4g37490) (0.2255)
CYCB1;3 (At3g11520) (0.1949)
CYCB1;1 (At4g37490)(0.2450)
CYCB1;4 (At2g26760) (0.2732)

Table 2-1: T-DNA or Transposone lines of B1-Type cyclins						
Name of gene	**Name of collection**	**Line ID**	**Segregation analysis (F2 seedlings)***	**T. hits/ border**	**RT-PCR result**	**Number of crosses to wild type**
cycb1;1 (At4g37490)	Koncz	-	45:15	Exon II/ (RB/LB)	null	1
cycb1,2 (At5g06150)	Koncz	-	26:5	Exon IV/ ($RB/LB_{N.D}$)	null	1
cycb1;3 (At3g11520)	Salk GABI-1 GABI-2 RIKEN (trasposone line)	016509 730C01 859B01 pst15850	N.D. N.D N.D. N.D	-137bp/(LB/LB) -190/($LB_{N.D}/RB_{N.D}$) -92 (LB/RB) Exon II ($LB/RB_{N.D}$)	Overexpression Overexpression Heterozygous Null	1 0 2 1
cycb1;4 (At2g26760)	Koncz GABI	- 386C01	74:20 45:15	Intron IV ($RB_{N.D}/LB$) Intron II($RB_{N.D}/LB$)	Overexpression null	0 1

* Antibiotic resistance: Antibiotic susceptible

N.D. Not Determined

T. hits: T-DNA or Transposone hits

2.1.2. B1-type cyclins mutants analysis

2.1.3. Transcription of B1-type cyclins knock out genes

To test whether the insertion resulted in a knock-out or knock-down of B1-type cyclin function, RT-PCR analyses were performed. No transcript could be detected in the homozygous mutants using primer combinations which annealed downstream of the T-DNA insertion and spanned the coding sequence of: *cycb1;1* and *cycb1;2* from the Koncz collection, *cycb1;3* from RIKEN and *cycb1;4* from the GABI collection. Thus, these lines represent null alleles of the respective genes.

However, in *cycb1;3* from GABI and Salk collections and *cycb1;4* from the GABI collection the respective RNA was expressed more strongly than in wild type control plants. This could be because the T-DNA contains the 35S promoter which might drive the expression of *CYCB1;3* or *CYCB1;4*. It can not be ruled out that the C-terminal transcript of *CYCB1;3* and *CYCB1;4* was properly translated and no further experiments were performed with these mutant lines.

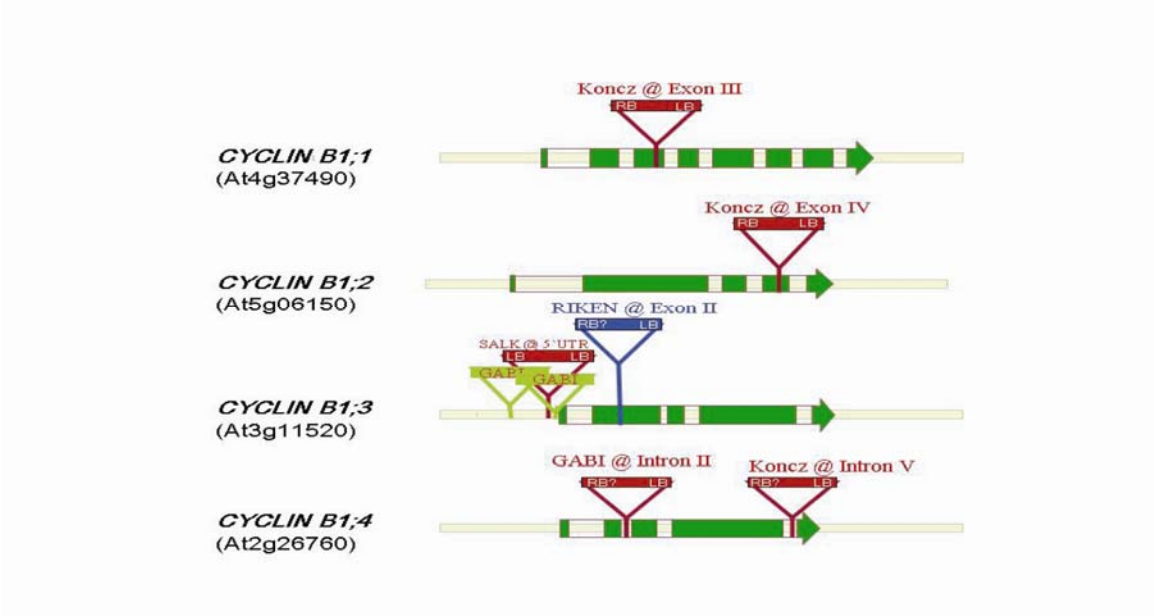


Fig 2-2. **The B1-type cyclins mutants:** Schematic drawing of the *B1-type cyclins* genes showing the T-DNA or Transposone insertion in the B1-type cyclins. Green box represents the Exon, colorless rectangular shows Intron.

2.1.4. Characterization of *B1-type cyclins* mutants

Phenotypic description of *cycb1* mutants

In order to classify phenotypes, flowering time, number of rosette leaves, side shoots, and auxiliary shoots were counted. Single mutants of *cycb1;1, cycb1;2, cycb1;4* and the *Colombia* ecotype as a control were grown in long day climate growth conditions (16 hours light /8 hours dark). The single mutants showed a wild-type like appearance under optimal growth conditions (green house).

Plant development can be regulated by B1-type cyclins

In wild-type plants almost all leaf axils, but not cotyledon axils, have the developmental potential to form an auxiliary meristem. The auxiliary branching and side shoot process generally involves two developmental stages: the formation of auxiliary meristems in the leaf axils and subsequent auxiliary bud growth. In many plant species, including *Arabidopsis*, the growth of auxiliary meristems is inhibited by the primary inflorescence, a phenomenon generally known as apical dominance. The plant hormones auxin and cytokinin appear to play a major role in controlling this process, with cytokinin as a key factor in promoting bud growth, whereas auxin has an inhibitory effect {Tantikanjana, 2001 #1480. Analysis of auxiliary and side shoots of mutants has shown that there is no significant difference between b1-type cyclin mutants and *Columbia* wild type.

Analysis of the number of rosette leaves produced prior to flowering of the *cycb1;1* mutant was not affected. However *cycb1;2* mutants displayed a reduced number of rosette leaves ($P<0.05$*) (Fig3-2). In *cycb1;4* mutants the number of rosette leaves increased relative to wild type in long day conditions ($P< 0.05$*) (Fig. 3-2 and table 2-2).

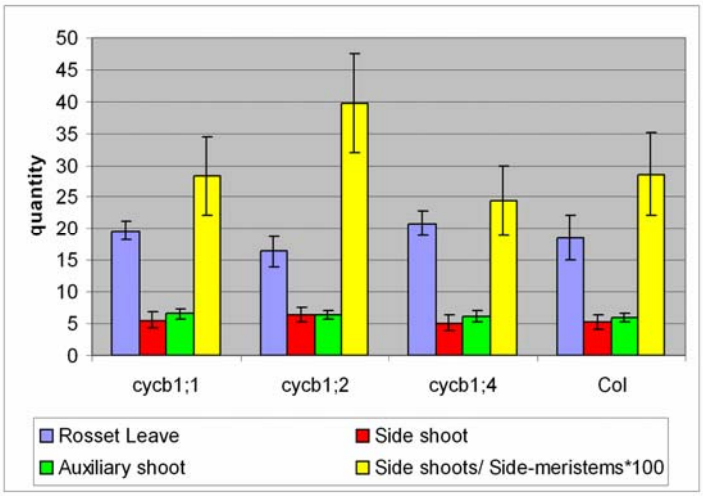


Fig. 3-2. Phenotype description of single mutant: Total Number of side shoots, auxiliary branches, number of rosette leaves and ration of side shoots/total side meristems of B1-type cyclins mutant and wild type *Columbia* ecotype. All values are means _ SE. For *cycb1; 1* and Col lines, *n* _ 14, *cycb1;2 n_ 17 and cycb1;4 n _15.*

In wild type, a meristem forms underneath a rosette leaf and a number of them will develop into side shoots depending on the environmental and genetic conditions of the plant. The ratio of side shoots to side meristems was calculated to analyze the relationship between these two features. In the *cycb1;2* mutant which has less meristems produced maximum number of side shoots were produced. Whereas the most side meristem were produced in cycb1;4 mutant but side shoot production was not affected (Table 2-2 and Fig.3-2).

Table 2-2: Morphological analysis of b1-type cyclin mutants										
	Auxiliary shoot number		side shoot number		Rosette Leave number			outgrowing bud/ meristem*100		
Name of mutant	n	M±SD	n	M±SD	n	M±SD	T-test	n	M±SD	T-test
cycb1;1	14	6,57±0,769	14	5,57±1,22	14	19,71±1,49	0,2812	14	28,28±6,19	0,8822
cycb1;2	17	6,29±0,69	17	6,41±1,12	17	16,35±2,37	0,04810*	17	39,74±7,84	0,00020***
cycb1;4	15	6,2±0,86	15	5,13±1,36	15	20,87±1,96	0,03997*	15	24,47±5,50	0,07041
Col	14	5,93±0,73	14	5,21±1,19	14	18,57±3,59	-	14	28,64±6,42	-
cycb1;1$^{-/-}$cycb1;2$^{+/-}$	16	5,75±1,18	16	6±2,1	16	15,19±1,60	0,00235**	16	39,22±12,11	0,0112*
cycb1;1$^{+/-}$cycb1;2$^{-/-}$	14	7±0,96	14	5,64±1,5	14	16,71±2,27	0,11371	14	34,57±11,27	0,09894

Mean and standard deviation of auxiliary shoot, side shoot rosette leave and out growing buds/meristems of *cycb1;1, cycb1;2, cycb1;4, cycb1;1*$^{-/+}$ *cycb1;2*$^{-/-}$*, cycb1;1*$^{-/-}$ *cycb1;2*$^{+/-}$ and Col wild type. M: Mean, SD: Standard deviation and n= number of plants in each experiment.
Significant differences of Rosette leave and outgrowing bud/ meristem*100 between wild type and *cycb1;1, cycb1;2, cycb1;4, cycb1;1*$^{-/-}$*cyc1; 2*$^{-/+}$ *and cycb1;1-/+ cycb1;2-/-* mutants are designed. *P<0,05, **P<0,01 and ***P<0,001. P values are determined by the Student T-test

Root growth analysis of b1-type cyclins

B1-type cyclin mutants and wild type control plants were grown on vertical plates in a growth chamber with controlled growth conditions. On odd days the plates were scanned and the length of the roots measured using Image J software. The data was analyzed using SPSS soft ware.

Growth analysis of root development revealed that knock outs of CYCB1;1 and CYCB1;2 have slightly decreased root growth but it is not significantly different from wild type root growth. While cycb1;4 mutant reduced root growth (Fig.4-2).

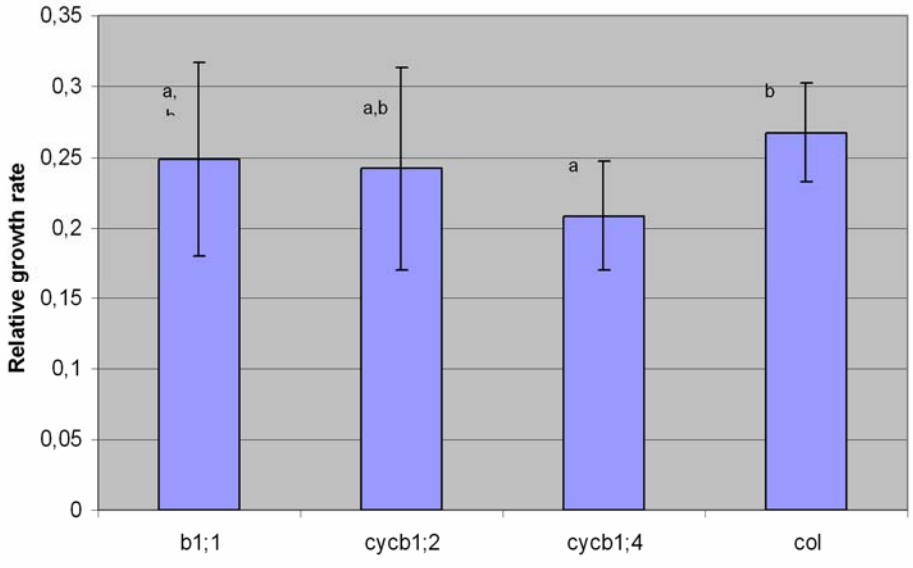


Fig. 4-2. Root growth analysis of *cycb1; 1, cycb1; 2, cycb1; 4* and Col Plants.
CYCb1;4 mutant and col clustered in Class a and Class b respectively while cycb1;1 and cycb1;2 grouped in a intermediated class a,b.

Rosette leaf growth analysis

Measurements of leaf area of single mutants showed that *cycb1;1* and *cycb1;2* mutants have a larger leaf area than wild type leaves. The number of rosette leaves was equal in *cycb1;1* and *Columbia*. And *cycb1;2* mutant produced the minimum leaf number within B1-type cyclins (Fig 5-2).

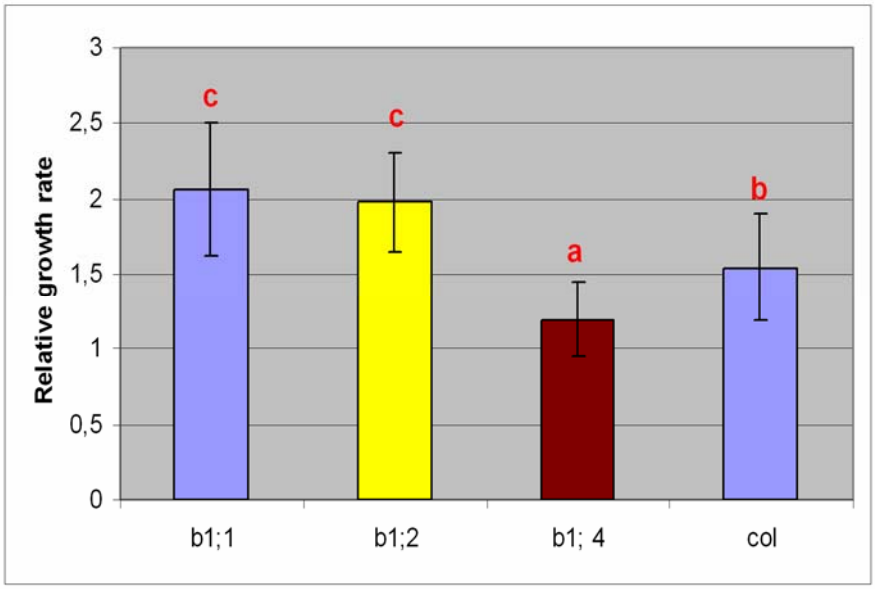


Fig. 5-2. Growth analysis of rosette leaves of *cycb1; 1, cycb1; 2, cycb1; 4* and Col Plants.
The mutants and wild type control are clustered in three sub groups: cycb1;4 belongs to group a, Col wild type group b and cycb1;1 and cycb1,2 contributed in group c.

Flowering time

Germination test of B1-type cyclins mutants showed that germination time was not affected by mutations in B1-type cyclins whereas vegetative growth of B1-type cyclin mutants is prolonged and flowering is delayed (Fig6-2 A and B).

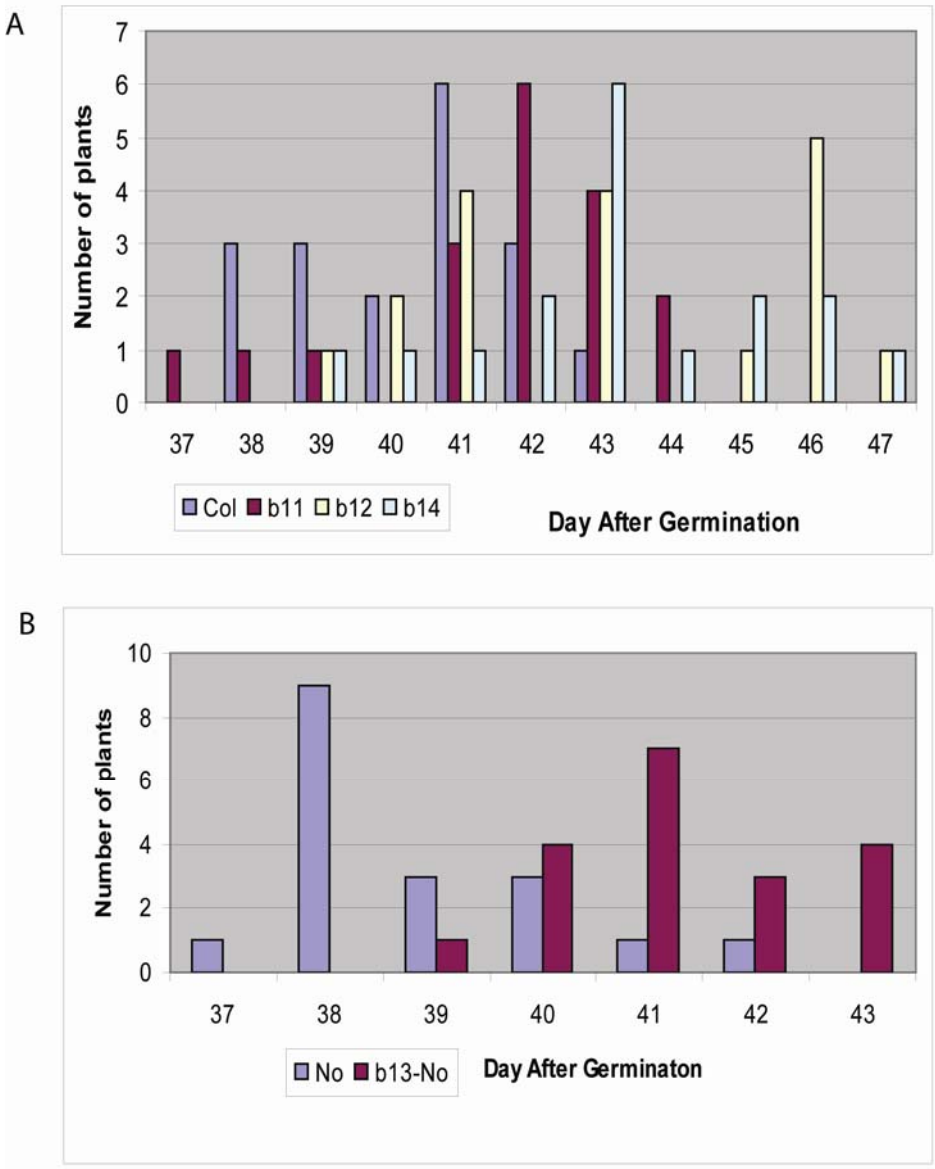


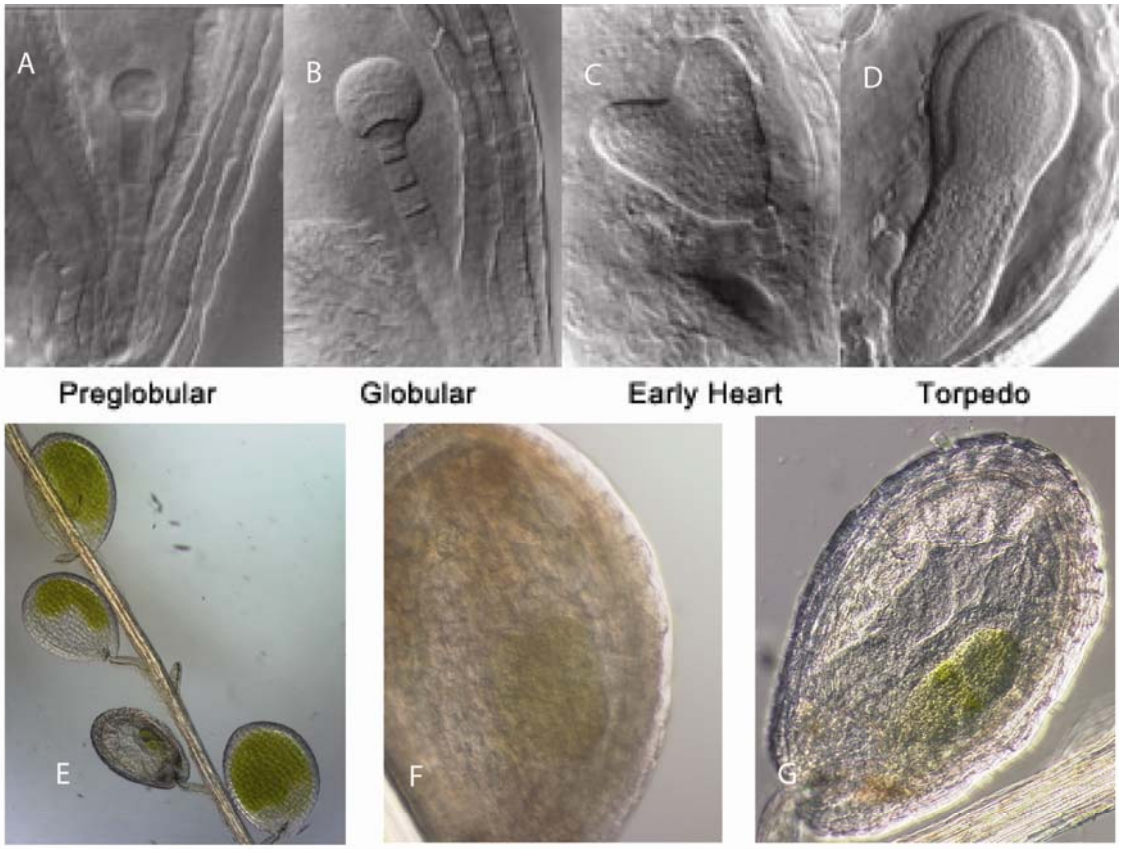
Fig. 6-2. Flowering time: Frequency distribution of flowering time of b1-type cyclin mutants in long day condition (16 hours light/ 8 hours dark). A. flowering time of *cycb1; 1, cycb1; 2, cycb1; 4* and wild type *Columbia* ecotype. Distribution of *cycb1; 3* in Nössen background and wild type Nössen were shown separately.

2.1.5. Redundancy within B1-type cyclins

No severe phenotype from wild-type was found in single mutant cyclins. One possibility is that the B1-type cyclins act redundantly. Therefore, Double mutants of *cycb1;1 cycb1;2* , *cycb1;1 cycb1,3* , *cycb1;2 cycb1;3* , *cycb1;1 cycb1;4, cycb1;2 cycb1;4* and *cycb1;3 cycb1;4* were created. No obvious phenotype could be detected in double mutants of *cycb1;1-cycb1;4* and *cycb1;2 –cycb1;4* under green house growth conditions. Double mutants were not analyzed any further.

In F2 progeny of *cycb1;1* and *cycb1;2* mutant combinations, no double homozygous line was found. Consistent with this observation in progeny of the *cycb1;1*$^{-/-}$ *cycb1;2*$^{-/+}$

mutant a quarter of the seeds were collapsed (Fig7-2 H). Analyses of these seeds showed that the embryo was arrested at late heart to torpedo stage. The same result has been observed for the progeny of *cycb1;1*$^{-/+}$ *cycb1;2*$^{-/-}$ mutant (Fig 7-2 A- G). Double mutants of cycb1;3 with other member of B1 type cyclin were not analyzed.



H

progeny	Abortion rate
cyc b1;1 CYCB1; 1& cyc b1;2$^{-/-}$	27.62%
cyc b1; 1$^{-/-}$& cyc b1;2 CYCB1; 2	25.14%

Fig. 7-2. **Embryo development** in **(A-D)** wild type plant and **(E-G)** *cycb1; 1*$^{-/-}$ *cycb1; 2*$^{-/+}$. Embryos of *cycb1; 1*$^{-/-}$ *cycb1; 2* $^{-/+}$.**(E)**Three normal embryos developed normal and one arrested embryo. **(F)**. arrested embryo at end of heart stage. **(G)** Arrested embryo at torpedo stage. **(H)** In selfed cycb1; 1$^{-/-}$cycb1; 2$^{-/+}$ or cycb1; 1$^{-/+}$cycb1; 2$^{-/-}$ plants in which one quarter homozygous cycb1; 1$^{-/-}$cycb1; 2$^{-/-}$ offspring is embryo arrested

2.1.6 Leaf growth analysis of cycb1;1$^{-/-}$cycb1;2$^{-/+}$ and cycb1;1$^{-/+}$cycb1;2$^{-/-}$

To descript the phenotype of *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ and *cycb1;1*$^{-/+}$*cycb1;2*$^{-/-}$ plants were grown with Columbia ecotype and single mutants of cycb1;1 and cycb1,2 in the same climate chamber and leave growth, number of rosette leaves, side shoot and sub branches were measured.

No significant deviation was observed in auxiliary or side shoots proving that CYCB1;1 and CYCB1;2 are not essential for auxiliary or side shoot formation (table2-2). In *cycb1;1*$^{-/+}$*cycb1;2*$^{-/-}$ number of rosette leaves was more reduced than *cycb1;2* mutants. *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ have shown reduction of rosette leave numbers but it is not a significant difference from wild type (table 2-2). It shows that CYCB1;1 has a redundant function with CYCB1;2 and the case is the same for the ratio of outgrowing buds to the total side meristem (table 2-2). While leaf area was increased in the cycb1;1 or cycb1;2 single mutant The removal of one allele of CYCB1;1 from cycb1;2 mutants did not change any further leaf growth (Fig. 8-2).

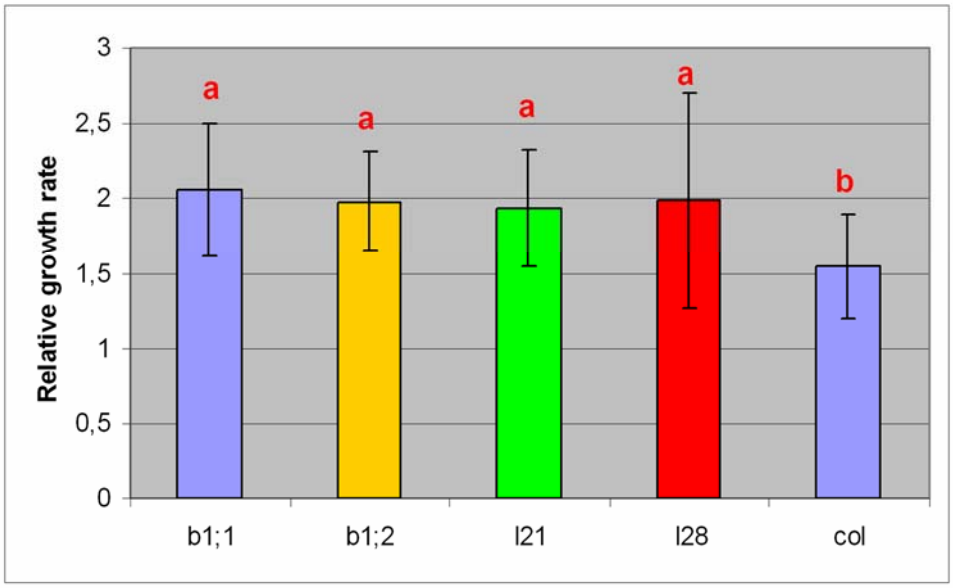


Fig. 8-2. Rosette leaves Growth analysis of cycb1*;1*$^{-/-}$ *cycb1;2*$^{-/+}$ *and cycb1;1*$^{-/+}$ *cycb1;2*$^{-/-}$ and compare with cycb1;1 cycb1;2, and Col Plants. Single and double mutants increased rosette leave area are clustered in group a and Col wild type was separated from the mutants and produced group b. I21: *and cycb1;1*$^{-/+}$ *cycb1;2*$^{-/-}$ and I28: cycb1*;1*$^{-/-}$ *cycb1;2*$^{-/+}$

2.1.4. Expression analysis of upstream region of B1-type cyclins

To analyze expression pattern of the B1-type cyclins, 1153 bps from the 5' region of *CYCB1;1*, 1050 bps from the 5' region of *CYCB1;2* and 1474 bps from the 5' region of *CYCB1;4* were cloned instead of the *CaMV 35S* promoter cassette of the pAMpAT binary gateway vector. GUS was fused and misexpressed in wild type plants. Expression patterns of B1-type cyclin promoters were then analyzed using GUS

activity. GUS activity was observed in shoot meristem and vascular systems of dividing region of leave, and young trichome (Fig 9-2).

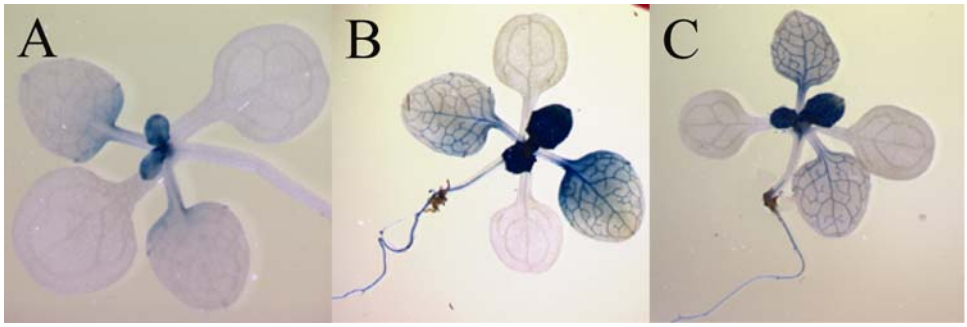


Fig. 9-2. Histochemical Analysis of *CYCB1;1*, *CYCB1;2* and *CYCB1;4* Promoters Activity. **(A)** *ProCYCB1;1:GUS* ,**(B)** *ProCYCB1 2:GUS* and **(C)** *ProCYCB1;4:GUS*

2.1.8. Rescue cycb1;1$^{-/-}$ cycb1;2$^{-/+}$ phenotype

ProCYCB1;1:CYCB1;2, ProCYCB1;1:CYCB1;2 and *ProCYCB1;1:CYCB1;3* could rescue the *cycb1;1*$^{-/-}$ *cycb1;2*$^{-/-}$ double mutant and double homozygous plants with normal growth have been found. *ProCYCB1;1:CYCB1;4* was not able to rescue the *cyc1;1 cycb1;2* double mutant. This data shows that there is a redundant function between CYCB1;1, CYCB1;2 and CYCB1;3, but no redundancy could be detected between CYCB1;4 and CYCB1;1 or CYCB1;2.

The B1-cyclins are essential for plant growth. There seems to be a matrix of redundantly acting cyclins.

2.1.9. Phenotype of cycb1;1 cycb1;2 double mutants

In the progeny of *cycb1;1 cycb1;2*, one double homozygous out of 200 plants was found. The vegetative phase of the double homozygous line is prolonged and the generative phase is started seventy days after germination. Double homozygous of *cycb1;1 cycb1;2* showed a dwarf and bushy phenotype which had problem in male and female gamete development.

2.1.10. Loss of CYCB1;1 and CYCB1;2 induce male and female development defects

cycb1;1-cycb1;2 double homozygous mutants produced 2-3 seeds per silique. To determine the origin of the sterility of the double mutant, double mutant pistils were

fertilized with wild type pollen, and few seeds were produced. A check for a defect in male gametophyte development was then done by fertilizing wild type pistils with double mutant pollen. This fertilization also produced only a low number of seeds.

To further define the cause of the sterility of the *cycb1;1-cycb1;2* double mutant and the role of CYCB1;1 and CYCB1;2 in pollen development, a search for pollen viability and gametophytic defects was performed. Pollen viability defined by Fluorescein Diacetate (FDA). In wild type 95.5% of wild type pollen were viable in contrast just 10.2% of double homozygous cycb1;1-cycb1;2 were viable. (Fig. 10-2). DAPI staining of pollen revealed trinucleated pollen.

My data showed that viability and formation of trinucleated pollen is not enough to fertilize an ovule and some how male gametophyte development is arrested later stages.

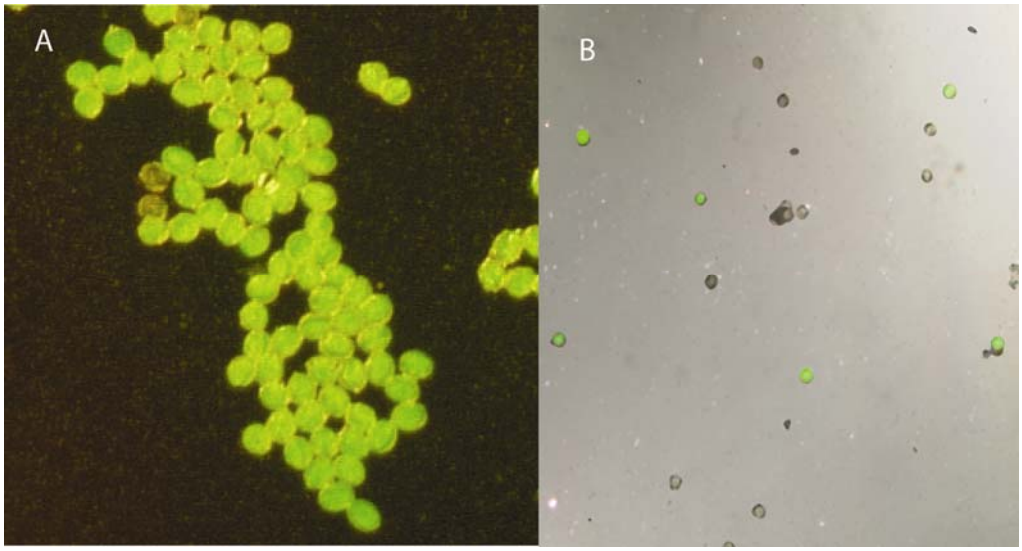


Fig. 10-2. FDA staining of pollen of **(A)** wild type and (B) double homozygous mutant of cycb1; 1 cycb1, 2. Pictures A and B have different scales

In order to explain the lack of seed production of double mutants with wild type pollen, ovule development of double mutants was investigated. It was shown that most ovules are arrested at early stages of embryo development or the integuments were formed but no embryo sac could be found (Fig.11-2).

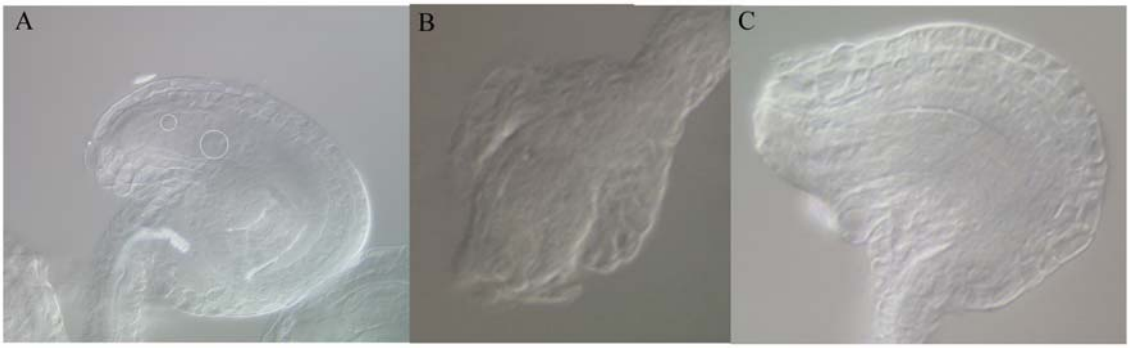


Fig. 11-2 Ovule development in wild type and cycb1;1$^{-/-}$ cycb1;2$^{-/-}$ double mutant. **(A)** Ovule of wild type the central cell and egg cell are shown by white circles. **(B)** and **(C)** ovule development in cycb1;1$^{-/-}$ cycb1;2$^{-/-}$ double mutant which **(B)** is arrested in integument development and **(C)** integuments are form but no cells are produced in embryo sac. The pictures have same age but different scales.

2.2. Gain of function analysis of B1-type cyclins

2.2.1 Misexpression of B1-type cyclins in endoreplicating cells

Previous experiments reported that CYCB1;2 misexpression produced multicellular trichomes but the closely related CYCB1;1 did not produce any multicellular trichomes {Schnittger, 2002 #530}.

To identify the domains of CYCB1;2 which induce ectopic mitosis in trichomes, double and triple fusion constructs were created by swapping domains of CYCB1;1 and CYCB1;2 (Fig. 12-2 A,B,C,D,E,F) and ectopically expressed them in trichomes under the control of the *GL2* promotor. No obvious phenotype was found in trichomes of transgenic lines with full length or domain swapped CYCB1;1 and CYCB1;2. In the process of further addressing what the reason for this apparent specificity among the cyclins, the sequence of CYCB1;2 which had been misexpressed in trichomes by Schnittger et al. 2002a was revisited. In this sequence a mutation was discovered that rendered the start codon inactive (the intial ATG was mutated to GTG). It seemed that the full length of CYCB1;2 did not produce any phenotype in trichomes. The next start codon in frame of the mutated cyclin sequence appears at a position 405 bp after the original start codon. This gave rise to the hypothesis that only the expression of a large N-terminally truncated cyclin, designated as CYCB1;2$^{\Delta 1\text{-}135}$ induces cell divisions in endoreplicating trichomes (Fig12-2 C and Fig.13-2). This theory was subsequently confirmed by the appearance of multicellular trichomes in plants upon transformation with an expression construct, in which a N-terminally cyclin was under the control of the *GL2* promotor (table 3-2).

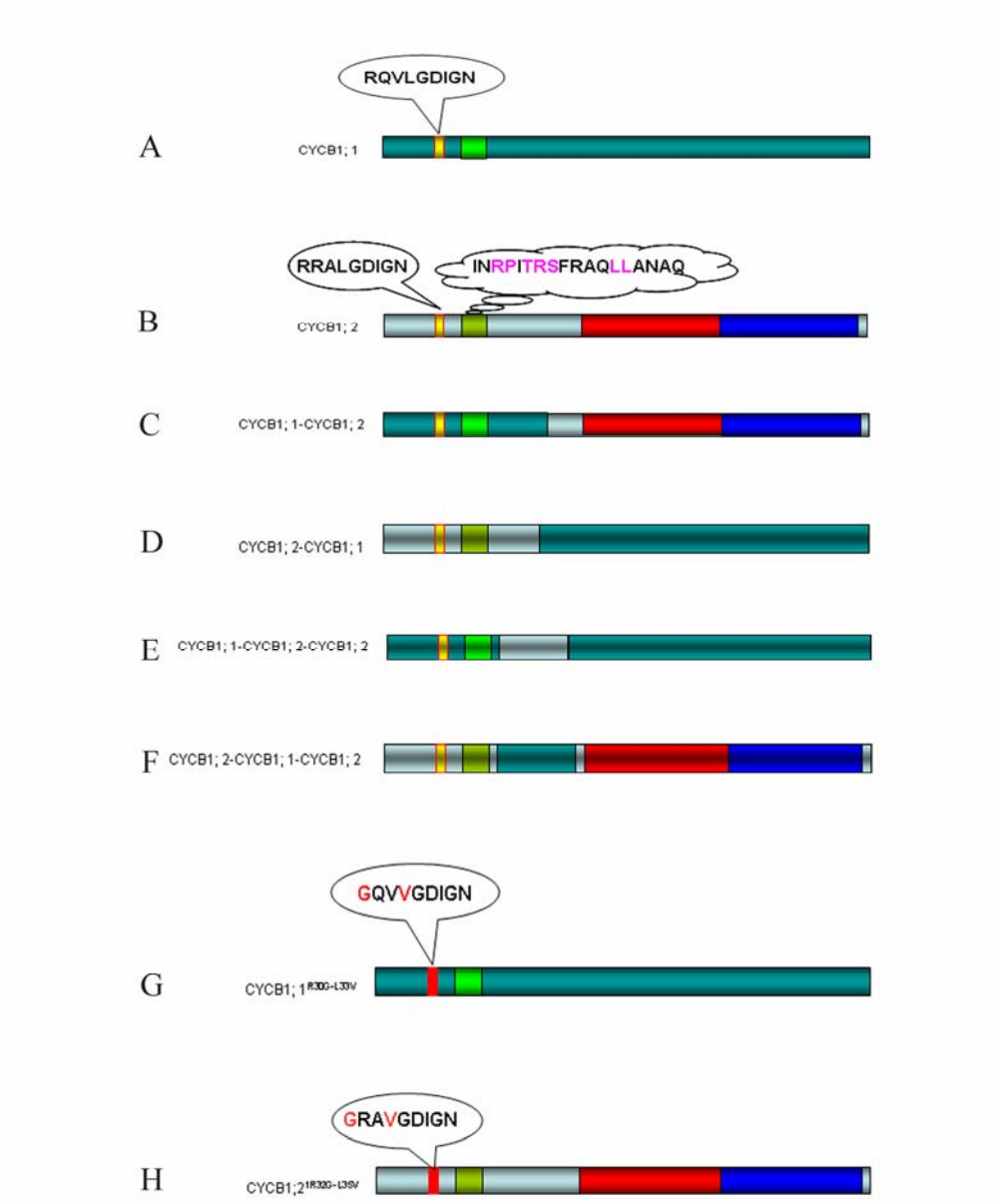


Fig.12-2 Schematics of the CYCB1;1, the CYCB1;2 and their fusions
(A). CYCB1;1, its destruction box (light yellow color) and the analogue fragment of the CYCB1;2 Barbie box in the CYCB1;1 gene (light green) **(B)** Schematic representation of the position of the destruction box, (Yellow color), Barbie box (green) cyclin boxes, CYCN (red color) and CYCC (blue color). **(C)** and **(D)**. Double fusion of the N-terminus of CYCB1;1 and C-terminus of CYCB1;2 and of the N-terminus of CYCB1;2 and C-terminus of CYCB1;1 respectively. **(E)** and **(F)** Triple fusion of CYCB1;1-CYCB1;2-CYCB1;2 and CYCB1;2-CYCB1;1-CYCB1;2 respectively. **(G)** and **(H)** Destruction box mutation of CYCB1;1 and CYCB1;2. Mutations in CYCB1;1 and CYCB1;2 destruction box and the exchanged amino acids are shown in red. 1 mm represents 4 amino

2.2.2. Misexpression of CYCB1;1 and CYCB1;2 destruction box mutation in endoreplicating cells

Degradation of B- type cyclins is dependent on a specific sequence element in its N-terminal region, termed the destruction box (Glotzer et al., 1991). Non-degradable cyclin versions of mitotic cyclins exhibit mitotic arrest in *D. melanogaster* (Rimmington et al., 1994) and Hela cells (Gallant and Nigg, 1992), in addition stabilization of different cyclins blocked the exit from mitosis at different steps (Sigrist et al., 1995; Su et al., 1998).

To define the function of the destruction box in destabilization of the CDK/CYCB complex and degradation of the B1-type cyclin in endoreplicating cells, the amino acids Argenine 31 and Leucine 35 were changed to Glycine and valine, respectively (Fig 14-2 A). Stewart et. al 1994 have shown that a mutation in argenine of the destruction box stabilizes cyclin A (Stewart et al., 1994). Criqui *et al* 2001 and Weingartner *et. al* 2004 have shown that deletion of destruction box prevent degradation of CYCB1;1 in BY2 cells and *N. tabacum* respectively (Criqui et al., 2001)(Weingartner et al., 2004).

To understand the function of the destruction box on degradation of CYCB1;1 and CYCB1;2 in endoreplicating cells CYCB1;1 and CYCB1;2 with a mutated destruction box were misexpressed in trichomes. No deviation from wild type trichome development was observed in CYCB1;1 mutated destruction box but CYCB1;2 destruction box mutation induced a mild phenotype in trichomes (table3-2).

Table 3-2
Trichome phenotype upon misexpression of CYCB1;2 and CYCB1;2 truncations

Name of construct	Multicellular trichome*	Cluster of trichome**	Wild type	Total number of plants analyzed	% of plants with an altered trichome phenotype
ProGL2:CYCB1;2	0	0	100	100	0
ProGL2:CYCB1;2$^{R32G-L35V}$-destruction box mutation	1		119	120	0.8
proGL2;CYCB1;2$^{\Delta 1-31}$	0	5	48	53	9.4
proGL2;CYCB1;2$^{\Delta 1-40}$	1	9	81	91	11
ProGL2:CYCB1;2$^{\Delta 1-135}$	17	3	5	25	80
ProGL2:CYCB1;2$^{\Delta 1-185}$	0	0	61	61	0
proGL2;CYCB1;2$^{\Delta 1-311}$	0	0	56	56	0

* More than one trichome per TIS, resulting in clusters of trichomes
** Multicellular trichome: multiple cells per trichome

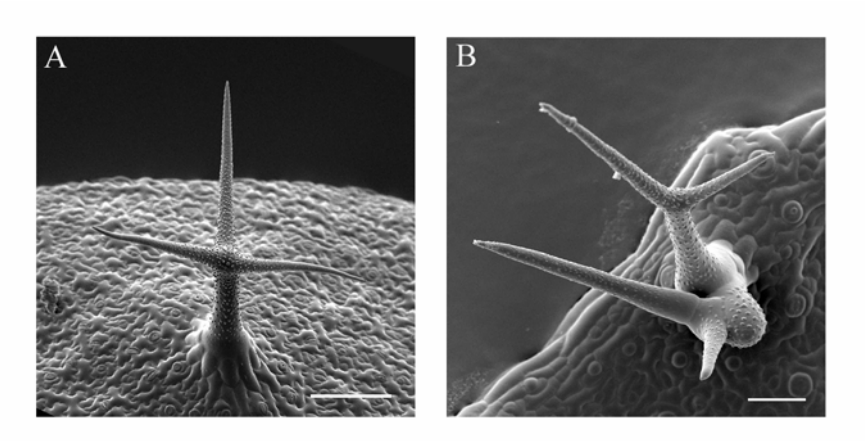


Fig. 13-2. Morphological Analysis
(A) Scanning electron micrograph of a wild-type trichome.
(B) Scanning electron micrograph of multicellular trichomes of ProGL2: CYCB1;2$^{\Delta 1\text{-}135}$.
Pictures taken from Schnittger et al. 2002

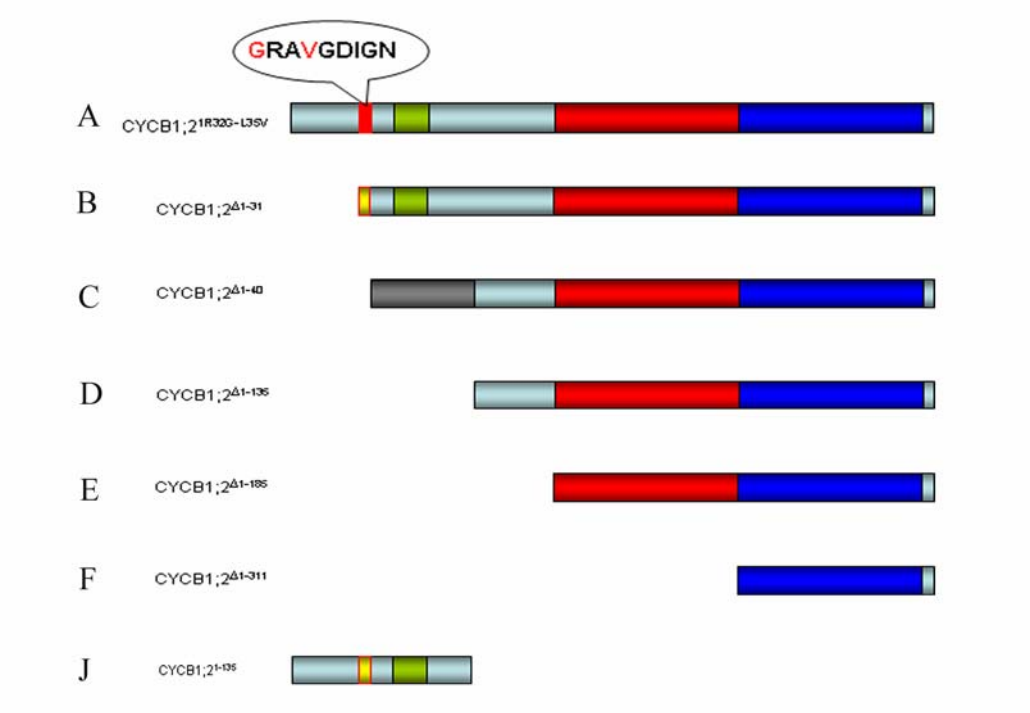


Fig. 14-2 Schematic of CYCB1; 2 truncations:
(A) CYCB1;2 destruction box mutation **(B)** CYCB1;2$^{\Delta 1\text{-}31}$ with destruction box. **(C)** CYCB1;2$^{\Delta 1\text{-}31}$ with out destruction box. Gray rectangle shows 95 amino acids which are important for destabilization of CYCB1;2. **(D)** CYCB1; 2$^{\Delta 1\text{-}135}$ which induces multicellular trichomes. **(E)** and **(F)** represent CYCB1;2$^{\Delta 1\text{-}185}$ and CYCB1;2$^{\Delta 1\text{-}311}$ **(J).** CYCB1; 2$^{1\text{-}135}$ contains the 95 amino acids which are important for destabilization of CYCB1; 2.

2.2.3. Different functions of destruction box in endoreplicating and dividing trichome cells

Misexpression of *ProGL2:GUS* and *ProGL2:CYCB1;1*$^{1\text{-}112}$*:GUS* in wild type:

The cyclin B destruction box is portable and chimeras containing the N-terminus of cyclin B attached to other proteins are rapidly degraded as they were cyclins (Glotzer et al., 1991; Amon et al., 1994; Brandeis and Hunt, 1996; Yamano et al., 1996; Yamano et al., 1998).

To precisely investigate the CYCB1;1 a destruction box function during an endoreplication cycle, GUS and GUS fusions with a N-terminal of CYCB1;1$^{1\text{-}112}$ was misexpressed under the control of the GL2 promotor in wild type *columbia*, in a cyclin D3; 1 misexpression line (Schnittger et al., 2002a) and in a sim mutant (Walker et al., 2000)

Expression of GUS with or without a CYCB1;1$^{1\text{-}112}$ both exhibited similar GUS activity patterns in wild type trichomes (Fig 15-2 A and B). Therefore the destruction box of CYCB1;1 does not target CYCB1;1 for degradation in trichomes.

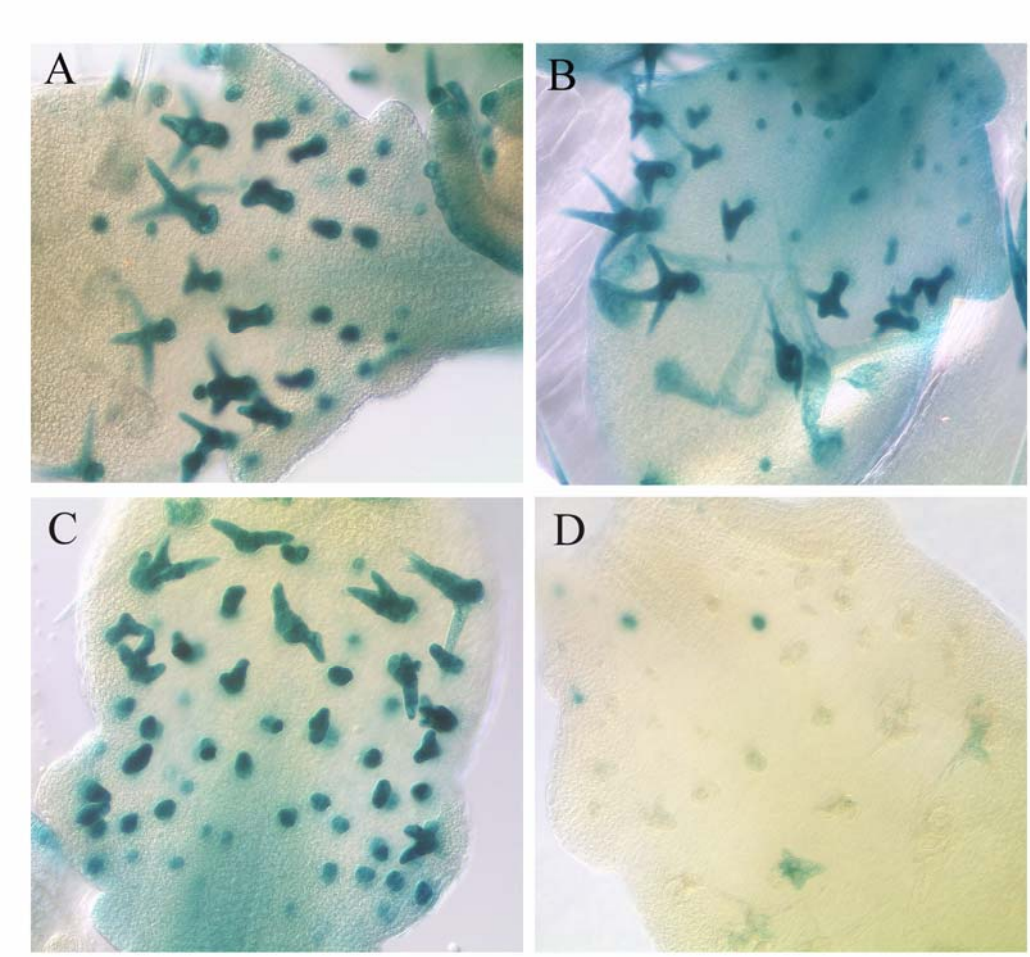


Fig.15-2. GUS analysis of CYCB1;1$^{1-112}$ in endoreplicating and dividing trichomes.
(A) and **(B)** Light micrograph of *ProGL2:GUS* and *ProGL2: CYCB1;1*$^{1-112}$:GUS in wild type. **(C)** and **(D)** Light micrograph of *ProGL2:GUS* and *ProGL2:CYCB1;1*$^{1-112}$: *GUS* in *ProGL2:CYCD3;1* misexpression line which has multicellular trichomes. Mitotic regulators could activate the destruction box pathway in dividing trichome cells and patchy pattern of GUS activity is produced.

Ectopic expression of the destruction box mutated version of CYCB1;1 did not produce any phenotype in trichome cells but mutations in the destruction box of CYCB1;2 produced a mild phenotype (table2-2). My data suggests that the destruction box is not important or is backuped by some other degradation signals.

ProGL2:CYCB1;1$^{1-112}$*:GUS* in *ProGL2:CYCD3;1* misexpression line and *siamese* mutant

To analyze the destruction box function for the degradation of B1-type cyclins in dividing cells, GUS and the GUS fusion with CYCB1;1$^{1-112}$ were misexpressed in

CYCD3;1 misexpression lines, and *sim* mutants which both display multicellular trichomes.

While similar activity of GUS without a destruction box was exhibited in single-celled wild type and mutant multicellular trichomes, a patchy GUS activity pattern was observed in multicellular trichomes expressing the CYCB1;1$^{1\text{-}112}$:GUS contains destruction box. This patchy pattern is typical for cell-cycle regulated genes and presumably only cells which are in the G2 or M-phase accumulate GUS (Fig15-2 C and D). This data proves that the CYCB1;1 destruction box pathway is not active in wild type single celled trichomes. Furthermore, it shows that this destruction box pathway is switched on in a mitotic cell cycle.

2.2.4. Novel degradation motifs in CYCB1;2

Loss of destruction box activity does not appear to be the reason why the expression of the truncated CYCB1;2 results in multicellular trichomes. To determine the significance of the CYCB1;2 domains, the CYCB1;2 amino acids were analyzed by searching pfam and PROSITE data bases. Besides the destruction box four further truncated cyclin B proteins were generated: the first truncation immediately before the highly conserved Cyclin box, CYCB1;2$^{\Delta 1\text{-}184}$, the second after the first half of Cyclin C, CYCB1;2$^{\Delta 1\text{-}310}$ the third truncation without the destruction box, CYCB1;2$^{\Delta 1\text{-}40}$ and finally a fourth truncation with the destruction box, CYCB1;2$^{\Delta 1\text{-}31}$ were created (Fig14-2 B-J).

The expression of *ProGL2:CYCB1;2*$^{\Delta 1\text{-}185}$ and *proGL2:CYCB1;2*$^{\Delta 1\text{-}311}$ truncations did not induce a multicellular phenotype indicating that the gene may be too small to remain functional (table 2-2).

The truncation CYCB1;2$^{\Delta 1\text{-}31}$ with DB Box truncations induced Cluster of trichomes in 9.4% of transgenic lines, the truncation of CYCB1;2 $^{\Delta 1\text{-}40}$ without the destruction box induced clustered trichomes in 10% of transgenic lines and one plant with few multicellular trichomes. CYCB1;2 destruction box mutation induced only multicellular trichome in 0.8% of transgenic lines.

Comparison of data for CYCB1;2$^{\Delta 1\text{-}135}$, which induced clusters and multicellular trichomes in 80% of transgenic lines, the truncation with destruction box CYCB1;2$^{\Delta 1\text{-}31}$, and the truncation of CYCB1;2 with out the destruction box, CYCB1;2$^{\Delta 1\text{-}40}$ shows that the presence of the 95 amino acids between the destruction box and the second

start codon after bp 405 are important for the regulation of CYCB1;2 function and the induction of multicelluarity in trichomes (Fig13 C and D). This region might harbor another element present in this part of the CYCB1;2 protein.

2.2.5. Stability of the CYCB1;2 full length, the destruction box mutation CYCB1;2 and the CYCB1;2$^{\Delta 1\text{-}135}$ in trichomes

Misexpression of CYCB1;2 or its destruction box mutation did not induce a phenotype in trichomes but the truncation CYCB1;2$^{\Delta 1\text{-}135}$ induced a multicellular phenotype. In order to monitor the localization and stability of CYCB1;2 versions, the Yellow Fluorescent Protein (YFP) was fused to the CYCB1;2 full length, the destruction box mutant CYCB1;2 and the truncated CYCB1;2$^{\Delta 1\text{-}135}$. Misexpression lines were generated using the *GL2* promoter. Plants expressing the fusion proteins were first analyzed with respect to their phenotypical strength in comparison with that of unfused CYCB1;2 versions. Phenotype analysis of transgenic lines showed that expression of the fusion protein containing the truncated *CYCB1;2*$^{\Delta 1\text{-}135}$ resulted in a phenotype of multicellular trichomes similar to the phenotype observed during misexpression of the unfused truncation CYCB1;2$^{\Delta 1\text{-}135}$.

Analysis of the transgenic lines by Confocal laser scanning microscopy did not reveal any YFP signal for plants misexpressing the full length and the destruction box mutated CYCB1;2 version. Nevertheless truncation CYCB1;2$^{\Delta 1\text{-}135}$fused to YFP showed a strong YFP signal in the nucleus and in the cytoplasm of trichomes (Fig16-2-A,B and C).

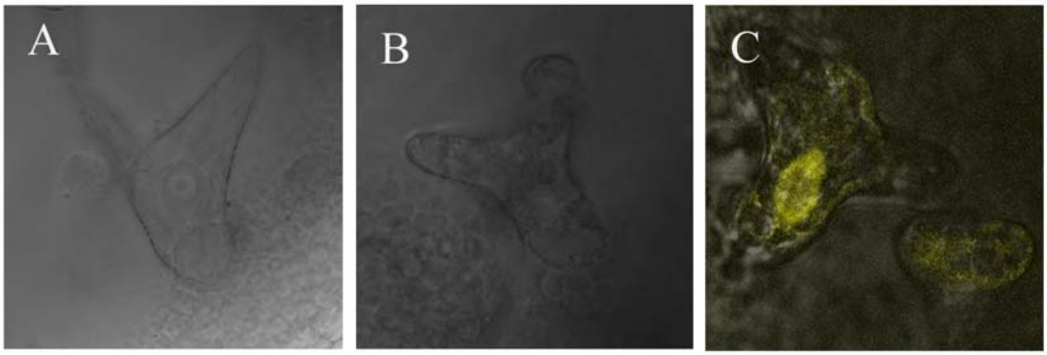


Fig. 16-2 Stability of CYCB1;2 protein in trichomes

Confocal laser scanning micrograph of a **(A)** *ProGL2:CYCB1;2* **(B)** *ProGL2:destruction box mutation CYCB1;2* **(C)** *ProGL2:CYCB1;2*$^{\Delta 1\text{-}135}$. Deletion of N-terminus could stabilize the CYCB1;2 protein.

To further analyze the involvement of the 95 aa region after the destruction box in the degradation of CYCB1;2, a CYCB1;2 N-terminal fragment, CYCB1;2$^{1\text{-}135}$ was fused to YFP and misexpressed under the control of the *GL2* promoter.

Thirty transgenic lines were analyzed, and no YFP signal was detected in trichomes. There are two possible explanations for the observation of the absence of a YFP signal of CYCB1;2$^{1\text{-}135}$: First, the construct is not functional. Secondly, the construct is functional and the fusion protein is degraded. To test these two possibilities, the constructs were brought into the background of dividing cells. In this case, *ProGL2:CYCB1;2*$^{1\text{-}135}$*:YFP* was crossed to *ProGL2:CYCD3;1* misexpression lines which have ectopically dividing trichome cells.

The F1 generation of these crosses was analyzed, the YFP signal was recovered in some cells as I had expected. The switch from endoreplication to cell division seems to activate the destruction box pathway in CYCB1;2$^{1\text{-}135}$.

So far, I have presented several lines of evidence that the destruction box mediated degradation is not active or it is not the main control pathway of B1-type cyclins degradation in trichomes. The destruction box of CYCB1;1 and CYCB1;2 are different in trichomes but they have similar functions in dividing trichome cells.

2.2.6. Analysis of B1-type cyclins in dividing cells

Misexpression of CYCB1;1, CYCB1;2 and CYCB1;3 in dividing epidermal cells

In order to analyze the function of B1-type cyclins in dividing cells and minimize interference with plant fertility and viability the *TMM* promoter was used (Weinl et al., 2005). *TMM* is expressed during early leaf development in cells of the stomatal lineage and some adjacent cells. Many of these cells will undergo at least one more cell division during leaf development. The stomata pathway begins with the selection and asymmetric division of the meristemoid mother cell. Asymmetric divisions in the stomatal pathway produce about half of all pavement cells and perhaps up to three-quarters of all epidermal cells in leaves (Nadeau and Sack, 2002a)

Analysis of transgenic lines showed misexpression of cyclins that caused deregulation of the cell division order. This resulted in leaves with fewer stomata, cells with a different shape and bigger size as well as some partial cell walls which represents cytokinesis defects. Furthermore induced clusters of stomata were observed (Fig. 17-2 A and B). Stomata are normally separated by at least one intervening cell (Nadeau and Sack, 2002b). Increased epidermal cell size and a decrease in the number of stomata are signs of cell division arrest.

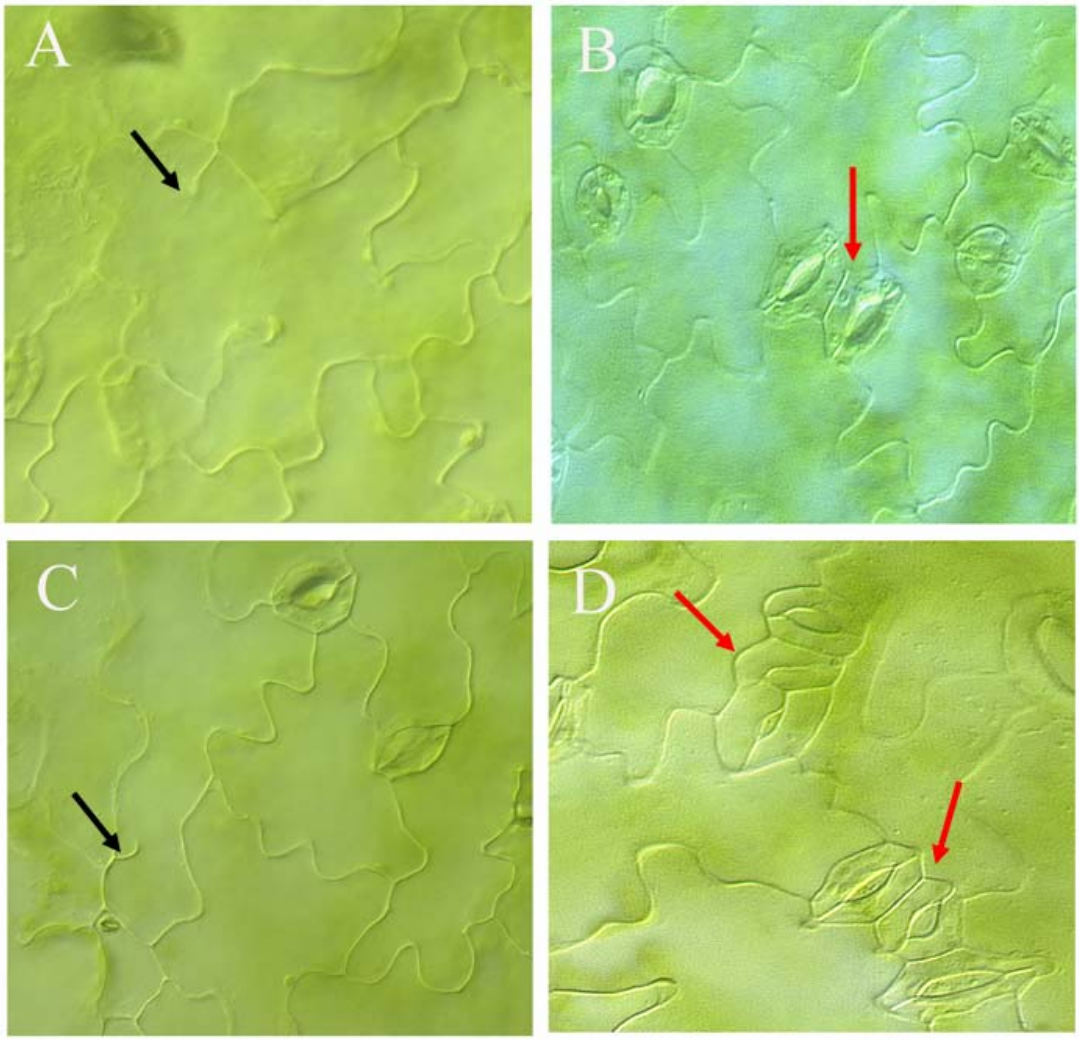


Fig.17-2. Light micrograph of CYCB1;2 and CYCB1;2 destruction box mutation in stomata lineage.
(A) and **(B)** Cytokinesis defects and cluster of stomata in *ProTMM:CYCB1;2* misexpression lines
(C) and **(D)** phenotype of *ProTMM:CYCB1;2 destruction box mutation* which induced Cytokinesis defect and more clusters of stomata.
Cytokinesis defects are shown with black arrows and Stomata clusters with red arrows.

Misexpression of the CYCB1;1 and CYCB1;2 destruction Box mutation in stomata lineage

To define functionality of the CYCB1;1 and the CYCB1;2 destruction box mutations they were transformed under control of the *TMM* promoter.

Analysis of transgenic lines revealed enlargement of epidermal cell size, cytokinesis defects, fewer stomata, and clusters of stomata. It was shown that the destruction box mutated CYCB1;1 or CYCB1;2 induced a stronger phenotype in comparison to CYCB1;1 or CYCB1;2 full length and induced cytokinesis defects, prevented cell wall formation, increased endoreplication in epidermal cells, and decreased the

number of cell divisions of the stomata lineage (Fig17-2 C and D). A detailed morphological analysis at the cellular level revealed that the number of stomata was drastically reduced in the strong *ProTMM:CYCB1;1* or *CYCB1;2* destruction box mutation lines, suggesting that cell division was blocked at early stages and cells did not develop into normal guard cells.

Misexpression of the CYCB1;2$^{\Delta 1\text{-}135}$ in cells of the stomata lineage as a model for dividing cell

Misexpression of CYCB1;2$^{\Delta 1\text{-}135}$ in trichomes induced cell division but over-expression of CYCB1;2 and the CYCB1;2 destruction box mutation in dividing cells induced different levels of cell sizes and clusters of stomata cells. Expression of CYCB1;2$^{\Delta 1\text{-}135}$ induced enlarged epidermal cells and induced clusters of stomata. This phenotype is similar to the phenotype induced by misexpression of CYCB1;2 with a destruction box mutation

Localization of *CYCB1;2* variants in dividing cells

To determine the localization of *CYCB1;2* and its variants in dividing cells, they were fused to YFP and expressed under the control of the *TMM* promoter.

ProTMM:CYCB1;2 and *ProTMM:CYCB1;2* with a destruction box mutation fused to YFP were found primarily in the nucleus but were also detected in the cytoplasm. CYCB1;2 destruction box mutation fused to YFP is stabilized due to a mutation in the destruction box and induced a YFP signal in more cells (Fig 18-2 A and B).

In *ProTMM:CYCB1;2$^{\Delta 1\text{-}135}$ YFP* misexpression lines in the stomata lineage showed that YFP fluorescent signal was detected only in the cytoplasm (Fig 18-2 C). In Xenopus, it was shown that the nuclear localization signal of CYCB is located in the N-terminus (Yoshitome et al., 2003) . In animal cells it was proven that nuclear localization of cyclin stimulates mitosis (Furuno et al., 1999) Hence stabilization of the truncated version of the CYCB1;2 is due to the deletion of the destruction box and localization in the cytoplasm. These are also reasons for the endoreplication and cytokinesis defects observed in these misexpression lines.

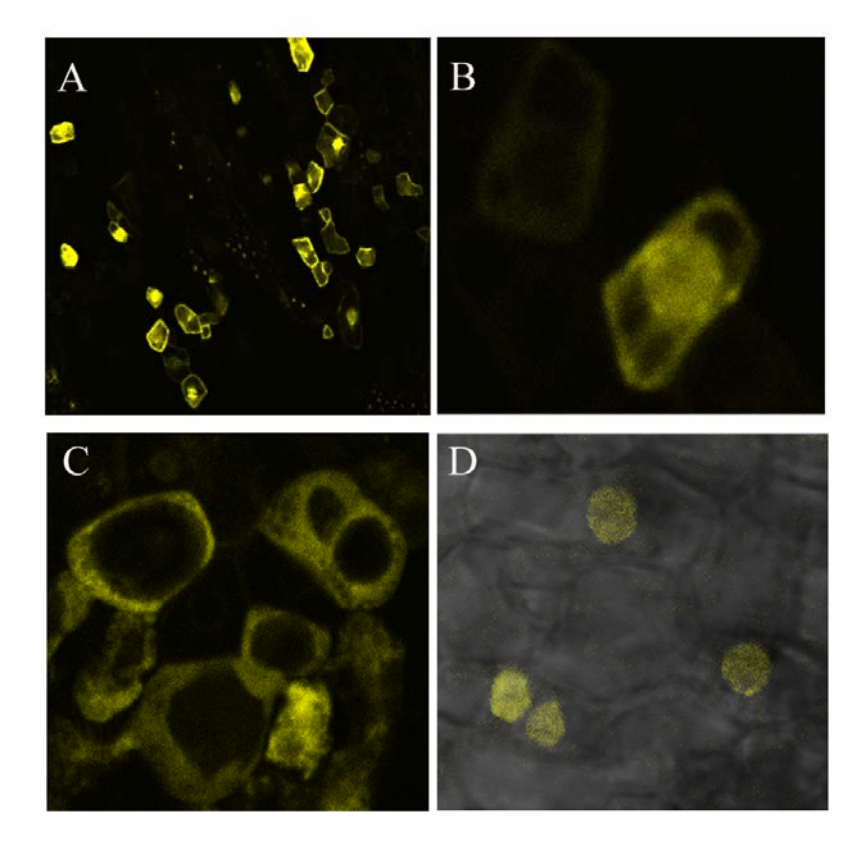


Fig. 18-2 Localization of CYCB1;2 and its variants in dividing cells
Confocal-laser-scanning micrographs a **(A)** *ProTMM:CYCB1;2* **(B)** *proTMM:CYCB1;2 destruction box mutation* **(C)** *ProTMM:CYCB1;2*$^{\Delta 1\text{-}135}$ and (D) ProTMM:CYCB1;2$^{1\text{-}135}$

Misexpression of *ProTMM:CYCB1;2*$^{1\text{-}135}$*:YFP* in dividing cells

An alignment of B-type cyclins of *Arabidopsis thaliana* and other plant species e.g. *Pisum sativum, Populus alba, Dictyostelium discoidium, Pneumocystis carinii* and *Cricetulus longcadatus* showed the nuclear export signal (NES) motif (DIDxxD) but no obvious nuclear localization signal (NLS) was found in plant B-type cyclins. To determine the NLS of *CYCB1;2,* sequences of *CYCB1;2*$^{1\text{-}135}$ were analyzed using PSORTII software. A 17 amino acid motif KKEVTKKEVAMSPKNKK was predicted as the NLS which is located 24 amino acids before NES.

CYCB1;2$^{1\text{-}135}$ containing the predicted NLS was fused to YFP and expressed in dividing cells under the control of TMM promoter. This resulted in a YFP signal specifically localized in the nucleus (Fig. 18-2 D).

Search for a novel degradation motif in CYCB1;2

Misexpression of CYCB1;2 and its variants in trichomes showed that a domain of 95 amino acids after the destruction box of CYCB1;2 plays a role in the destabilization of cyclin in trichomes.

First, the ninety five amino acids after the destruction box were analyzed in different data bases but no known motif was identified in this region. Subsequently CYCB1;2 was compared with B-type cyclins of other plant species available in NCBI. Using a ClustalW alignment algorithm a highly conserved region spanning about eighteen amino acids in some plant species was identified (Fig19-2 A).

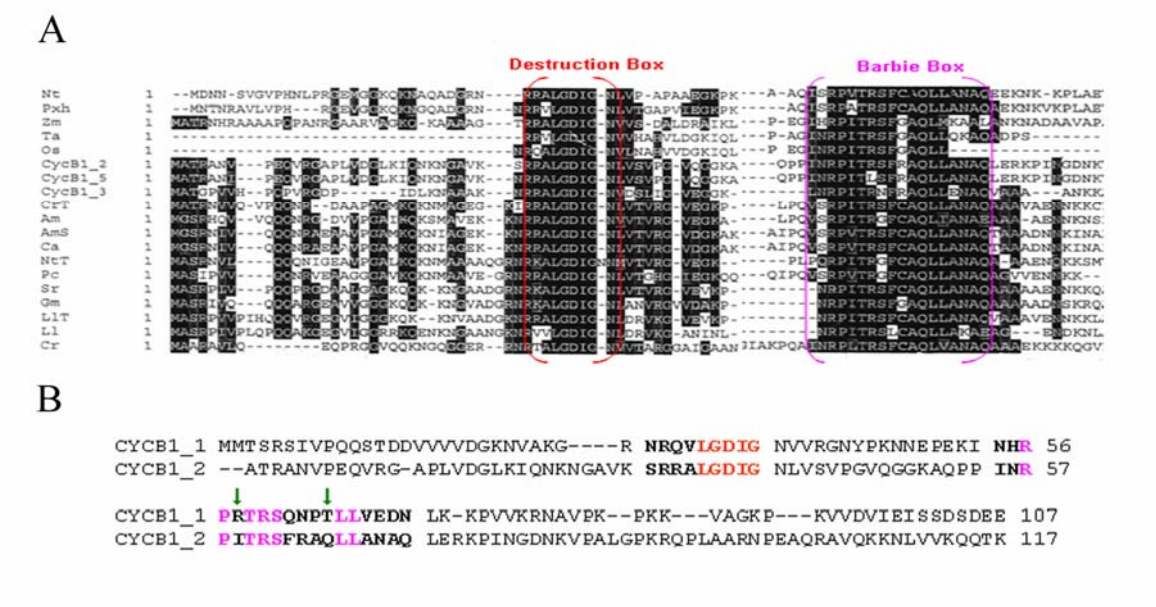


Fig: 19-2:
(A) Alignment of CYCB1; 2 N-terminal sequences with cyclins of other plant species. Two conserved motifs were found, Destruction box and Barbie box.
(B) Alignment of CYCB1; 1 and CYCB1; 2 N-termini. The two bold sequences correspond to the Destruction box and Barbie box, respectively. Red and purple letters indicate homologous sequences.

So far, two degradation motifs have been discovered in the protein which is active in M phase, the destruction box (Glotzer et al., 1991) and the KEN box (Pfleger and Kirschner, 2000). Some proteins e.g. Cdc20, contain a KEN box. B-type cyclins contain the destruction box but do not contain the KEN box. An 18 amino acid motif which seemed to be a degradation motif in CYCB1;2 was considered as an analog of the KEN box. In toy dolls the analog of Ken is called Barbie. Therefore the 18 amino acid motif of CYCB1;2 which is conserved in some B-type cyclins of plant species was called the Barbie box.

Barbie Box is a novel degradation box in plant cyclins

Several strategies were designed to better understand the function of the Barbie box:

Misexpression of CYCB1;2$^{1-135}$ containing mutations in the Barbie Box in trichomes

I60R or I60D exchange in CYCB1;2$^{1-135}$ Barbie box

The alignment of the Barbie box of *CYCB1; 2* with analogous amino acids of *CYCB1;1* revealed several amino acid exchanges (Fig. 19-2 B). While a *CYCB1;1$^{1-112}$* is stable in trichomes, no YFP signal has been detected in the *ProGL2: CYCB1;2$^{1-135}$YFP* transgenic lines. It was considered that the exchanged amino acids of Isoleucine 60 and Glutamine 67 in Barbie box of CYCB1;2 may play a role in destabilization of *CYCB1;2$^{1-135}$*. To test this, the amino acid Isoleucine 60 and Glutamine 67 of CYCB1;2 was changed to Arginine and Threonine or Aspartate, respectively. The constructs were misexpressed under the control of the *ProGL2* and the transgenic lines were analyzed. Analysis of transgenic plants revealed that both the I60R and I60D exchanges were able to partly restore the YFP fluorescent signal of the *CYCB1;2$^{1-135}$* YFP in young trichomes and showed similar pattern expression (Fig 20-2 A).

Q67T or Q67D exchange in Barbie box of CYCB1;2$^{1-135}$

To examine the function of another candidate; Glutamine 67 on stabilization of the N-terminus of CYCB1;2 it was changed to Threonine or Aspartate, respectively. Both exchanges were able to restore the YFP signal of CYCB1;2$^{1-135}$:YFP. The YFP signal of CYCB1;2$^{1-135,Q67T}$:YFP was comparable with the signals expressed by CYCB1;2$^{1-135,I60R}$YFP and the CYCB1;2$^{1-135,I60D}$YFP (Fig 20-2 B). CYCB1;2$^{1-135,Q67D}$ also exhibited YFP signal expression but its expression was weaker than that expressed by the other *CYCB1;2$^{1-135}$* YFP constructs with exchanged amino acids. Isoluecine 60 and Glutamine 67 are important amino acids for the destabilization of *CYCB1;2,* and exchange of Isolucine 60 and Glutamine 67 stabilize the N-terminus of the CYCB1;2 protein.

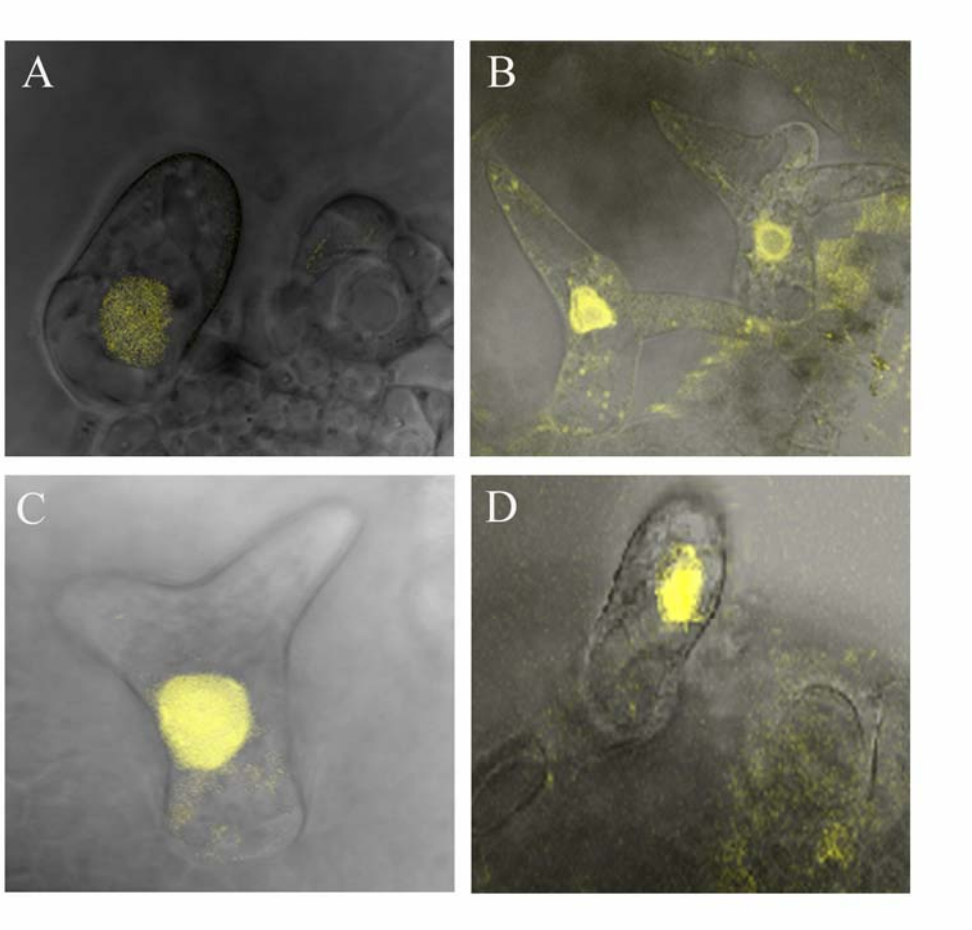


Fig. 20-2. Mutation in I 60 and Q67 able to restore YFP signal of CYCB1;2$^{1-135}$:YFP
Confocal laser scanning micrograph of **(A)** *ProGL2:CYCB1;2$^{1-135,I60R}$* **(B)** *ProGL2:CYCB1;2$^{1-135,Q67T}$* **(C)** *ProGL2:CYCB1;1$^{1-135,I60R,Q67}$* and **(D)** *ProGL2:CYCB1;2$^{1-135}$Barbie box analogous region of CYCB1;1*

Expression of CYCB1;2$^{1-135}$YFP with I60R and Q67T exchanges in trichomes

Because the single mutation of I60R or Q67T showed stabilization of the N-terminus of the CYCB1;2, a I60R and Q67T mutant was created to study the function of double mutations. Confocal laser scanning microscopy data showed that double mutations stabilized the N-terminus of the CYCB1;2 protein more than a single mutation of I60R or Q67T (Fig. 20-2 C). This experiment revealed that two amino acids have additive effect on stabilization of the N-terminus of CYCB1;2.

Expression of the CYCB1;2$^{1-135}$:YFP with the Barbie Box analogous region of CYCB1;1 in trichomes

Isolucine 60 and Glutamine 67 are key amino acids for the destabilization of CYCB1;2$^{1-135}$. A region in CYCB1;1 was identified as an analog to the Barbie Box in CYCB1;2. To find out the role of the analogous region in stabilization of the

CYCB1;2$^{1-135}$, the analogous region of *CYCB1;1* was expressed in CYCB1;2 in place of the Barbie Box. Confocal laser scanning microscopy analysis of transgenic lines revealed that the majority of transgenic lines showed a YFP signal similar to the I60R or Q67T YFP signal (Fig. 20- 2 D).

Ectopic expression of CYCB1;2^{I60R}, CYCB1;2^{Q67T} and CYCB1;2$^{I60R, Q67T}$ in trichomes

To assess the function of I60R, Q67T and I60R-Q67T exchanges in stability of CYCB1;2 full length, the CYCB1;2 with single and double mutations in position I60 and/or Q67 were created (schematic).

Analysis of the transgenic lines revealed that none of the mutations were able to induce clusters or multicellular trichomes. It seems that induction of clusters or multicellular trichomes requires higher amounts of cyclin that can not be recovered by the single or double mutations. This suggests that the destruction box mediates cyclin degradation or that these mutations interfere with the functionality of the cyclin.

Misexpression of CYCB1;2 without Barbie box in trichomes and stomata lineage cells

To find out the role of the *CYCB1;2* Barbie box*, CYCB1;2* cDNA without the Barbie box, the CYCB1;2$^{\Delta 57-75}$ was created and transformed under the control of the *GL2* and the *TMM* promoters.

The CYCB1;2$^{\Delta 57-75}$did not induce any clusters or a multicellular phenotype in trichomes. And scanning electron microscopy revealed that the misexpression of CYCB1;2$^{\Delta 57-75}$ in the stomata lineage did not induce any phenotype in stomata linage (Fig. 21-2 A and B).

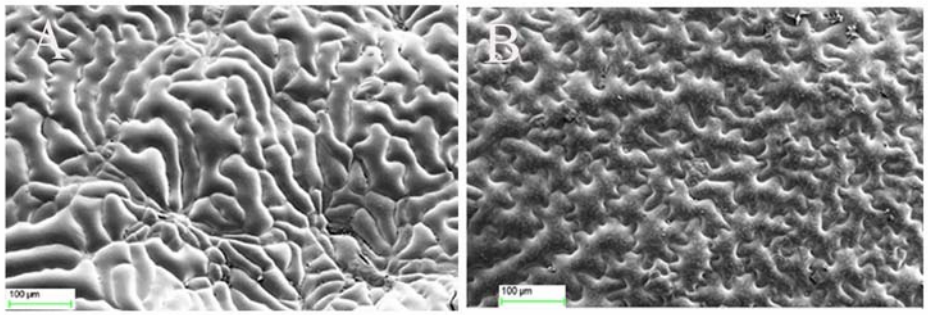


Fig 21-2. Morphological analyses of *ProTMM:CYCB1;2* and *ProTMM:CYCB1;2*$^{\Delta 57\text{-}74}$ Plants.
Scanning electron micrograph of a **(A)** *ProTMM:CYCB1;2* and **(B)** *ProTMM:CYCB1;2*$^{\Delta 57\text{-}74}$ misexpression lines

Misexpression of CYCB1;2$^{\Delta 57\text{-}75}$ in the *siamese* mutant

Triple fusion of *CYCB1;1* and *CYCB1;2* did not change the sim phenotype whereas *CYCB1;1-CYCB1;2* and *CYCB1;2-CYCB1;1* double fusions increased the *sim* phenotype faintly. However, *CYCB1;2* without the Barbie box gave rise to slightly stronger multicellular trichome phenotype than CYCB1;1 and CYCB1;2 fusions. Nevertheless misexpression of CYCB1;2$^{\Delta 57\text{-}75}$ induced cell death in multicellular trichomes of the *sim* mutant plants (Fig. 22-2 A, B, D and E).

Analysis of the misexpression lines of the *proGL2:CYCB1;2*$^{\Delta 1\text{-}135}$ in the *sim* mutant showed that CYCB1;2$^{\Delta 1\text{-}135}$ increased the multicellular phenotype of the sim mutant more than CYCB1;2$^{\Delta 57\text{-}75}$ misexpression, and at the end induced cell death in multicellular trichomes of the *sim* mutant lines (Fig. 22-2 C and F).

It was shown that that overexpression of cyclin E enhances cytokine mediated apoptosis in breast cancer cells (Dhillon and Mudryj, 2003) Or ectopic expression of cyclin B3 in mouse testa induces abnormal round spermatids and increased apoptosis in the testa (Refik-Rogers et al., 2006) All these data show that deregulation of cyclins induce more cell divisions and finally led to cell death in mitotic cells. Taken together, these data suggest that the CYCB1;2$^{\Delta 1\text{-}135}$ or CYCB1;2$^{\Delta 57\text{-}75}$ stabilizes CYCB1;2 protein and deregulate the cell cycle in *sim* mutants. Even though, the CYCB1;2$^{\Delta 1\text{-}135}$ induced more cell divisions than CYCB1;2$^{\Delta 57\text{-}75,}$ both induced cell death in the *sim* mutant.

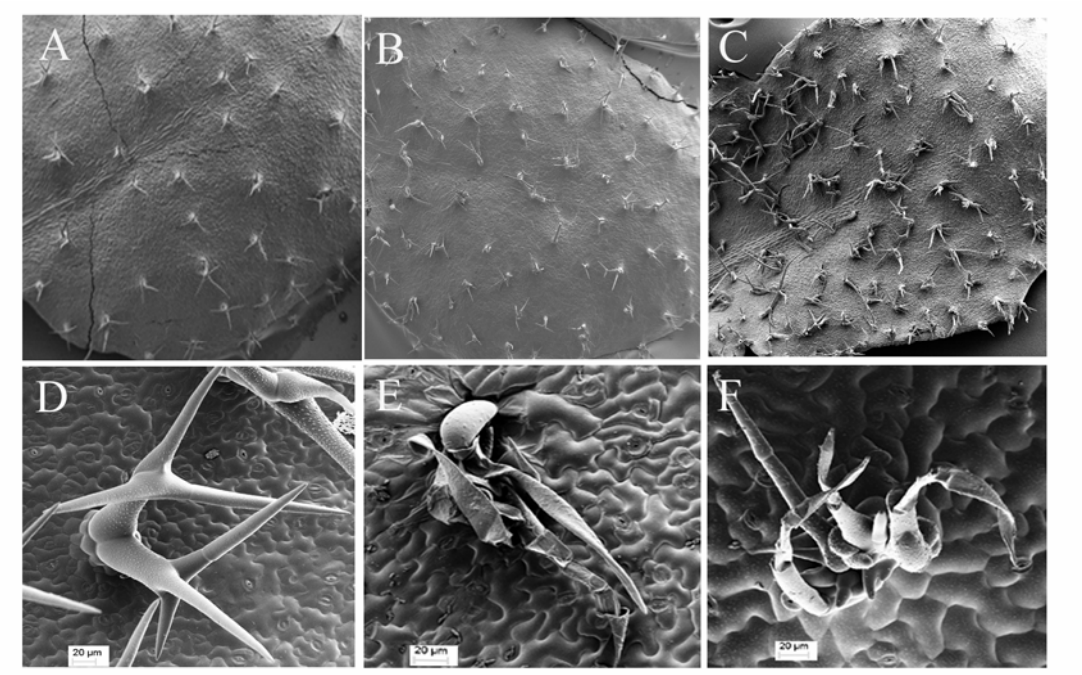


Fig. 22-2. Scanning electron micrograph:

Overview of **(A)** sim mutant trichomes **(B)** and **(C)** Enhanced multicellular trichomes in sim mutant by misexpression of CYCB1;2$^{\Delta 57-75}$ and CYCB1;2$^{\Delta 1-135}$ respectively.
(D) A mature multicellular trichomes of sim mutant **(E)** and **(F)** Cell death induced in enhanced multicellular trichomes of sim mutant of **(B)** and **(C)**

Rescue of *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ with *ProCYCB1;2:CYCB1;2*$^{\Delta 57-75}$

The double homozygous mutant of the *CYCB1;1* and the*CYCB1;2* proved to be lethal. To investigate the function of the CYCB1;2 without Barbie box, it was transformed in *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ plants under the control of the *CYCB1;2* endogenous promoter. Genotyping of the T2 generation of the transgenic lines revealed *ProCYCB1;2:CYCB1;2*$^{\Delta 57-75}$ could not rescue *cycb1;1*$^{-/-}$ *cycb1;2*$^{-/-}$ mutant showed presence of Barbie box is required for functionality of CYCB1;2 in gametogenesis.

CYCB1;2$^{\Delta 57-75}$ and CYCB1;2$^{\Delta 1-135}$ induced multicellular trichomes in *ccs52a1* mutant

The best documented molecular mitosis-to-endocycle switch is the CCS52A protein, a plant ortholog of the yeast and animal Cdh1/Fzr in Drosophila proteins that acts as a substrate-specific activator of the APC/C. Mitotic cyclins are likely candidates for substrates of CCS52A-mediated proteolysis (Kondorosi and Kondorosi, 2004) In green house growth conditions the knock out lines of ccs52a1 produced small and two

branched trichomes but other tissues were not affected by the ccs52a1 mutant, suggesting that the presence of CCS52A1 is necessary for endoreplication of trichomes. To investigate the function of CYCB1;2 and its variants in the ccs52a1 mutant background, the CYCB1;2 full length, CYCB1;2 mutated destruction box, CYCB1;2$^{\Delta 57\text{-}75}$ and CYCB1;2$^{\Delta 1\text{-}135}$ were transformed in the ccs52a1 mutant under the control of the *GL2* promoter. The *ProGL2:CYCB1;2* or *CYCB1;2 mutated destruction box* did not induce a phenotype in the *ccs52a1* mutant lines. In contrast, *proGL2: CYCB1;2*$^{\Delta 57\text{-}75}$ and *CYCB1;2*$^{\Delta 1\text{-}135}$ induced clusters and multicellular trichomes.

This data suggest that the CYCB1;2 full length or Destruction box mutation misexpression cannot induce any phenotypes. It may be possible that a different degradation pathway removed the full length or the mutated CYCB1;2 destruction box from trichomes and no phenotype was induced.

Furthermore, the phenotypes obtained by expressing the *CYCB1;2 without Barbie box, CYCB1;2*$^{\Delta 57\text{-}75}$ in *ccs52a1* mutant plants suggested that the block of the destruction box mediated pathway using mutation of ccs52a1 and deletion of Barbie box removed two different degradation pathways were able to stabilize CYCB1;2 and induce multicellular trichome. Presence of either the destruction box or the Barbie box is sufficient to remove CYCB1;2 from trichomes.

Misexpression of CYCB1;2$^{\Delta 1\text{-}135}$ which does not have the N-terminus of CYCB1;2 containing the destruction box and the Barbie box induced similar phenotype in wild type and *ccs52a1* mutants

APC/C dependent degradation in trichomes

The Destruction Box appeared not to be involved in CYCB1;1 degradation in trichomes and it has a redundant role with the Barbie box for the removal of CYCB1;2 in trichomes. This raises the question of what role APC/C dependent degradation plays during trichome development. To assess the functionality of the APC/C dependent degradation of B1-type cyclins, an artificial RNAi was designed against the *ANAPHASE PROMOTING COMPLEX11 (APC11)* subunit of the APC/C. The APC11 is a RING-H2 finger protein and functions as the catalytic core of the APC/C complex by mediating the transfer of ubiquitin from an ubiquitin-conjugating enzyme (E2) to the substrate (Chang et al., 2004).

The APC11 is structurally related to the RBX1 component of another E3 ligase class, called the Skp1, CDC53/Cullin, F-box (SCF) complex. (Gmachl et al., 2000 Capron, 2003 #947) (Fig. 23-2).

To analyze the effect of APC11 loss of function in trichomes, an attempt was made to silence APC11 activity by expressing RNAi constructs against it.

```
RBx1-1 AT3G42830  MASLNSDVIM---GESSSISVPSSSSKNSKRFELKKWSAVALWAWDIVVDNCAICRNHIM 57
RBX1-1 AT5G20570  MATLDSDVTMIPAGEASSSVAASSSNKKAKRFEIKKWSAVALWAWDIVVDNCAICRNHIM 60
APC11  AT3G05870  ------------------------------MKVKILRWHAVASWTWDAQDETCGICRMAFD 31

RBx1-1 AT3G42830  DLCIECLANQASATSEECTVAWGVCNHAFHFHCISRWLKTR---QVCPLDVCEWEFQKYGH 115
RBX1-2 AT5G20570  DLCIECQANQASATSEECTVAWGVCNHAFHFHCISRWLKTR---QVCPLDNSEWEFQKYGH 118
APC11  AT3G05870  GCCPDCKL-----PGDDCPLIWGACNHAFHLHCILKWVNSQTSQAHCPMCRREWQFKE--- 84
```

Fig. 23-2. Alignment of APC11 (AT3G05870) and RBX1 (AT3G42830 and AT5G20570).
APC11 showed 37.3% similarity and 26.3% identity with RBX1 (AT3G42830) and 36.4% similarity and 26.4% identity with RBX1-2 (AT5G20570) . Alignment was done using ClustalW software.

Misexpression of *APC11* RNAi did not induce any phenotype in trichomes

APC11 RNAi was designed against the whole *APC11* ORF and expressed under the control of the *GLABRA2* promoter in trichomes. No deviation of trichome development was observed in *APC11 RNAi* transgenic lines.

YFP:APC11 over expression in *Arabidopsis thaliana*

The absence of phenotypic alterations in the *APC11 RNAi* lines can be explained by two reasons: either the RNAi construct was not functional or APC11 is not important

during trichome development. To discriminate between these two possibilities, *APC11* was fused to *YFP* and under the control of the constitutive *CaMV 35S* promoter transformed in wild type *Columbia* plants. A YFP signal of epidermal cells of the obtained transgenic lines was checked using Confocal laser microscopy (Fig 24-2 A).

When transgenic plants had six leaves, the YFP signal could be detected in leaves one and two. In leaves three and four, the YFP signal was detected at the tip of the leaves but no signal could be detected in younger epidermal cells at the base of the leaf adjacent to the petiole. In leaves five and six no fluorescence signal was observed (Fig. 24-2 B). This expression pattern was a common phenomenon in all transgenic lines. To determine whether the age dependent YFP fluorescence was caused by the 35S promoter, by GFP or by the APC11 protein, control plants were analyzed in which GFP is expressed under the *35S promotor*. In these control plants, a GFP signal was detected in all young and mature epidermis cells including trichomes. Moreover, the GFP signal was detectable in all leaves of these plants (Fig. 24-2 C). This shows that age dependent expression of *Pro35S:YFP:APC11* is dependent on the expression of the YFP:APC11 fusion protein and not the 35S promotor or YFP.

Expression of *APC11 RNAi* in *YFP:APC11 over expression line*

The YFP signal of *Pro35S:YFP:APC11* was detected in all epidermal cells of mature leaves, including trichomes. When it was crossed to wild type *Columbia* plants, the same expression pattern was observed in the F1 plants. In contrast, after crossing the YFP:APC11 misexpression plants with the previously generated *APC11 RNAi* lines, the YFP signal was detected only in epidermal pavement cells and stomata cells but not in trichomes (Fig. 24-2 D). This data shows that *APC11 RNAi* is active and can cause a degradation of *YFP:APC11* transcript in trichomes. Since the APC11 RNAi lines showed no phenotypic alteration of mature trichomes, it is plausible that APC11 is not required at this stage in trichomes.

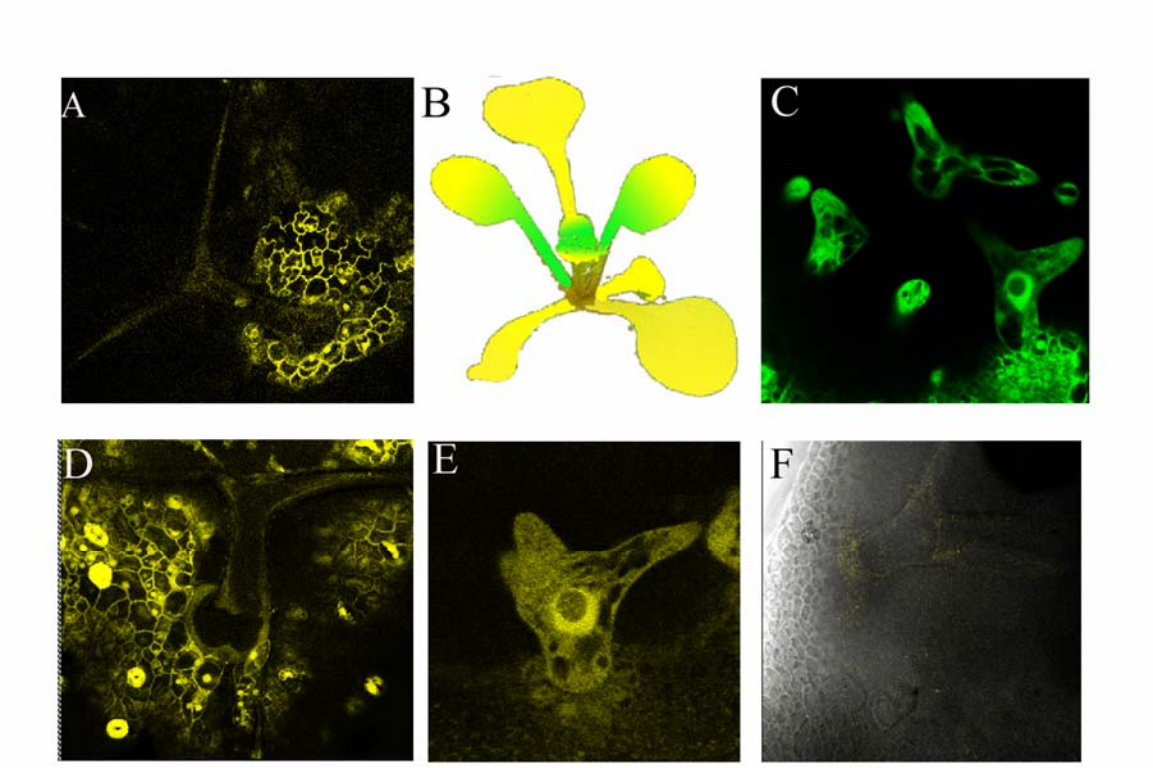


Fig. 24-2 Analysis of the APC11 RNAi function in trichomes.
(A), **(C)**, **(D)**, **(E)**and **(F)** Confocal laser scanning micrographs of (A) expression of *Pro35S:YFP:APC11* in mature trichome and epidermal cells**. (B)** Schematic of age dependent expression pattern of *Pro35S:YFP:APC11* in wild type Col plants.YFPAPC11 was detected in old leaves but it was observed in the apex of younger leaves. Newly developed leaves did not show any YFP signal.
(C) Expression of GFP under the control of the 35S promoter. GFP signal was detected in all leaves showing that age dependent expression of Pro35S:YFP:APC11 is dependent on APC11 protein not promoter or YFP.
(D) *proGL2:APC11 RNAi* in *Pro35S:YFP:APC11* plants silenced the YFPAPC11 in trichomes. Other cells showed the YFPAPC11 signal.
(E) YFP expression pattern of *ProGL2:YFPAPC11* in a young trichome.
(F) APC11 RNAi in *ProGL2:YFPAPC11* silenced the YFPAPC11.

Expression of *APC11 RNAi* in *ProGL2:YFP:APC11* line

To determine activity of *APC11 RNAi* in young trichomes, the YFP:APC11 fusion protein was expressed under the control of the *GLABRA2* promoter. Analysis of transgenic lines revealed that the YFP:APC11 fusion protein accumulates in both, young and mature trichomes (Fig. 24-2 E). Next, *ProGL2:YFP:APC11* was crossed to wild type *Columbia* and *ProGL2:APC11RNAi.*

In control crosses, a YFP signal was seen on trichomes of *ProGL2:YFP:APC11* crossed to wild type *Columbia*. However, no YFP fluorescent signal was detected in

trichomes in the progeny of the *ProGL2:APC11 RNAi* crossed to the *ProGL2:YFP:APC11* (Fig. 24-2 E and F).

Hence *APC11RNAi* is active not only in mature trichomes but also in young trichomes and can remove *YFP:APC11* RNA from the trichomes. Consequently, APC11 and likely the APC/C are not necessary for protein degradation during trichome development.

ProGL2:APC11 RNAi in siamese mutant

Silencing of *APC11* using RNAi has revealed that APC11 is not essential for trichome development. To analyze the function of APC11 in mitotic cells, *APC11 RNAi*, was transformed in the *sim* mutant which displays multicellular trichomes (Walker et al., 2000). (Fig. 25-2 B)

The analysis of transgenic lines showed that *APC11 RNAi* could reduce the number of cells in sim trichomes almost restoring a wild-type phenotype (Fig A, B and C).

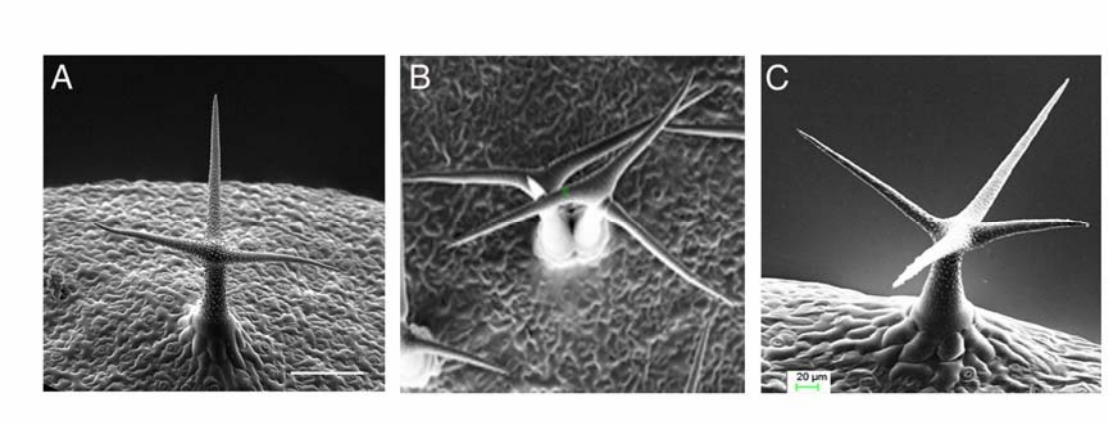


Fig. 25-2 Analysis of trichomes of wild type, sim mutant and *ProGL2:APC11 RNAi* in sim mutant.
(A) Scanning electron micrographs showing a wild type trichome. taken from Schnittger et al. 2002. **(B)** Trichome of sim mutant. **(C)** Scanning electron micrographs showing the proGL2:APC11 RNAi blocked multicellular trichomes induction of sim mutant. Presence of APC/C complex is required for cell division of sim mutant

Presence of Cdh1/Fizzy related, activator of APC/C in trichomes

APC11 appears not to be essential during trichome development. If the APC/C is not active in trichomes, its activators should also not be required for trichome development.

Cdh1/Fzr activates the APC/C complex in endoreplicating cells of *M. truncatula* (Cebolla et al., 1999). Cdh1 which is called CCS52 in plants is highly conserved in plant species. The *Arabidopsis* genome contains three *CCS52* genes: *ATCCS52A1*, *ATCCS52A2* and *ATCCS52B* (Capron et al., 2003a ; Fulop et al., 2005).

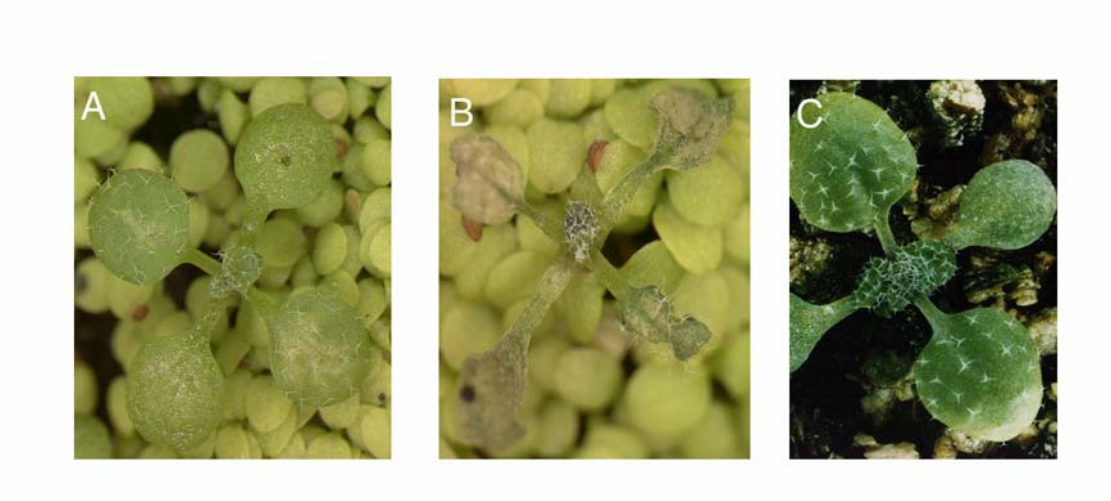


Fig. 26-2 Ethanol inducible *CCS52B RNAi* in *Arabidopsis thaliana*
Induction of *CCS52B RNAi* under the control of the ethanol inducible promoter
Light micrograph of transgenic line **(A)** and **(B)** and a wild type control **(C)**.
Transgenic lines were exposed to the ethanol for 18 hours. (A) 24 hours after exposure necroses were observed **(B)** 48 hours after exposure the transgenic plants died. **(C)** Wild type Col as control.

To study the function of the CCS52 in trichomes, an RNAi construct was made against *CCS52B* (table 4-2).

Table 4-2: RNAi CONSTRUCTS TO KNOCK OUT *CCS52B*

Name of RNAi	Primer sequences (5´-3´)	Length of RNAi
FZR11	Forward primer: AGGTCTTGGATGCTCCTTCTTTACAAG Reverse primer: GAACATTCTGTGAACTCCAGTCCAC	72
FZR22	Froward primer: ACCAAATCATGCTCTGGAAGTACCC Reverse primer: CCCGTAAGTGTTGCAACCTTTGAC	55
FZR33	Froward prime: TCACCTGATGGCCAGACTATAGTGAC Reverse primer: GACGTTCCAAAACCGCAGGG	63

No phenotype was revealed in trichomes of the T1 generation of *ProGL2:CCS52B RNAi*. To test functionality of *CCS52B RNAi* it was transformed under the control of an ethanol inducible promoter. The T1 plants were exposed to ethanol for 18 hours. Twenty four hours after exposure, necroses were observed on leaves of transgenic lines (Fig. 26-2 A) and after 48 hours the plants died (Fig. 26-2 B).
While the knockout plants of ccs52A were smaller than wild type plants no phenotypic alteration was found in trichomes (John Larkin personal communication).

In contrast, the knockout line of CCS52A1 reduced the size and number of branches of trichomes while the rest of the plant appears to be wild-type like. While APC11 and APC10, two main subunits of APC/C are not active in trichomes, it seems that CCS52A1 function is independent from the APC/C complex or there is a difference between entry into endocycle and control of its progression.

3. Discussion

3.1 B1-type cyclins in *Arabidopsis thaliana*

Plants contain a large number of cyclin genes but their functional distinction remain largely unclear. A Typical example aris the 11 B-type cyclins found in Arabidopsis. They have been categorized into three subclasses B1, B2, and B3 based on their primary sequence (Vandepoele et al., 2002). A gene expression analysis with microarrays has shown that the transcript levels of B1-type cyclin genes have a distinct peak at early mitosis in synchronized *Arabidopsis* cell cultures (Menges et al., 2005). To analyze the function of B1-type cyclins in *Arabidopsis thaliana*, their mutants were identified from T-DNA and transposon line collections.

In the second part, I showed that the downregulation of B1-type cyclins is important for the execution of an endocycle in plants. B1-type cyclins are expressed in emerging trichome cells and cyclin activity can drive the cell into M phase. Importantly, a new degradation motif, designated the Barbie box, was found in a subgroup of B1-type cyclins.

In third part it was shown that higher levels of APC/C activity are required for mitosis than for endoreplication. Furthermore the study on APC11 showed cell type specific involvement of ubiquitin-protein ligases.

3.2. A regulatory role of B1-type cyclins in *Arabidopsis thaliana*

In my study, CYCB1;1, CYCB1;2 and CYCB1;4 promoters fused with GUS were found to be active not only in dividing cells as shoot meristems and vascular systems of dividing regions as expected but also in endoreplicating cells as young trichome. This type of redundancy often becomes a major hindrance in genetic analysis. Nevertheless, the redundancy allowed analyzing the loss of function effect of B1-type cyclins, which would have led to lethality if the genes were unique and indispensable.

Results based on number of rosette leaves and size of rosette leaves indicate that B1-type cyclins are important not only for cell cycle regulation but also play an indirect role in the development of rosette leaf number.

Underneath each rosette leaf a meristem forms. The number of out growing side shoots was not affected by mutation of CYCB1;1 or CYCB1;4 whereas mutation of

CYCB1;2 increased number of out growing shoots. Results of meristem growth indicates that CYCB1;2 has negative regulatory affects on side shoot growth.

The out growing of axillary buds into side shoots is influenced by hormonal signals (Tantikanjana et al., 2001) In many plant species, the development of lateral shoots is inhibited by signals that are derived from the main shoot tip, a phenomenon named apical dominance or correlative inhibition (Horvath et al., 2003). In many plant species, the inhibitory effect of the main shoot can be relieved by decapitation, but apical application of Auxin can restore apical dominance. Therefore it is possible that auxin controls out growing axillary buds via CYCB1;2. If that would be the case spraying the auxin on the cycb1;2 mutant could reduce the number of out growing of axillary buds to wild type level.

3.3. Redundancy of B1-type cyclins

Expression analysis of B1-type cyclin promoters suggested redundant function of B1-type cyclins and growth analysis of single mutants of B1-type cyclins showed that B1-type cyclin genes are highly redundant. To further analysis of individual B1-type cyclins, double mutants were made. Double mutants of *cycb1;1 cycb1;4* and *cycb1;2 cycb1;4* did not show any obvious phenotype in green house growth conditions. This indicates that CYCB1;1 and CYCB1;2 are able to compensate for each other function. Embryo collapse and lethality of the *cycb1;1 cycb1;2* double mutant shows CYCB1;1 and CYCB1;2 are essential for development after the heart stage.

Analysis of transcription data of genvestigator data has hinted that until heart stage CYCB1;4 and or CYCB1;3 have high levels of transcription and they can compensate for the lose of function of *cycb1;1* and *cycb1;2*. When the transcription of CYCB1;3 and CYCB1;4 decreased, the cell cycle suffered from lack of cyclin and embryo development was arrested (Fig. 3-1).

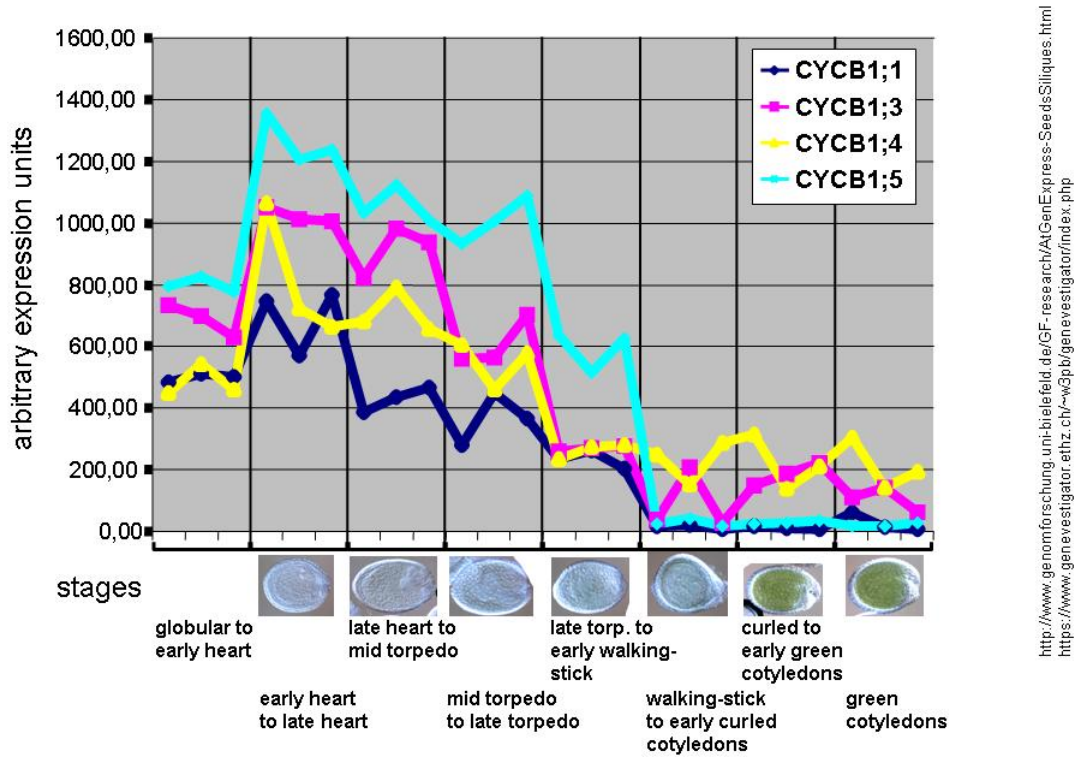


Fig. 3-1 Expression profile of B1-type cyclins during embryogenesis. Data was extracted from genevestigator – B1;2 is not on the affy chip and the probes used for B1;5 also recognize B1;2, thus the depicted B1;5 might reflect a mixture of B1;2 and B1;5 or even only B1;2 if B1;5 is a pseudogene.

Leaf growth analysis has shown that *cycb1;1* and *cycb1;2* single mutants as well as *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ and *cycb1;1*$^{-/+}$ *cycb1;2*$^{-/-}$ double mutants have the same leaf area. This shows that other cyclins have redundancy with CYCB1;1 and CYCB1;2.

A lack of both CYCB1;1 and CYCB1;2 causes much more severe mitotic defects., A double homozygous mutants appeared in the progeny of *cycb1;1*$^{-/-}$*cycb1;2*$^{-/+}$ plants with a frequency of about 0, 05% (expected 25%) and displayed severe phenotypes. Male and female developmental defects of *cycb1;1 cycb1;2* mutant reveal a high level of redundancy function between CYCB1;1 and CYCB1;2 in gametogenesis.

It may be that the amount of CYCB1;1 or CYCB1;2 distributed from sporophytic tissues is sufficient to drive cell division or other cyclins partially compensate for CYCB1;1 and CYCB1;2 function that allowed for double mutant viable pollen to develop into mature pollen in the one plant identified as a double mutant. 10% of pollen was FDA positive and DAPI staining of pollen revealed trinucleated pollen but formation of trinucleated pollen does not guarantee fertility of pollen and pollination

of the wild type ovule with double mutant pollen produced 2-3 seeds per siliques. This shows that after formation of trinucleated pollen most of the pollen is not fertile.
CYCB1;1 and CYCB1;2 are essential genes for cell division in gametogenesis. But *cycb1;1 cycb1;4* or *cycb1;2 cycb1;4* were not, implying that CYCB1;4 is not an essential gene in gametophytic development and it has distinct functions.

3.4. Distinct roles for CYCB1;1 and CYCB1;2 with CYCB1;4

Two obvious hypotheses could explain the difference between the B1-type cyclins mutant phenotypes, given the extensive overlap in protein expression and their high degree of similarity. First, in spite of their similarity, these proteins may have acquired novel biochemical functions during evolution and so fulfill different roles. Alternatively, the proteins could be biochemically equivalent, but the divergence in the temporal expression pattern between the genes may have resulted in the expression of a single cyclin B gene, at different times (Hanks et al., 1995). Hence, mutation of B1-type cyclins would be largely compensated for by the presence of another B cyclin. In order to distinguish between these two hypotheses, I replaced CYCB1;1 functionality with either CYCB1;2, CYCB1;3 or CYCB1;4 by inserting their coding sequences into the CYCB1:1 locus. The targeting event brought the integrated sequences under the control of the CYCB1;1 promoter and endogenous regulatory elements. Genotyping of the T2 generation of transgene double mutants has revealed that replacement of the CYCB1;1 cDNA with CYCB1;2 or CYCB1;3 could rescue the double mutant phenotype but CYCB1;4 did not. This shows that CYCB1;1, CYCB1;2 and CYCB1;3 have redundant functions but CYCB1;4 which has diverged from other members of B1-type cyclins does not.

3.5. Complementation of *cycb1;1$^{-/-}$ cycb1; 2$^{+/-}$*

For demonstrating that the mutant phenotype is the result of a mutation in the gene of interest, a gene rescue experiment is commonly performed. To show that the mutant phenotype arose from CYCB1;1 and CYCB1;2 mutations, *cycb1;1$^{-/-}$ cycb1;2$^{+/-}$* mutants were transformed with *ProCYCB1;1:CYCB1;1* and *ProCYCB1;2:CYCB1;2*. Genotyping of the T2 generation of transgenic double mutants yielded double homozygous mutants that exhibited wild type like growth.

3.6. Function of B1-type cyclins in endoreplicating and dividing cells

During endoreplication cycles, nuclear DNA is replicated without cytokinesis, resulting in cells with DNA content greater than 2C. The primary functional features of the endocycle appear to be the absence of G2/M phase CDK activity, prevention of mitosis, and oscillations of G1/S CDK activity that allow relicensing of replication origins between each round of DNA replication ((Larkins et al., 2001). Misexpression of CYCB1;1, CYCB1;2, CYCB1;3 and CYCB2;1 did not induce any phenotypes in trichomes. However, it has been well known in fungi and animals that CDK inactivation is affected essentially through proteolysis of the B-type cyclins by a multi subunit ubiquitin protein ligase, termed the anaphase-promoting complex or cyclosome (APC/C) (Harper et al., 2002; Peters, 2002). B-type cyclin degradation is dependent on a specific sequence element in its N-terminal region, termed the destruction box (D-box) (Glotzer et al., 1991). The misexpression of CYCB1;1 with a mutated destruction box did not induce any phenotypes while the expression of CYCB1;2 with a mutated destruction box resulted in a faint phenotype (table3-2).

To determine whether the CYC B1;1 destruction box is active in trichomes, a CYCB1;1^{1-112}-GUS fusion gene including the CYCB1;1 N-terminal portion of the coding region that encodes the cyclin destruction box was created. This fusion is thought to mimic the expression pattern of CYCB1;1 and has been used in other studies to identify G2/M phase cells (Colon-Carmona et al., 1999). CYCB1;1^{1-112}-GUS was misexpressed in trichomes of the wild type plants. The activity of GUS was detected in all trichomes which shows that the destruction box of CYCB1;1 is not sufficient for degradation of CYCB1;1.

On the other hand the CYCB1;2^{1-135} which was fused to YFP was degraded and did not show any YFP signal in trichomes. To test the functionality of CYCB1;1^{1-112} and CYCB1;2^{1-135} N-termini, they were fused to GUS and YFP respectively and transformed under control of the GL2 promoter, were transferred to the proGL2:CYCD3;1 line. Surprisingly both N-termini showed patchy patterns, revealing that a switch from endoreplication to division induces the destruction box dependent degradation pathway, even though they have shown different patterns in endoreplicating trichome cells.

Results reveal that something more than the destruction box is required for degradation of CYCB1;2 in trichomes.

3.7. Expression of CYCB1;1 and CYCB1;2 in dividing cells

Misexpression of CYCB1;1 and CYCB1;2 did not induce any phenotypes in trichomes. To test whether the constructs are functional CYCB1;1 and CYCB1;2 were expressed in dividing plant cells, CYCB1;1 and CYCB1;2 were expressed under control of the *TMM* promoter. This misexpression induced clusters of stomata and cytokinesis defects of epidermal leaf cells. Misexpression of CYCB1;1 and CYCB1;2 with a mutated destruction box induced a severe phenotype, showing epidermal cells with more clusters of stomata and big cells with partial cell walls consistent with previous data (Weingartner et al., 2004) (Fig 2-17). Overexpression of CYCB2 in Hela cells dysregulated mitosis, and induced cell arrest. However, the effect produced by mutant cyclin B2 was considerably more severe than that produced by the wild-type protein (Gallant and Nigg, 1992). Nondegradable versions of mitotic cyclins also produce mitotic arrest in *D. melanogaster* (Rimmington et al., 1994; Sigrist et al., 1995), and in *S. cerevisiae* (budding yeast), (Surana et al., 1993). This data shows that degradation of Cyclin B by the destruction box is required for exit from mitosis.

3.8. The Barbie box: a novel degradation motif in plant cyclins

Expression of destruction box mutations or the CYC B1;2 $^{\Delta 1\text{-}31}$ with destruction box or the CYC B1;2$^{\Delta 1\text{-}40}$ without destruction box slightly induced clusters of trichomes and one plant with a few multicellular trichomes was found. Nevertheless, the truncation CYCB1;2$^{\Delta 1\text{-}135}$ which lost not only the destruction box but also the 95 amino acids after the destruction box, induced multicellular and clustered trichomes in 80% of transgenic lines. Localization analysis of the CYCB1;2 full length, the destruction box mutated CYCB1;2 and the CYCB1;2$^{\Delta 1\text{-}135}$ in trichome cells showed that transgenic lines of full length or destruction box mutated CYCB1;2 did not show any YFP signal. But truncated CYCB1;2 fused to YFP induced a YFP signal and multicellular trichomes.

This data together proves that a domain with in 95 amino acids after the destruction box is important for the stabilization and induction of multicellular trichome cells. Destruction box mutated and truncated CYCB1;2 induced similar phenotypes in the stomata lineage.

The E3 ubiquitin ligase, APC/C, regulates the exit from mitosis, late mitotic events, and events in G1 (Irniger and Nasmyth, 1997). It was shown that Cdc20/Fizzy and Cdh1/Fizzy-related can activate the APC/C (Schwab et al., 1997; Visintin et al., 1997; Fang et al., 1998). All known Cdc20– APC substrates contain a destruction box. In late mitosis Cdh1 activates APC. Cdh1–APC recognizes both D-box and KEN-box-containing substrates (Pfleger and Kirschner, 2000). Analysis of 95 amino acids after the destruction box did not show any known destruction box or KEN box structure. – The presence of the KEN box can be found in APC/Cdh1 substrates e.g. Cdc20 but has not been identified in B-type cyclins.

But alignment algorithm of the CYCB1;2 with other plants cyclins revealed a conserved motif in some plant species, which we called Barbie box. The name Barbie box arose because it is considered to be an analog of the KEN box, and in toy dolls the partner of Ken is Barbie. No Barbie box sequences were found in mammalian or fungi DNA sequences. It seems it is a conserved motif in some plant species cyclins (Fig. 19-2 A).

To find out the conservation of the Barbie box in B1-type cyclins, B1-type cyclins were aligned. The alignment algorithm showed that the Barbie box is not present in

CYCB1;1 (Fig 192 B). This provides an explanation as to why the CYCB1;$2^{1\text{-}135aa}$ N-terminus is degraded in trichomes and the CYCB1;1 N-terminus is not.

3.8.1 Barbie Box function in dividing and endoreplicating cells

Expression of CYCB1;$2^{\Delta 57\text{-}75}$ without Barbie box under the control of the endogenous CYCB1, 2 promoter in cycb1;$1^{-/-}$ cycb1;$2^{-/+}$ mutants was not able to rescue the double mutant phenotype and misexpression of the CYCB1;$2^{\Delta 57\text{-}75}$ did not induce any phenotype in dividing cells.

Barbie box is a degradation motif and that is divergent from CYCB1;1. Point mutations were induced in I60R and Q67T. Both mutations were able to restore the N-terminus YFP signal of CYCB1;$2^{1\text{-}135}$. The double mutation of I60R-Q67T exhibited an even stronger YFP signal than the single mutations of I60R or Q67T.

So far several lines of evidence have shown that the Barbie box is important for the degradation of the CYCB1;2 in trichomes.

3.8.2 CYCB1;$2^{\Delta 57\text{-}75}$ without Barbie box and the truncation CYCB1;$2^{\Delta 1\text{-}135}$ induce cell death in *siamese* mutant

Misexpression of CYCB1;2 or its double and triple fusions with CYCB1;1 faintly increased the phenotype of the sim mutant. CYCB1;$2^{\Delta 57\text{-}75}$ raised the phenotype of sim slightly more than CYCB1;2 full length and cell death was induced in dividing trichome cells.

In human Hela cells which are highly susceptible to apoptosis, Non-degradable forms of sea urchin cyclin B induces activation of the maturation promoting factor, in addition the activation of the cdc2 kinase led to caspase-3 activation and apoptosis (Gu et al., 2003).

It is conceivable that there is a selection pressure against cells with abnormal cdc2 kinase as it induces apoptosis. Cyclin E overexpression enhances apoptosis in breast cancer cells (Dhillon and Mudryj, 2003) and ectopic expression of cyclin B3 in mouse testa induces abnormal round spermatids and increased apoptosis in the testa (Refik-Rogers et al., 2006). This data points towards an important cell cycle deregulation of cyclins that induces cell division and finally cell death. However, the molecular base for the observed cell death in siamese mutants remains to be explored.

Taken together, this data suggest that the truncation CYCB1;2$^{\Delta 1\text{-}135}$ or CYCB1;2$^{\Delta 57\text{-}75}$ without Barbie box stabilized the CYCB1;2 protein and deregulates cell cycle in the *sim* mutant. However, the truncation CYCB1;2$^{\Delta 1\text{-}135}$ induces more cell division. Nevertheless both versions induce cell death.

3.8.3 Barbie box mediates degradation of CYCB1;2 independent from FIZZY related (CCS52A1):

The APC/C is an E3 ubiquitin ligase that, by targeting substrates for proteasomal degradation, plays a major role in cell cycle control (Fry and Yamano, 2006). The APC is required to induce progression and exit from mitosis by inducing proteolysis of different cell cycle regulators including Pds1/ securin and cyclin B (Castro et al., 2005). The proper timing of APC/C's activation and its substrate specificity are regulated, at least in part, by two associated proteins, CDC20/FIZZY and CDH1/FIZZY- related (Vodermaier, 2001). Mitotic cyclins are recognized as APC/C substrates by both the CDC20 and CDH1 proteins (Eloy et al., 2006) Finally, several studies have shown that CCS52A, the ortholog of FZR, is essential for endoreduplication (Kondorosi and Kondorosi, 2004).

In *M. nodules*, endoreduplication cycles are accompanied by the expression of CCS52A. Down-regulation of CCS52A in transgenic *M. truncatula* plants drastically affected nodule development, resulting in lower ploidy and reduced cell size (Vinardell et al., 2003).

Knock outs of CCS52A1 but not CCS52A2 or CCS52B in *Arabidopsis thaliana* produced small trichomes with two branches (Jahn Larkin personal communication). Expression of the CYCB1;2 full length or destruction box mutated CYCB1;2 in *ccs52a1* mutant plants did not induce any further phenotype showing that somehow CYCB1;2 is removed from trichomes and only the *ccs52a1* mutant phenotype was observed. Whereas the deletion of the Barbie box blocked a second degradation pathway and stabilized CYCB1;2 inducing multicellular trichomes.

This data and expression of CYCB1;2 destruction box mutations or CYCB1;2 without Barbie box which did not induce any phenotypes in trichomes shows that the presence of the Destruction box or the Barbie box is sufficient to degrade CYCB1;2 in trichomes. Removal of CCS52A1 which recognizes APC/C substrates and mutations in the destruction box which must be recognized by the APC/C complex did not

enhance the phenotype of the ccs52a1 mutant. This shows that they function in one pathway and CYCB1;2 is degraded by the Barbie box pathway. When the CCS52A1 pathway was blocked, CYCB1;2$^{\Delta 57\text{-}75}$ without Barbie box which not induce any phenotype in wild type plants, induced multicellular trichomes in the *ccs52a1* mutant. All together this data suggests that CYCB1;2 has two recognition boxes for degradation and they are redundant and independent from each other.

Model of function of Destruction and Barbie box in trichomes

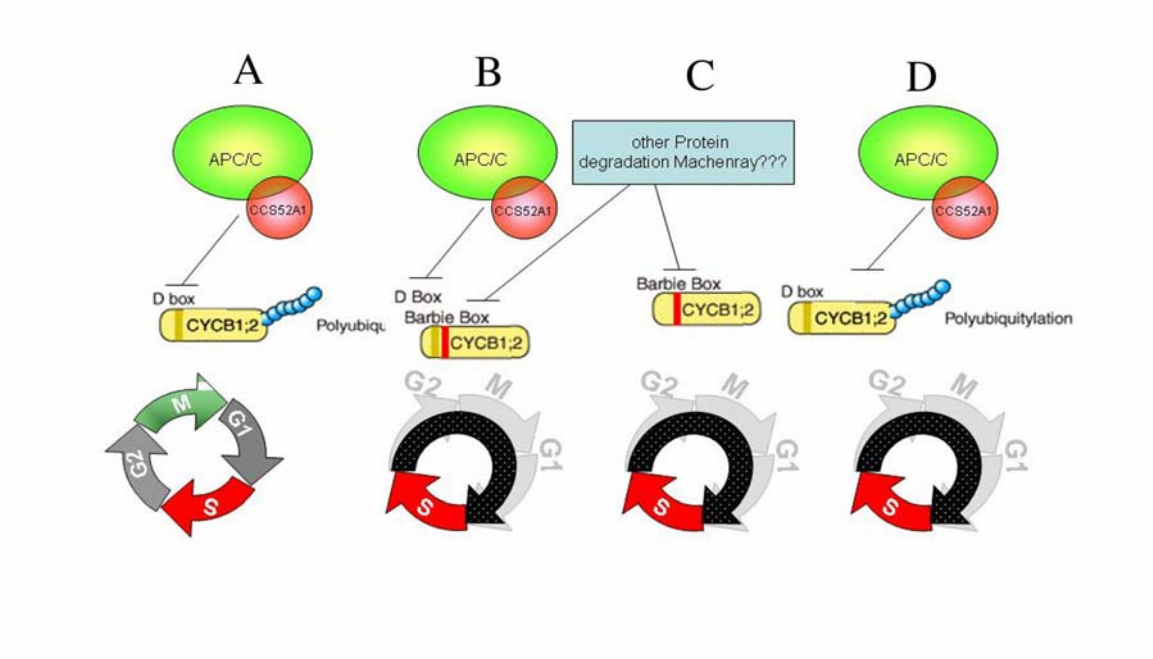


Fig. 2-3:

In dividing cells APC/C with activators mediates degradation of CYCB1;2 via recognition of the destruction box **(A)**. In endoreplicating cells degradation of CYCB1;2 occurs via the destruction box which is APC/C dependent and or the Barbie box which is independent from CCS52A1 **(B)**. If mutations are induced in the destruction box, degradation of CYCB1;2 can still occur via the Barbie box **(C)**. If the Barbie box is removed degradation of CYCB1;2 will still occur via the destruction box **(D)**.

3.9 APC/C degradation machinery

The APC/C is a multisubunit E3 ubiquitin ligase that targets cell cycle-related proteins for degradation, regulating progression from prometaphase to exit from mistosis(Passmore et al., 2003).

More than a dozen different groups of proteins are degraded by the APC/C pathway; including mitotic A and B type cyclins (Sudakin et al., 1995; Zur and Brandeis, 2002). Most of the *APC/C* targets carry a short peptide motif of nine amino acids called the destruction box (D-box) (Irniger, 2002). The first recognized target of this multiple-subunit protein complex was cyclin B (Irniger, 2002).

In the budding yeast, human and plant genome, 11 core subunits of *APC/C* have been found (Capron et al., 2003b; Peters, 2006). TheAPC11, RING finger subunit and APC2 the cullin domain-containing subunit form the APC catalytic center. APC11 recruits E2 to the *APC/C* (Gmachl et al., 2000; Leverson et al., 2000).

Knock out of the APC11 in *Saccharomyces cerevisiae* and embryo of *C. elegans* (Leverson et al., 2000; Moore and Boyd, 2004) showed that it is an essential gene in both organisms.

Ectopic expression of APC11 did not induce any phenotypes in *Arabidopsis thaliana* transgenic plants. It may be that the presence of a subunit is not enough to recruit the other 10 subunits and regulators of the *APC/C* therefore no phenotype was detected, or we only got weak misexpression lines that only show a signal late in development. In Human HT2-9 cells, it was shown that the *APC/C is* activated in endoreplication cells (Laronne et al., 2003). And APC/C regulator CCS52A1 expression is essential for endoreduplication of *Medicago truncatula* Nodules (Vinardell et al., 2003).

RNAi of *APC11* did not show any phenotypes in trichomes. Nevertheless *APC11* is an essential gene in development of Yeast and *C. elegans*. Two possibilities can explain the results of *APC11 RNAi*: first silencing of *APC11* did not work in trichomes or the APC11 levels were not sufficiently reduced. Secondly silencing worked but the APC11 is not essential for trichome development.

Analysis of progenies of the *APC11 RNAi* line crossed to *Pro35S:YFP:APC11* or *ProGL2YFP:APC11* showed that *APC11 RNAi* is active and can degrade the *YFP:APC11* in trichomes While the YFP:APC11 signal could be detected in epidermal cells of *ProGL2:APC11 RNAi* crossed to *Pro35S:YFPAPC11* progenies.

Previously it was shown that APC11 alone can ubiquitinate the APC/C substrates with the use of Ubc4 as the E2 enzyme.

APC11 does not have any functional paralogue in the *Arabidopsis* genome, APC11 is an essential subunit of the APC/C complex and activation of APC/C requires the presence of APC11. RNAi of *APC10* another subunit of APC/C did not induce any phenotype in trichomes (unpublished data of Sebastian Marquardt and Arp Schnittger).

Whilst the presence of APC/C subunits are suspected in trichomes ccs52a1 mutants showed small and two branched trichomes which might be the result of less endoreplication. In my thesis, I could show that the *ProGL2:CYCB1;2* destruction box mutation could not induce any phenotype in the *ccs52a1* mutant while the *ProGL2:CYCB1;2*$^{\Delta 57\text{-}75}$ induced multicellular trichomes.

This shows that CCS52A1 mediates destruction box dependent degradation of CYCB1;2 in trichomes.

All together this data suggests that APC/C is not active in trichomes and CCS52A1 functions with other E3 ligases e.g. SCF in trichomes even though interaction of CCS52A1 with other E3 ligase has not been reported so far.

4 MATERIALS & METHODS

4.1 Materials

4.1.1 Chemicals and antibiotics

All used chemicals and antibiotics of analytical quality have been used from Sigma (Deisenhofen, Germany), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Duchefa (Haarlem, Netherlands).

4.1.2 Enzymes, primers and kits

Restriction enzymes were used from MBI-fermentas (St.Leon-Rot, Germany) and New England Biolabs (Frankfurt/Main, Germany). Modifying enzymes were used from MBIfermentas (St.Leon-Rot, Germany), Invitrogen (Karlsruhe, Germany), Roche (Mannheim, Germany), usb (Cleveland, USA), Qbiogene (Heidelberg, Germany), TaKaRa (Otsu, Japan).

Primers were generated by Metabion (München, Germany), Invitrogen (Karlsruhe, Germany), MWG (Ebersberg). Kits were supplied from peqlab (Erlangen, Germany), Roche (Mannheim, Germany) and QIAGEN (Hilden, Germany).

4.1.3 Cloning vectors and constructs

The following vectors and constructs were used in this work:

• *pGEM-T* easy (Promega) for the cloning of the promoter region of *CYCB; 1, CYCB1; 2 CYCB1;3 and CYCB1; 4*

• *pDONR 201* vector (Invitrogen) was used as a donor in gateway based clonings

• *pAM-PAT-GW* as a binary gateway target vector containing a *CaMV 35S* promoter cassette and BASTA resistance (GenBank accession AY027531)

•*pGL2- pAM-PAT-GW* as a binary gateway target vector containing a *Glabrata2* promoter cassette and BASTA resistance (GenBank accession AY027531)

pTMM-pAM-PAT-GW as a binary gateway target vector containing a *Too Many Mouth* promoter cassette and BASTA resistance (GenBank accession AY027531)

pGL2_pAM-PAT-GW-RNAi as a RNAi binary gateway target vector containing a *Glabrata2* promoter cassette and BASTA resistance (GenBank accession AY027531)

pINO as a RNAi binary gateway target vector containing a *CaMV 35S alcohol inducible* promoter cassette and BASTA resistance (GenBank accession AY027531)

• *pAM-PAT-GW* as a binary target vector containing the promoter region of *CYCB; 1, CYCB1; 2 CYCB1;3* and *CYCB1; 4* instead of the *CaMV 35S* promoter cassette cassette and BASTA resistance (GenBank accession AY027531)

4.1.4 Plant material

Arabidopsis plants used in this study were derived from the Columbia (Col) and the Nossen (No) accessions. The *cycb1; 1 and cycb1; 2* were obtained from Koncz collection. cycb1; 3 T-DNA lines collected from GABI-Kat (http://www.gabi-kat.de/) and SALK T-DNA (http://signal.salk.edu) collections, and transposone line from RIKEN (http://www.brc.riken.jp/lab/epd/Eng/) collection. cycb1; 4 collected from GABI-Kat and Koncz collections.

4.1.5 Bacterial strains

For standard cloning the *Escherichia coli* strains DH5alpha was used, the DB3;1 strain, which is resistant to the *ccdB* gene, was used for the Gateway Entry, Donor and Destination vectors. For plant transformation *Agrobacterium tumefaciens* strain GV3101 was used. For all gateway vector based plant transformation GV3101+pMP90RK was used.

4.2 Methods

4.2.1 Plant work

Plant growth conditions

Arabidopsis thaliana seeds were germinated on soil or half-strong MS-2 medium containing 0,5 % sucrose and 0,8 % agarose. Plants were grown between 18 and 25 ºC under standard greenhouse conditions or in culture rooms or Percival growth chambers under long-day conditions with a 16/8 h light/dark cycle at 18 ºC or 20 °C, respectively.

Crossing of plants

At a stage when the flowers were closed and the pollen of the anthers was not ripe the anthers of the acceptor flower were removed completely using very fine forceps. All remaining older and younger flowers were also removed. After two days the stigma of the carpels were pollinated with pollen from the donor plant.

Plant transformation

Plants were transformed according to the "floral dip" method (Clough and Bent, 1998). To gain strong plants, these were allowed to grow at 18 °C until the first flowers appeared at stalks of approximately 10 cm in length. Four days before plant transformation a 5 ml Agrobacterium preculture was incubated for two days at 28 °C. This preculture was used to inoculate the final 500 ml culture which was then incubated again for two days at 28 °C. Before transformation 5 % sucrose and 0.05 % Silwet L-77 were added to the culture. Plants were dipped in this solution for approximately 20 seconds and then horizontally placed in a moist chamber. The plants were transferred to the greenhouse on the following day.

Seed surface sterilization

The surface of the seeds was sterilized by a five min incubation in 95 % ethanol followed by a 10 min incubation in a 20 % Klorix solution (containing 0.1 % Triton X-100). Afterwards the seeds were washed two to three times with 0.01 % Triton X-100 solution and than plated under the clean bench on MS-Agar plates (1 % Murashige-Skoog salts (MS), 1 % sucrose, 0.7 % agar, pH 5.7). Alternatively the seeds were sterilized in a small vacuum container. In this container, 20 ml of bleach (DanKlorix by Colgate-Palmolive, Hamburg) were placed in a 50 ml glass beaker. 2 ml of concentrated hydrogen chloride were added to the bleach and the lid of the vacuum container was closed immediately afterwards. The chlorine that was produced by this reaction was used to sterilize the surface of the seeds for approximately 4 hours to 12 hours. The seeds were then plated as indicated above.

Selection of transformants

The seeds of transgenic plants carrying in their T-DNA a kanamycin or a hygromycin resistance were selected on half strong MS-Agar plates with 50 µg/ml kanamycin or 25 µg/ml hygromycin, respectively. Transgenic plants containing the BASTA resistance were grown on soil for 10 to 15 days. The seedlings were sprayed with a 0.001 % BASTA solution, the spraying was repeated after 3 to 7 days.

4.2.2 Microscopy and cytological methods

Microscopy

Light microscopy was performed with an Axiophot microscope (Zeiss, Heidelberg, Germany) equipped with differential interference contrast (Nomarski) and epifluorescence optics. The DISKUS software package (Carl H. Hilgers-Technisches Büro, Königswinter, Germany; version 4.30.19) was used to quantify the fluorescence intensity of DAPI stained pollen to determine nuclear DNA content. Confocal-laser-scanning microscopy was performed with Leica TCS SP2 AOBS (Leica, Wetzlar, Germany) or LSM 510 META (Zeiss, Heidelberg, Germany).

Whole-Mount preparation of seeds

Pistils and siliques of different developmental stages were prepared as described previously (Grini et al., 2002). Dissected siliques were fixed on ice with FAA (10:7:2:1 ethanol:distilled water:acetic acid:formaldehyde [37 %]) for 30 minutes, hydrated in a graded ethanol series to 50 mM NaPOH4 buffer, pH 7.2, and mounted on microscope slides in a clearing solution of 8:2:1 chloral hydrate:distilled water:glycerol. The specimens were cleared 1 hour at 4 °C before inspection. Light microscopy was performed with a Zeiss Axiophot microscope using Differential Interference Contrast (DIC) optics.

GUS staining

GUS-activity was assayed according to Sessions and Yanofsky (Sessions and Yanofsky, 1999). To allow complete penetration of the X-Gluc-solution plants or parts of plants were vacuum infiltrated in staining buffer (0.2 % Triton X-100, 50 mM NaPO4 pH 7.2, 2 mM potassium-ferrocyanide K4Fe(CN)6*H2O, 2 mM potassium-ferricyanide K3Fe(CN)6 containing 2 mM X-Gluc) for 15 to 30 minutes and afterwards incubated at 37 °C over night. Clearing was performed in 70 % ethanol at 37 °C over night.

Pollen preparation for fluorescence analysis

Anthers were prepared from flowers of different developmental stages and put in a droplet (15 µl) of DAPI working solution (watery solution containing 2.5 µg/ml of 4',6-Diamidino-2- phenylindole (DAPI), 0.01 % Tween, 5 % DMSO, 50 mM PBS buffer [pH 7.2]) on a microscopy slide. The anther was then covered with a covering glass (18 mm x 18 mm) and slightly squashed to free the pollen or microspores. The slides were then placed in the dark until the rim of the covering glass was dry, and

then sealed with nail polish. After 4 hours to 12 hours of incubation in the dark at 4 °C, the preparations were checked for DAPI fluorescence then check under a fluorescence microscope with a UV-filter.

Pollen viability assay

For pollen viability staining, mature pollen at the stage of anther dehiscence was transferred onto microscope slides covered with a thin film of germination medium (0.4 mM CaCl2, 0.4 mM Boric Acid, 0.5 % Agarose, 10 % Sucrose in distilled water, pH adjusted to 7). The pollen was allowed to rehydrate in a moist chamber at 4 °C for 2 hours. Subsequently, fluorescein diacetate (FDA) staining solution (2 mg of FDA in 1 ml acetone as stock solution, which was added drop by drop to 10 ml of a 0,5 M sucrose solution) was applied to the pollen. After 10 minutes of incubation at room temperature, the samples were observed under a fluorescence microscope with a FITC filter.

4.3 Molecular-biological methods

All primers and probes are listed in Table 4-1 at the end of the Materials and Methods section.

4.3.1 Genomic DNA preparation from plant tissue I

To gain high-quality genomic DNA, the CTAB-preparation was used (Rogers and Bendich, 1988). Plant material (single rosette or cauline leave) was grinded and 200 µl of extraction buffer (2 %(w/v) CTAB, 1,4 M NaCl, 20 mM EDTA, 100 mM Tris/HCl pH 8.0, 0.2 % bmercaptoethanol) was added and incubated for 30 minutes at 65 °C. After addition of 150 µl Chloroform/Isoamylalcohol (24:1) and careful shaking, the probes were centrifuged for 15 minutes at 4000 rpm. The aqueous phase was transferred into a new tube and mixed with 200 µl isopropanol and centrifuged for 15 min. at 4000 rpm. The pellet was washed with 70 % Ethanol and dried, afterwards the pellet was resolved in 20 µl 20 mM Tris/HCl pH 8.0.

4.3.2 Genomic DNA preparation from plant tissue II

For PCR based genotyping, genomic plant DNA was isolated following a modified protocol from Berendzen (Berendzen 2005). A small amount of young plant material (e.g. a cotelydon) was put in a 2 ml reaction tube and 300 µl of DNA extraction buffer (5 ml 1 M Tris/HCl pH 7.2 + 6 ml 5 M NaCl + 10 g sucrose and adjusted to the final

volume of 100 ml with water) was added. The plant tissue was ground by adding a metal bead and shaking the reaction tubes for 1 min at a high frequencey in the Mixer Mill MM 301 by Retsch (Haan, Germany). 1 µl of this solution (no centrifugation required) was used as template for PCR using standard Taq-Polymerase and the following 10 times PCR buffer: Tris/HCl pH 8.7: 200 mM / KCl: 500 mM / MgCl2: 20 mM. The DNA preparation could be stored at –20 °C for further use.

4.3.3 Plasmid DNA preparation from bacteria

Plasmid preparation was performed using a column pEQ-LAB Plasmid Miniprep KitI (PEQLAB Biotechnology GmbH, Erlangen) according to the manufacturer's protocol.

4.3.4 DNA-manipulation

DNA manipulation and cloning were carried out according to Sambrock et al.(Sambrook et al., 1989) or Ausubel (Ausubel, 1994), using standard procedures. All PCR-amplified fragments were sequenced prior to further investigation.
PCR-Primers and constructs were designed using the VectorNTI-suite 7.1 software (Invitrogen, Karlsruhe).

4.3.5 Cloning of complementation and reporter constructs

For the rescue construct 1153bp upstream of the CYCB1; 1, 1059 bp upstream of the CYCB1, 2 and 1.5 kb upstream of the CYCB1; 4 start codon together with the B1-type cyclins cDNAs were used. Alternatively, the same CYCB1; 1 promoter region was used
to rescue the cycb1, $1^{-/-}$-cycb1; $2^{-/+}$ double mutants with a fusion construct consistent of the proper cDNA or CYCB1; 2, CYCB1; 3 and CYCB1; 4 cDNAs. To obtain a B1-type cyclins promoters reporter construct, again the same 5´ region was fused to the ß-glucuronidase gene (GUS).

4.3.6 RNA isolation, reverse transcription and RT-PCR

Isolation of RNA, DNAse digest, reverse transcription and RT-PCR was performed according to Weinl et al., 2005.

4.3.7 Identification of *b1-type cyclin* mutants by PCR

To analyze T-DNA insertion lines for *CYCB1;2, CYCB1;2, CYCB1;3* and *CYCB1;4* Allele-specific PCRs were carried out to determine the T-DNA insertion sites using the T-DNA or Transposone border primer in combination with cyclin-specific primer. To identify homozygous knockout plants PCR was done using cyclin gene specific primers. All screening and T-DNA primers are listed in table 4-1.

Table 4-1: Primers of T-DNA and Transposone lines

Name of Primer	Target and orientation of primer	Sequences
F120-B11-U	CYCB1; 1S	AAG CCC GAT TTC GTG TCT CTA CAG C
F119-B11-L	CYCB1; 1 AS	GCA GTG TTT GGG AAT GAA TCA TGC TAA AGG
F117-B12-U	CYCB1; 2S	GTA AAA TTA TCC AGT GCC AGC GCA TTC C
F118-B12-L	CYCB1; 2 AS	ACA ATA AGT CGG AAA CTA ATT GCA CGA TCT G
F151-B13-UTR61-Fr	CYCB1; 3S	ACG AAC GAC CAA TCT TTA ATC GTT TAC ATG AAC TG
FK37-B13-1020	CYCB1; 3 AS	TTC TCC AGT TTC TGG TCA GAA CC
B1;4 –U	CYCB1; 4 S	GATTTTTGATTCAGAGAGAGTGGAGTCTG
B1;4 –L	CYCB1; 4 AS	GACAGCTACATGATCAACAAAAGCAGACTAGG
F156-Ds5-2a	Left Border-Transposone	TCC GTT CCG TTT TCG TTT TTT AC
F157-DS5-3	Left Border Transposone	TAC CTC GGG TTC GAA ATC GAT
F158-Ds3-2a	Right Border Transposone	CCG GAT CGT ATC GGT TTT CG
F159-DS3-4	Right Border Transposone	CCG TCC CGC AAG TTA AAT ATG
F114-GABI-RB	RB-GABI	GTG GAT TGA TGT GAT ATC TCC
F115-GABI-LB	LB-GABI	ATA TTG ACC ATC ATA CTC ATT GC
F136-Fish1-2	Right Border-T-DNA Koncz collection	TCA CTC AGG GTC AAT GCC AGC G
Fk62-FISH2	Left Border-T-DNA Koncz collection	CAG TCA TAG CCG AAT AGC CTC TCC A
J507-SALK-RB	Right Border-SALK	CTC CGC TCA TGA TCA GAT TGT CGT TTC CCG
F104-504 SALK_LB	Left Border -SALK	GCG TGG ACC GCT TGC TGC AAC TCT CTC AGG

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6. Appendix

6.1 Constructs

Name of Constructs

Name of Construct	Cloning/Sources	Resistance
B1;1	in pBuescript from Arp	Amp
B1;2	in pBuescript from Arp	Amp
B1;1	pDONR201	Kan
B1;2	pDONR201	Kan
B1;1-B1;2	pDONR201	Kan
B1;2-TF	pDONR201	Kan
B1;1TF	pDONR201	Kan
B1;1DB	pDONR201	Kan
B1;2 DB	pDONR201	Kan
B1;4	pDONR201	Kan
B1;3	pDONR201	Kan
B1;2HA	pDONR201	Kan
B1;1HA	pDONR201	Kan
Truncation B1;2 with UTR	pDONR201	Kan
Truncation B1;2 with out UTR	pDONR201	Kan
pGl2:B1;1	pGL2-pAM-pAT-GW	Amp
pGl2:B1;2	pGL2-pAM-pAT-GW	Amp
pGl2:B1;1-B1;2	pGL2-pAM-pAT-GW	Amp
pGl2:B1;2-B1;1	pGL2-pAM-pAT-GW	Amp
pGl2:B1;1TF	pGL2-pAM-pAT-GW	Amp
pGl2:B1;2TF	pGL2-pAM-pAT-GW	Amp
pGl2:B1;1DB	pGL2-pAM-pAT-GW	Amp
pGl2:B1;2DB	pGL2-pAM-pAT-GW	Amp
pGl2:B1;1 HA	pGL2-pAM-pAT-GW	Amp
pGl2:B1;2 HA	pGL2-pAM-pAT-GW	Amp
pTRY:B1;3	pTRY-pAM-PAT-GW	Amp
B2;1	pDONR201	Kan
pTRY:B12 Tra-UTR	pTRY-pAM-PAT-GW	Amp
pTRY:B12 Tra+UTR	pTRY-pAM-PAT-GW	Amp
pGl2:B12 Tra+UTR	pTRY-pAM-PAT-GW	Amp
pGl2:B12 Tra-UTR	pTRY-pAM-PAT-GW	Amp
pTRY:B1;1	pTRY-pAM-PAT-GW	Amp
pTRY:B1;2	pTRY-pAM-PAT-GW	Amp
pTRY:B1;1-B1;2	pTRY-pAM-PAT-GW	Amp
pTRY:B1;2-B1;1	pTRY-pAM-PAT-GW	Amp
pTRY:B1;1TF	pTRY-pAM-PAT-GW	Amp
pTRY:B1;2TF	pTRY-pAM-PAT-GW	Amp
pTRY:B1;1DB	pTRY-pAM-PAT-GW	Amp
pTRY:B1;2DB	pTRY-pAM-PAT-GW	Amp
pTRY:B1;1 HA	pTRY-pAM-PAT-GW	Amp
pTRY:B1;2 HA	pTRY-pAM-PAT-GW	Amp
YFP	in PDONR201from Christina weinl	Kan
CFP	in pDONR201from Christina weinl	Kan
pAMPAT GW	from Arp	Amp+Chl
pAMPAT(2x 35S)	from Arp	Amp+Chl
pAMPAT pGl2	from Arp	Amp+Chl

pAMPAT pTRY	from Arp	Amp+Chl
pGL-pAM_PAT_RNAi	from Arp	Amp+Chl
pTMM-pAM_PAT_RNAi	from Arp	Amp+Chl
pB1;2	pGEMT	Amp
pB1;1	pGEMT	Amp
pTRY:B1;2 3'UTR	pTRY-pAM-PAT-GW	Amp
pGL2:B1;2 3'UTR	pGL2-pAM-PAT-GW	Amp
B1;2 3'UTR	pDONR201	Kan
B1;2_N	pDONR201	Kan
pB11-pAMPAT(FK)	pAM-PAT-GW, 35S promoter replaced	Amp+Chl
pB12-pAMPAT(FK)	pAM-PAT-GW, 35S promoter replaced	Amp+Chl
pB1;1:B1;1	pB11-pAMPAT(FK)	Amp
pB1;1:B1;2	pB11-pAMPAT(FK)	Amp
pB1;1:B1;3	pB11-pAMPAT(FK)	Amp
pB1;1:B1;4	pB11-pAMPAT(FK)	Amp
pB1;2:B1;1	pB12-pAMPAT(FK)	Amp
pB1;2:B1;2	pB12-pAMPAT(FK)	Amp
pB1;2:B1;3	pB12-pAMPAT(FK)	Amp
pB1;2:B1;4	pB12-pAMPAT(FK)	Amp
B11YFP	pDONR201	Kan
B1;2YFP	pDONR201	Kan
DB1;2YFP	pDONR201	Kan
Trb1;2YFP	pDONR201	Kan
wDB1;2	pDONR201	Kan
TrDb1;2	pDONR201	Kan
CYC B1;2_N	pDONR201	Kan
CYC B1;2_C	pDONR201	Kan
B1;2RFP	pDONR201	Kan
B1;4	pDONR201	Kan
pB1;2: B12YFP	pB12-pAMPAT(FK)	Amp
pB1;2:DB12YFP	pB12-pAMPAT(FK)	Amp
pB1;2:TrB1;2 YFP	pB12-pAMPAT(FK)	Amp
pGL2:TrB12	pGL2-pAM-pAT-GW	Amp
pGL2:CYCB1;2-C	pGL2-pAM-pAT-GW	Amp
pGL2:CYCB1;2_N	pGL2-pAM-pAT-GW	Amp
APC11	pDONR201	Kan
YFPAPC11	pDONR201	Kan
pGl2:YFPAPC11	pGL2-pAM-pAT-GW	Amp
pGL2:APC11RNAi	pGL-pAM_PAT_RNAi	Amp
pTMM:APC11 RNAi	pTMM-pAM_PAT_RNAi	Amp
pTMM-pAMPAT	from Arp	Amp+Chl
pTMM:B1;1	pTMM-pAMPAT-oliver hofman	Amp
pTMM:B1;2	pTMM-pAMPAT-oliver hofman	Amp
pTMM:DB Mutation B11	pTMM-pAMPAT-oliver hofman	Amp
pTMM:DB Mutation B1;2	pTMM-pAMPAT-oliver hofman	Amp
pTMM:Truncation B1;2 with out UTR	pTMM-pAMPAT	Amp
pTMM:B1;3	pTMM-pAMPAT	Amp
pTMM:DB1;2YFP	pTMM-pAMPAT	Amp
pTMM:TrYFP	pTMM-pAMPAT	Amp

pTMMB1;2	pTMM-pAMPAT	Amp
pB1;2:DB-YFP	pB12-pAMPAT(FK)	Amp
pB1;2:DB-CFP	pB12-pAMPAT(FK)	Amp
NI-N-terminus of CYCB1;2 (405bp)	pDONR201	Kan
NII N-terminus of CYCB1;2 (924bp)	pDONR201	Kan
NIYFP	pDONR201	Kan
NI(IR)YFP	pDONR201	Kan
NI(QT)YFP	pDONR201	Kan
NI+B11Barbei Box	pDONR201	Kan
B12-BB	pDONR201	Kan
B12(IR)	pDONR201	Kan
B12(QT)	pDONR201	Kan
B12(IR-QT)	pDONR201	Kan
NI(IR-QT)	pDONR201	Kan
pGL2:NIYFP	pGL2-pAM-pAT-GW	Amp
pGL2:N(IR)YFP	pGL2-pAM-pAT-GW	Amp
pGL2:NI(QT)YFP	pGL2-pAM-pAT-GW	Amp
pGL2:NI(IR_QT)YFP	pGL2-pAM-pAT-GW	Amp
pGL2:NI+B11Barbei Box-YFP	pGL2-pAM-pAT-GW	Amp
pGL2:B12-BB	pGL2-pAM-pAT-GW	Amp
pGL2:B12(QT)	pGL2-pAM-pAT-GW	Amp
pGL2:B12(IR)	pGL2-pAM-pAT-GW	Amp
pGL2:NI(QD)YFP	pGL2-pAM-pAT-GW	Amp
pGL2:Ni(ID)YFP	pGL2-pAM-pAT-GW	Amp
pGL2:B12IR-QT	pGL2-pAM-pAT-GW	Amp
B13 genomic	pDONR201	Kan
pB12:NIYFP	pB12-pAMPAT(FK)	Amp
PB12:NI(IR)YFP	pB12-pAMPAT(FK)	Amp
pB12:NI(QT)YFP	pB12-pAMPAT(FK)	Amp
pB12:NI(IR_QT)YFP	pB12-pAMPAT(FK)	Amp
pB12:DBmNIYFP	pB12-pAMPAT(FK)	Amp
FZR-RNAi-11	pDONR201	Kan
FZR-RNAi-12	pDONR201	Kan
FZR_RNAi-22	pDONR201	Kan
FZR-RNAi-33	pDONR201	Kan
FZY RNAi	pDONR201	Kan
pGL2:FZR-RNAi-11	pGL2-pAM-pAT-GW	Amp
pGL2:FZR-RNAi-12	pGL2-pAM-pAT-GW	Amp
pGL2:FRR-RNAi-22	pGL2-pAM-pAT-GW	Amp
pGL:FZR-RNAi-33	pGL2-pAM-pAT-GW	Amp
pINo RNAi -gateway	Christina Nue	Amp+Chl
pINO:FZR11-RNAi	pINo RNAi -gateway	Amp
pINO:FZR12-RNAi	pINo RNAi -gateway	Amp
pINO:FZR22-RNAi	pINo RNAi -gateway	Amp
pINO:FZR33-RNAi	pINo RNAi -gateway	Amp
pGL2:FZY-RNAi	pGL-pAM_PAT_RNAi	Amp
pINO:FZY-RNAi	pINo RNAi -gateway	Amp
pAMPAT-MCS	in pAMPAT-GW from Ralph Panstruga	Amp
pGEXp6	from chuann kwan	kan
pDONR201	from Arp	Kan

6.2 Primers

List of Primers which were used in this study

Name of Primer	sequences(5'-3)
F189-B12-1240-Fr	CTC GAA GGC AGA GAA TGG AG
F187-B12-1295Fr	TCT TGA GTG CTG CTG CTG AT
F188-YFP-251-rev	AAG TCG TGC TGC TTC ATG TG
F128-YFP128S	GCT GAC CCT GAA GTT CAT CTG
F186-YFP485AS	TGA TAT AGA CGT TGT GGC TGT TG
F185-b13downattb2	GGG GAC CAC TTT GTA CAA GAA AGC TGG CAC TAC TAG GAA ACA TAC CGG TGA TGC
Fk62-FISH2	CAG TCA TAG CCG AAT AGC CTC TCC A
F120-B11-U	AAG CCC GAT TTC GTG TCT CTA CAG C
F119-B11-L	GCA GTG TTT GGG AAT GAA TCA TGC TAA AGG
F183-I60R-Q-T-FR	CAA CCT CCG ATT AAT CGA CCC AGA ACT CGA AGC TTC CGT GCC ACT TTA TTA GCG AAT GCC CAA C
F184-I60R-Q-T-Rev	GTT GGG CAT TCG CTA ATA AAG TGG CAC GGA AGC TTC GAG TTC TGG GTC GAT TAA TCG GAG GTT G
F181-B1;1-NotI-rev	AAA GCG GCC GCC TAA ACA ATC TTT TCT TTC TGT TTC TTC TCT AAA C
F182-B1;2-NotI-Rev	AAA GCG GCC GCC TAC GCT ACT TCC TTT TTA GTC ACC TCC TTC
F177-B11-EcoRI-FR	TTT GAA TTC ATG ATG ACT TCT CGT TCG ATT GTT CC
F178-B11-NotI-rev	AAA GCG GCC GCA ACA ATC TTT TCT TTC TGT TTC TTC TCT AAA C
F179-B12-EcoRI-Fr	TTT GAA TTC ATG GCG ACG AGA GCA AAC GTA CC
F180-B12-NotI-Rev	AAA GCG GCC GCC GCT ACT TCC TTT TTA GTC ACC TCC TTC
F175-B13DownHind-Fr	TCA GAA GCT TAC AAG GCT TGA AAG ATG GCT
F176-B13downSpeI-Rev	CAC TAG TCA CTA CTA GGA AAC ATA CCG GTG
F169-FZR11920Fr	CAA AAA AGC AGG CTC CAC CAT GGA AGA AGA TGA ATC AAC AAC ACC G
F170-FZR11920Rev	CAA GAA AGC TGG GTT CCG GAT TGT TGT TCT ACC AAA AGA TAA TG
F171-FZR22910Fr	CAA AAA AGC AGG CTC CAC CAT GGA AGA AGA AGA TCC TAC AGC AAG
F172-FZR22910rev	CAA GAA AGC TGG GTT CCG AAT TGT TGT TCT ACC AAA GAA AG
F173-FZR13840Fr	CAA AAA AGC AGG CTC CAC CAT GGC ATC GCC ACA GAG TAC CAA AAC
F174-FZR13840rev	CAA GAA AGC TGG GTT TCG GAT CTG TGT CCT CCC CAA TGA C
F164-FZR840Fr	CAG AGC GAT TTT GTC AGC AA
F165-FZR840Rev	GGA TGG GTA CTT CCA GAG CA
F166-FZR920Fr	AAA TGG TCT TCG GAC AAT CG
F167-Fzr920rev	TGA CAC CGC AAG GTA CAG AA
F168-FZY260Fr	GAT CGT TCT GTC GCT TCC TC
F168-Fzy260Rev	AGC TCA GCC ATT TTC ACC AT
F163-B13-494Fr	GCT GCT GTG GAA TAC GTT GA
F162-B1;3Gen-rev	TGG CGC GCC GAT GAA ATC GAT GAA CGG TGA GAA AAC C
F160-B1;3Gen-Fr	CAC TAG TCA CTA CTA GGA AAC ATA CCG GTG ATG CG
F161-B1;3Gen-rev	GGT TTT CTC ACC GTT CAT CGA TTT CAT CGG CGC GCC C
FK37-B13_1020	TTC TCC AGT TTC TGG TCA GAA CC
F115-GABI-LB	ATA TTG ACC ATC ATA CTC ATT GC
F152-B13Intron-rev	CAA AAA GCA ACC TAT CAA GAT TTG GAT CCT TG
F151-B13-UTR61-Fr	ACG AAC GAC CAA TCT TTA ATC GTT TAC ATG AAC TG
F156-Ds5-2a	TCC GTT CCG TTT TCG TTT TTT AC
F157-DS5-3	TAC CTC GGG TTC GAA ATC GAT
F158-Ds3-2a	CCG GAT CGT ATC GGT TTT CG
F159-DS3-4	CCG TCC CGC AAG TTA AAT ATG
F128-YFP126_S	GCT GAC CCT GAA GTT CAT CTG
F127-J408	CCT TAT CTG GGA ACT ACT CAC ACA TTA TTC
F154-B12Re-B11barbei	ATT TTG AGA TCG TGT ACG AGG ACG ATG AAT CGG AGG TTG AGC CTT TCC
F155-B12Fr-B11barbei	CGT ACA CGA TCT CAA AAT CCC ACG CTT CTT GTG GAG GAT AAT CTC GAA AGA AAG CCA ATC AAT GG
F115-GABI-LB	ATA TTG ACC ATC ATA CTC ATT GC

F117-B12-U	GTA AAA TTA TCC AGT GCC AGC GCA TTC C
F118-B1;2L	ACA ATA AGT CGG AAA CTA ATT GCA CGA TCT G
F153-B12Fr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CAT GGC GAC GAG AGC AAA CGT ACC TGA AC
F117-B12-U	GTA AAA TTA TCC AGT GCC AGC GCA TTC C
F116-Fish2	CAG TCA TAG CCG AAT AGC CTC TCC A
F118-B12L	ACA ATA AGT CGG AAA CTA ATT GCA CGA TCT G
F152-B13Intron-rev	CAA AAA GCA ACC TAT CAA GAT TTG GAT CCT TG
F151-B13-UTR61-Fr	ACG AAC GAC CAA TCT TTA ATC GTT TAC ATG AAC TG
F147-Q-D-HindIII-R	GAG TTG GGC ATT CGC TAA TAA ATC GGC ACG GAA GCT TCG AGT AAT
F148-Q-D-HindIII-F	ATT ACT CGA AGC TTC CGT GCC GAT TTA TTA GCG AAT GCC CAA CTC
F150-I-D-ApaI-FR	CAA CCT CCG ATT AAT CGA CCC GAT ACT CGA AGT TTC CGG GCC C
F149-I-D-ApaI-R	GGG CCC GGA AAC TTC GAG TAT CGG GTC GAT TAA TCG GAG GTT G
F145-barbie-pfoI-Fr	ATC TTG TTT CTG TTC CCG GAG
F146-Barbie-AvaII-R	GAG GTT GTC TCT TTG GAC CAA G
F141-ITR-RAR-Fr	GCT CAA CCT CCG ATT AAT CGT CCC AGA ACT CGA AGT TTC CGT GCC C
F142-ITR-RAR-Rev	GGG CAC GGA AAC TTC GAG TTC TGG GAC GAT TAA TCG GAG GTT GAG C
F143-RAQ-RAT-Rev	CGA GTT GGG CAT TCG CTA ATA AAG TGG CAC GGA AGC TTC GAG TAA TGG GTC GAT TAA TC
F144-RAQ-RAT-Fr	GAT TAA TCG ACC CAT TAC TCG AAG CTT CCG TGC CAC TTT ATT AGC GAA TGC CCA ACT CG
F139-b12motFR	GAC AAC AAG GTT CCA GCT CTT GG
F140-B12motailREV	GAG CTG GAA CCT TGT TGT CTT GAG CCT TTC CTC CTT GAA CTC C
F134-Fish2-1	AGA TCC TCG CCG TCG GGC AT
F135-Fish2-2	CGG GTA GCC AAC GCT ATG TCC TGA TA
F136-Fish1-2	TCA CTC AGG GTC AAT GCC AGC G
F137-Fish1-3	TGG TCA CTG ATG CCT CCG TGT AAG GG
F138-B12_2401	GGT GAG AGC AGG CTA CGT GCA GTG
F133-RB2-GABI	CGC CAG GGT TTT CCC AGT CAC GAC G
F131-LB3-GABI	GAA CCC TAA TTC CCT TAT CTG GG
F132-LB2-GABI	GGG CTA CAC TGA ATT GGT AGC TC
F127-J408	CCT TAT CTG GGA ACT ACT CAC ACA TTA TTC
F128-YFP126_S	GCT GAC CCT GAA GTT CAT CTG
F129-B1;2-CYCC	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGC CGA CTC AAT ACG TCT TCC TTG
F130-B12-970S	GCT TCG ATG TCT GAT CCA GAA ATG G
F125-B12_C-RT	CCC GAC TCA ATA CGT CTT CCT TGT C
F123-B12C-RT-Fr	CCC GAC TCA ATA CGT CTT CCT TG
F121-35S_terRev	CCT TAT CTG GGA ACT ACT CAC ACA TTA TTC
F122-EF1_Rev	TTG GCG GCA CCC TTA GCT GGA TCA
F124-EF1-Fr	ATG CCC CAG GAC ATC GTG ATT TCA T
F116-Fish2	CAG TCA TAG CCG AAT AGC CTC TCC A
F117-B12-U	GTA AAA TTA TCC AGT GCC AGC GCA TTC C
F118-B12-L	ACA ATA AGT CGG AAA CTA ATT GCA CGA TCT G
F120-B11-U	AAG CCC GAT TTC GTG TCT CTA CAG C
F119-B11-L	GCA GTG TTT GGG AAT GAA TCA TGC TAA AGG
F114-GABI-RB	GTG GAT TGA TGT GAT ATC TCC
F115-GABI-LB	ATA TTG ACC ATC ATA CTC ATT GC
112F-Ntr_IB2Re	CAA GAA AGC TGG GTT CGC TAC TTC CTT TTT AGT CAC C
F113-Ntr_IIB2Re	CAA GAA AGC TGG GTT TGT CAA ATA CCA TTC GAG GTT TC
F112-NTr_IB2Re	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC GCT ACT TCC TTT TTA GTC ACC
F111-Ntr_IIB2Re	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT GTC AAA TAC CAT TCG AGG TTT C
F108-35S_terRev	CCT TAT CTG GGA ACT ACT CAC ACA TTA TTC
F110-J611	GGG GAC CAC TTT GTA CAA GAA AGC TGG GT
105F-NtrIRev	CTC GCC CTT GCT CAC CAT CGC TAC TTC CTT TTT AGT CAC C
106F-Nt_IIR	CTC GCC CTT GCT CAC CAT TGT CAA ATA CCA TTC GAG GTT TC

107F-YFR_Fr	ATG GTG AGC AAG GGC GAG G
F104-504salk	GCG TGG ACC GCT TGC TGC AAC TCT CTC AGG
K100-B12-418AS	AAT AAG CAA GGC ACT GAT TCC C
K101-B12_455AS	TAA CCT GAG GTG GCC AGA TTT C
K102-B1_2_465	AAT AAG CAA GGC ACT GAT TCC C
K103-GL2_S	GAG GAG AAG AGG GAA GAG ATC ATA A
K104-GL2_AS	CTC TTT CTC TTA TTA GTG CCC TTG T
Fk98-APC11_rev	CTC TTT GAA CTG CCA TTC TCT TC
Fk99-YFPreAPC	GAA TGG CAG TTC AAA GAG ATG GTG AGC AAG GGC GAG
Fk97-APC_as	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAC TCT TTG AAC TGC CAT TCT CTT C
Fk95-B12_689S	TCA ACC TTG AAA CTC TGT ACC TCA CCG TCA ACA T
FK96-B12_689AS	ATG TTG ACG GTG AGG TAC AGA GTT TCA AGG TTG A
Fk89-APC_Fr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGA AAG TCA AGA TCT TGC GAT GG
Fk90-APC_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AAG AGA ATG GCA GTT CAA AGA GTA A
Fk91-APC_FR_YFP	GCT GTA CAA GAT GAA AGT CAA GAT CTT GCG
Fk92-APC_Rev_YFP	GCT CAC CAT CTC TTT GAA CTG CCA TTC TC
FK93-YFP_Fr_APC	GTT CAA AGA GAT GGT GAG CAA GGG CG
Fk94-YFP_Rev_APC	GAC TTT CAT CTT GTA CAG CTC GTC CAT GC
Fk83-pB12F	GTG GCG CGC CTG ATC ACA TCC TCT TGA TGA AAT CTT C
Fk84-pB12_Rev	GTC TCG AGA TCG CTC TCC CAA TGA TTC TTA CTC TTC
FK85-B1;5Rev1	GCC GGT TAT CGT CCG GCA AAT CCA GC
FK86-B1;5Rev2	GGC ACG CCG ATT CTT CAC AGC ACC G
FK87-B1;5_Fr1	CAG GAG AGA CAA GGT GGC TTT GAT GTC TGT GG
Fk88-B1;5_Fr2	GCT GGA TTT GCC GGA CGA TAA CCG GC
Fk82-B12revRFP	GGA GGC CAT AGA AGA AAC AGG CTT C
FK81-pAM_Rev	GGT GCC TAA TGC GGC CGC CAT AG
Fk80-RFP_Fr_B12	GTT TCT TCT ATG GCC TCC TCC GAG G
Fk69-Fzyi_F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG TGA CCA TTG ATG AGG AGA AGG G
Fk70-FZYi_R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TAC TCC AAA AAC GTT CCA AAA CC
Fk71-FZYRi1_F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CCA AAT CAT GCT CTG GAA GTA CCC
Fk78-FZYRi_F2	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAC CTG ATG GCC AGA CTA TAG TGA C
Fk72-FZYR3_F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA GGT CTT GGA TGC TCC TTC TTT ACA AG
FK73-FZYRi1_R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CCG TAA GTG TTG CAA CCT TTG AC
Fk74-FZYRi2_R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG ACG TTC CAA AAC CGC AGG G
Fk79-FZYRi3_R	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG AAC ATT CTG TGA ACT CCA GTC CAC
FK75-Db1;2Str_F	AAC GGT GCT GTG AAG AGT GGG CGT GCC GTC GGT GAC ATC GGA AAT C
Fk76-DB1;2Str_R	GAT TTC CGA TGT CAC CGA CGG CAC GCC CAC TCT TCA CAG CAC CGT T
FK76-Hyg_Anti	CTA CAT CGA AGC TGA AAG CAC GAG ATT C
Fk77-wDB1;2Tr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGC GGC GTG CCC TCG GTG AC
FK65-DB12Tr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGC CGA CTC AAT ACG TCT TCC TTG
FK66-TrDb12	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGC TTG TTT CTG TTC CCG GAG TTC
FK67-B12N_Tr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG ATA TGT ACT CGT TCT ATA AAG AAG TTG AG
FK68-B12C_Tr	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGC CGA CTC AAT ACG TCT TCC TTG
FK63-DB1;2T	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC GGC GTG CCC TCG GTG ACA TC
FK64-TaDB1;2	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TTG TTT CTG TTC CCG GAG TTC AA
Fk62-FISH2	CAG TCA TAG CCG AAT AGC CTC TCC A

Fk60-VenusFr_B12	GTT TCT TCT ATG GTG AGC AAG GGC G
Fk61-B12Re_Venus	GCT CAC CAT AGA AGA AAC AGG CTT C
Fk58-CFPv2R	GTC GGC GAG CTG CAC GCC GCC GTC CTC GAT GTT GTG G
FK57-B11Rep273F	CCG AAG AAA GTG GCT GGG AAA CCA AAG GTA GTA GAC GTG ATT G
FK59-B11Rep273R	CAA TCA CGT CTA CTA CCT TTG GTT TCC CAG CCA CTT TCT TCG G
Fk51-CFPv1F	ACG GCA AGC TGA CCC TGA AGC TGA TCT GCA CCA CCG GCA AGC
FK52-CFPv1R	GCT TGC CGG TGG TGC AGA TCA GCT TCA GGG TCA GCT TGC CGT
FK53-CFPv2F	CCA CAA CAT CGA GGA CGG CGG CGT GCA GCT CGC CGA C
Fk54-CFPv2R	GTC GGC GAG CTG CAC GCT GCC GTC CTC GAT GTT GTG G
Fk55-B11Rep1	ACG CTT CGC TGT GGC TTT GAT CCC TCC GGC CAA A
Fk56-B11Rep2	TTT GGC CGG AGG GAT CAA AGC CAC AGC GAA GCG T
FK49-B13RevUTR	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TAG AAG AGA AGA AGA ATT TCA TTA TAA TAC TTT TGA ATT C
FK50-B22F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG TTA ATC CAG AGG AGA ACA ATC GTA AT
Fk48-B12Tranc	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGT CAC CTA AGA ATA AGA AAG TGA CGT AC
Fk43-B12FUTR	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC GAA AGA GAA ATA TCA AGA CTA AGA AAC AAG AAG AG
Fk44-B12RevUTR	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA ACA TTA ACT ATT TTA TGC ATT TCA TTT ATC ATC ATT C
FK45-DB12S1	GGG CGT GCC GTC GGT GAC ATC GGA AAT CTT GTT TC
FK48-DB12S2	GAA ACA AGA TTT CCG ATG TCA CCG ACG GCA CGC CC
FK46-DB11S1	CCA ACA ACT TGA CCG TTT CTT CCT TTC GCT ACG
Fk47-DB11S2	CGT AGC GAA AGG AAG AAA CGG TCA AGT TGT TGG
FK41-B11FUTR	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA GAC GCC CCC ACT ACT TAG ACT TTT TC
FK42-B11RUTR	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG TAC TAA AGA AGT TAA ACC ACA GAA TAT TAT ATC TAA GG
Fk38-B13F	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA TGG CGT CTT CTA GAG TCT CTG ATC TTC C
FK30-B13Rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT CAT GCA CAA GAA ACA GAG AAG TCG TC
FK31-B14FUTR	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTA CCA TTC GCA ATT TCA CAG TCG AG
FK34-B14reUTR	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG ACA AAA AGA GTT AAA AAT TCC AAA ACG TAA
FK33-B13_355	GGT TCA AAA GAA AGC AAG
FK37-B13_1020	TTC TCC AGT TTC TGG TCA GAA CC
FK40-B21_1201	CGT GAA CAT TCC ATG AGC
Fk35-B21_371	CGA CTT CGG TGA TTG TAT
Fk36-B22_432	GGA GGA TAT GGA AGA AGA
Fk24-B11Pro_Fr	GGC GCG CCC GAG AGA TGA CTA AAT TTG AAA G
Fk29-B11Pro_Rev	CTC GAG CTT AGT GTT CTC TTC TCT TTC TCT CAG ACT
Fk25-B11rev_YFP	CAC CAT AGC AGA TTC AGT TCC G
Fk26-B12Rev_YFP	CAC CAT AGA AGA AAC AGG CTT C
FK27-YFP_B11	TCT GCT ATG GTG AGC AAG GGC G
Fk28-YFP_B12	TCT TCT ATG GTG AGC AAG GGC G
Fk30-YFP_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT TAC TTG TAC AGC TCG TCC ATG CC
FK10-B1;3F	ATG GCG ACA GGA CCA GTT GTT CAT
FK11-B1;3Rev	TCA TGG AGC AGA TGA CAT AAG AGA CTT G
FK12-B1;3FUTR	AAG CGC TCT CTC TCA AAT CTC AAT CAT
FK13-B1;3RUTR	ATA GAA GAG AAG AAG AAT TTC ATT ATA ATA CTT TTG AAT TC
FK14-B2;1F	ATG GTT AAC TCA TGC GAG AAC AAA ATC TTC
FK15-B2;1Rev	TTA AGA ATG ATG AGA CTC AGA CAC TAG AAA GTG TG
FK16-B2;1FUTR	CTA CTC TCT TTG TGG GTT TCT CTT GTA ATA ATC
FK19-B2;1RUTR	GTT GTT TTT GTA TTT AGC TCA AAT AAG ATA CAA AAT AC
FK20-B2;2	ATG GTT AAT CCA GAG GAG AAC AAT CGT AAT
FK21-B2;2R	TTA GTG AGA ATC TGA CAC AAG AAA GTG TGC
FK22-B1;1-1240	CCA AGG ACG AAC GCT TCG CTG TGG CTT TGA TCC CTC

FK23-StypDONR	ATC GCG AGC CCA TTT ATA CCC ATA TA
FK8-Adapt1	GTC GGC GCG CCT CTG CA
FK9-Adapt2	ACG TCA GCC GCG CGG AG
FK6-B11-150	TCA ATC ATC GTC CTC GTA CAC G
FK7-B11-453	CGA TTC TGC TGA TGT TGA GAA TG
FK4-aatB1	GGG GAC AAG TTT GTA CAA AAA AGC AGG C
FK5-aatB2	GGG GAC CAC TTT GTA CAA GAA AGC TGG

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