Regulation of development and cell cycle through a retinoblastoma like protein, RBR1, in Arabidopsis

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Regulation of development and cell cycle through a retinoblastoma like protein, RBR1, in Arabidopsis

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### Table of Contents

**Abstract**

Zusammenfassung

1. **Introduction**

2. **Review of Literature**
   2.1. Retinoblastoma- the gene and the protein
   2.2. Plant cell cycle
   2.3. Plant development and the Shoot Apical Meristem (SAM)
   2.4. RNA interference (RNAi)

3. **Materials and methods**
   3.1. Materials
   3.2. Methods

4. **Results**
   4.1. Low transformation efficiency with all the *RBR1(RNAI)* constructs
   4.2. *Promoter::RBR1* lines had no apparent phenotype
   4.3. *Promoter::RBR1(RNAI)*
      4.3.1. CLV1::RBR1(RNAI)
      4.3.2. CLV3::RBR1(RNAI)
      4.3.3. AP1::RBR1(RNAI)
      4.3.4. STM::RBR1(RNAI)
      4.3.5. WUS::RBR1(RNAI)
   4.4. *Promoter::GFP*
   4.5. *Promoter::GUS*
      4.5.1. AP1::GUS
      4.5.2. CLV3::GUS
      4.5.3. STM::GUS
      4.5.4. WUS::GUS
   4.6. Vector Control (VC)
   4.7. 35S::RBR1(antisense)
   4.8. 35S::RBR1(RNAI)
      4.8.1. Phenotypic description
      4.8.2. Scanning Electron Microscopy (SEM) of the SAM
      4.8.3. Histology of the SAM
4.8.4. Histology of the leaves 57  
4.8.5. Effect on gametophyte development causes sterility 59  
4.8.6. Segregation and DNA blot analysis 62  
4.8.7. Effect on RBR1 transcript level 62  
4.8.8. Effect on RBR1 protein level 64  
4.8.9. Upregulation of several cell cycle marker genes 65  
4.8.10. CYCLIN B1.1 promoter was deregulated in presence of 35S::RBR1(RNAi) 66

5. Discussion  
5.1. Low transformation efficiency with RBR1(RNAi) constructs 67  
5.2. “Promoter”:::RBR1 lines had no apparent phenotype 67  
5.3. CLV3::RBR1(RNAi) and STM::RBR1(RNAi) lines showed similar but not identical phenotypes with 35SRi lines 67  
5.4. Most of the AP1::RBR1(RNAi) transformants were sterile 68  
5.5. WUS::RBR1(RNAi) lines had fasciated stems 68  
5.6. Leaf development of several 35SRi lines was affected 69  
5.7. Ectopic differentiation of SAM of several RNAi lines 70  
5.8. Gametophytic development was affected in many 35SRi lines 72  
5.9. Effect of RNAi on RBR1 and GAPDH expression 73  
5.10. Several cell cycle regulated genes were deregulated in 35SRi lines 74  
5.11. Upregulation of CYCLINB1.1 promoter in presence of 35SRi 75

6. References 78

7. Acknowledgements 101

8. Curriculum Vitae 102
Abstract

The retinoblastoma tumor suppressor gene is currently the only known gene whose mutation is necessary and sufficient for the development of human cancer. It's highly conserved among almost all the metazoans, tested so far. It's a key regulator of the cell cycle and differentiation and also influences many other physiological and cellular processes like apoptosis, virus infection, endoreduplication, chromatin remodeling, DNA polymerase III mediated transcription and maintenance of telomere length. Many of its functions are cell type or tissue specific.

The discovery of the homologues of this important tumor suppressor gene in corn plants (Zea mays) about 8 years ago came as a surprise to many plant biologists. Our group was the first to clone it from the model plant, Arabidopsis thaliana. Cloning of this single copy gene, RBR1, in Arabidopsis opened up a new and attractive opportunity to study its role in plants. Until now there are a few reports suggesting its role in G1-S transition in the plant cell cycle and infection by Geminivirus. I suspect that this protein is much more important than just controlling G1-S transition and plays a major role in plant development and differentiation. It became more challenging considering the fact that there are many significant differences between animal and plant developmental programs.

I mainly used the newly emerging RNA interference (RNAi) technology. I could not have any homozygous line with any of the RBR1(RNAi) constructs driven by different promoters. None of the RNAi lines maintained the typical characteristic phenotype that I observed among the independent, primary transformants in subsequent generations. Hence, all the data presented in this thesis were obtained mainly from the primary transformants.

Here I have shown, for the first time, that ectopic expression of RBR1(RNAi) within different domains of shoot apical meristem (SAM) resulted in specific phenotypes, while constitutive expression of RBR1(RNAi) using 35S CaMV promoter had a wide range of phenotypes. My results indicate that the plant orthologue of the retinoblastoma tumor suppressor gene indeed plays a major role during various aspects of plant development, viz gametophyte, leaf and shoot meristem development. Data presented in this thesis also suggest a very complex mode of regulation of RBR1 in Arabidopsis.
Zusammenfassung


Retinoblastoma (RB) was the first tumor suppressor gene identified about 20 years ago. The best understood function of the protein (pRB), encoded by this gene is to control re-entry into the cell cycle and G1-S phase transition in actively growing cells (Weinberg, 1995). pRB is highly conserved among higher eukaryotes, from human to plants, suggesting the conservation of the core cell cycle machinery among them (Gutierrez, 2000; Dewitte et al., 2003). pRB has also been implicated in several other physiological processes, such as chromatin remodeling (Brehm et al., 1999; Williams et al., 2000), DNA endoreduplication (Jiang et al., 2000), development and differentiation (Lee et al., 1994; Sage et al., 2000; MacPherson et al., 2003), apoptosis (Hsieh et al., 1999; Decary et al., 2002; Hickman et al., 2002), RNA Pol-III mediated transcription (Hirsch et al., 2000; Ciarmatori et al., 2001) and regulating telomere length (Garcia-Cao et al., 2002). RB homologues seem to be suitable candidates to integrate different external and internal signals into the cell cycle (Dewitte et al., 2003) and the cell cycle itself with development (Gutierrez, 1998; Meijer et al., 2001). It is also a key target for many viruses in both plants and animals (Chellappan et al., 1992; Jansen-Durr, 1996; Gutierrez, 2000; Kong et al., 2000).

Knock-out of both the RB alleles in mice results in death of the embryo during mid gestation, around 14.5 days, due to severe developmental abnormalities (Sage et al., 2000). Cells lacking all the RB homologues undergo uncontrolled cell division, though with shorter cell cycle (Sage et al., 2000; Garcia-Cao et al., 2002) but can never overcome the developmental defects. Till now most of the studies on RB or RB related proteins (RBRs) were performed with cell cultures, even in animals. We now know that cells behave very differently in cultures as compared to in-vivo (Cooper et al., 2001). Thus it becomes imperative to study the role of RB in the context of the whole organism. I hypothesized that RB plays a more important role during development and differentiation than in the cell cycle. Plant RB homologues can provide a unique opportunity to study the role of the retinoblastoma protein from a developmental perspective.

All aerial parts of a plant arise from the shoot apical meristem (SAM). The SAM can be subdivided in several distinct cell layers and zones. Different cells of SAM have very unique patterns as well as rates of cell division. It is presently assumed that specific cell division patterns and division rates are related to their developmental fate (Wolpert et al., 2002). In other words, pRB has the potential to control plant development through cell division in the SAM as well.

Our lab was among the few to clone first the plant RB homologue from maize (ZmRBR1) and first to clone from Arabidopsis (AtRBR1) (Durfee et al., 2000). The molecular and biochemical properties of pRB and/or its homologues are still largely unknown, particularly in plants. Most of the metazoans possess more than one copy of RB homologues but Arabidopsis has only one copy,
RBR1 (Durfee et al., 2000). This provides an added advantage to the use Arabidopsis to study the role of this important protein in plants.

The primary objective of my doctoral research focuses on understanding the function of RBR1 in controlling development and the cell cycle in Arabidopsis. In my working hypothesis I predicted that up or down-regulation of RBR1 activity will disturb normal cell cycle and developmental control, particularly in those regions of the SAM that control the initiation of pattern formation and development of new organs. Specifically, I have designed the following experimental approaches;

A: Down-regulation of RBR1.
   - Constitutively: using a constitutive promoter.
   - Cell type/tissue specific: using promoters that are active only in certain cells/zones of SAM.

B: Up-regulation of RBR1 in a cell type/tissue specific manner.

For down-regulation of RBR1 expression, I applied antisense-RNA and RNA interference (RNAi) strategies. Antisense-RNA lines were expected to result in partial suppression of RBR1, while RNAi was expected to give stronger suppression. The aim was to generate plants with different dosage of RBR1.

For constitutive expression of RBR1, I used the 35SCaMV promoter. The cell type-specific suppression and over-expression of RBR1 in specific regions of the SAM was supposed to be achieved by using selected upstream promoter sequences to express the RBR1(RNAi) construct and the full-length AtRBR1 cDNA respectively. Several genes had been identified that are expressed in cells/zones of specific regions of the SAM. The same upstream "promoter" sequences were also used to express the reporter gene encoding GFP (Green Fluorescent Protein) and GUS (β-glucuronidase). I have chosen the upstream "promoter" sequences of five genes, viz. APETALA1 (AP1), CLAVATA1 (CLV1), CLAVATA3 (CLV3), SHOOTMERISTEMLESS (STM), and WUSCHEL (WUS). Expression patterns of these genes are used as markers for different important regions of the SAM (Brand et al., 2001).

This thesis encompasses several important yet quite different aspects of biology, mainly related to plants. It would be helpful to discuss these topics separately to have a better understanding of the research that I am going to present later. Hence, current status of our knowledge on the retinoblastoma gene and the protein; the plant cell cycle; plant development with a special emphasis on the SAM; RNA interference and its use to down-regulate genes have been discussed in detail in the next review-of-literature chapter.
Review of literature

Chapter 2

Review of literature

Retinoblastoma - the gene and the protein:

Retinoblastoma is the most common intraocular malignancy during infancy and childhood. Prior to this century, retinoblastoma was a uniformly fatal disease (Albert, 1987). It represents the phenotypic expression of an abnormal or absent tumor suppressor gene known as retinoblastoma (RB). Prior to the knowledge of RB, children having retinoblastoma used to be classified as having either sporadic or inherited retinoblastoma, though clinically and histologically these forms are indistinguishable from one another (Bishop et al., 1975). About 17 years ago, the gene (RB) was cloned (Lee et al., 1987; Fung et al., 1987). Shortly after the cloning, Kundson's "Two-Hit hypothesis" (Kundson, 1971) was validated by demonstrating that mutational inactivation of both the alleles of RB is necessary and sufficient for both hereditary and sporadic retinoblastoma. Later it was demonstrated that RB was mutated or deregulated in nearly every type of human cancer examined to date. Deregulation of RB is so frequent that many had argued it is a prerequisite for almost all human cancers (Johnson et al., 1995; Goodrich, 2003).

The retinoblastoma protein (pRB), also called p105, is the founding member of the family of so called "pocket proteins" that includes the p107 and p130 in human. These two other members of "pocket proteins" share many biochemical and structural properties with pRB. In-vivo studies with p107 and p130 revealed that their functions overlap extensively with one another and with pRB. Analysis of mice and cell lines derived from animals deficient in these proteins shows that individual members of this family also have distinct functions (Classon et al., 2001). The three members of the pocket protein family are differentially expressed during mouse development. Targeted disruption of all the three RB related genes in mouse embryonic fibroblast (MEF) cells make the cells immortal with a shorter cell cycle and also become resistant to G1 arrest following DNA damage, serum starvation and contact inhibition (Sage et al., 2000). RB<sup>−/−</sup>p107<sup>−/−</sup> and RB<sup>−/−</sup>p130<sup>−/−</sup> mice embryos die earlier than RB<sup>−/−</sup> with more pronounced cell cycle defects and increased cell death (Sage et al., 2000). MEF cells doubly or triply deficient in RB (rb1), p107 (rb1) and p130 (rb2) have remarkably elongated telomeres compared to those of wild type or RB<sup>−/−</sup> cells. The double and triple knockout MEF cell lines remain functional. They retain the same level of telomerase activity as well as maintain the telomere end capping function (Garcia-Cao et al., 2002). RB<sup>−/−</sup> mice embryos die at midgestation with inefficient erythropoiesis, abnormal cell cycle entry, extensive cell death in the liver, lens and nervous system as well as many other developmental defects leading to embryonic death by day 14.5 (Lee et al., 1992; Jacks et al., 1992; Lee et al., 1992; Sage et al., 2000).

Different types of cells appear to have different levels of pRB. Fibroblasts contain the lowest level of pRB while blood and neuronal cells contain amounts that are more easily detectable (Bignon et al., 1993). Overexpression of this protein resulted in dwarf mice and the degree of
dwarfism correlated roughly with the level of pRB. This demonstrates that the effect of pRB on overall development is closely dependent on its dosage (Bignon et al., 1993).

The first molecular function identified for pRB was as a repressor of G1-S phase transition in the mammalian cell cycle (Weinberg, 1995). The eukaryotic cell cycle consists of 4 major phases, G1, S, G2, M, in actively growing cells. A landmark to this scenario had been added. The "restriction (R) point transition", which occurs during mid-late G1 phase and thus subdivides G1 into two phases: early/mid G1 phase and late G1 phase. To many people, the R-point transition would imply another "check point". The later concept derived initially from the studies of the yeast cell cycle. The difference between R-point and other "check points" are very real. During a "check point", the cell ascertains that its metabolic household is in order, its genome is intact and the previous cell cycle step had been executed properly before it can move ahead to the next step in the cell cycle. The R-point transition, in contrast, implies the workings of an entirely different process. Here the cell assesses its cumulative exposure to specific signals, mostly extra-cellular, received over a period of time. All such signals are processed. Then the cell decides whether to proceed (i.e to pass the R-point). Alternatively the cell will cease its advance through G1 and will eventually exit the cell cycle, either proceeding towards G0 or into a post mitotic, possibly more differentiated state (Planas-Silva et al., 1997). The level of pRB does not seem to change during different stages of the cell cycle (Classon et al., 2001), but it undergoes extensive phosphorylation at a time when the cell passes through the R-point as defined by its acquisition of serum dependence in animal cell culture. Specific amino acids of pRB are sequentially phosphorylated. First by cyclin D-CDK4/6 during mid G1 and then by cyclin E-CDK2 during late G1 (Bonitti et al., 2001; Cooper et al., 2001). Hyperphosphorylated pRB loses its affinity towards many of its interacting partners. The most important among them are the E2F and DP class of transcription factors. The E2F class of proteins control transcription of many target genes by binding to their promoters in a sequence specific manner. Six members of the mammalian E2F family can be subdivided into activators (E2F-1, E2F-2 and E2F-3) and repressors (E2F-4, E2F-5 and E2F-6) (for review: Trimarchi et al., 2002). Most of the E2F target genes are involved in nucleotide metabolism and DNA replication. The members of the E2F family function as heterodimers with two members of the DP family of proteins. At the end of S phase, Cyclin A-CDK2 phosphorylates DP proteins. This leads to dissociation of E2F from DP and shuts off E2F dependent transcription of its target genes (Taya, 1997). It is also been suggested that the Cyclin A-CDK2 complex maintains a hyperphosphorylated state of pRB after S phase till pRB is dephosphorylated by PP16 phosphatase during mitotic exit or early G1 phase (Gutierrez et al., 2001; Bianchi et al., 2001, Cooper et al., 2001). A simplistic model of the roles of pRB, E2F and DP in the cell cycle is shown in figure-1. Human pRB has 16 phosphorylation sites, out of which 9 are phosphorylated by Cyclin E and the remaining by Cyclin D (Taya, 1997). Ectopic expression of cyclin E, but not cyclin D, was able to rescue the G1 block without affecting the RB-E2F pathway (Lukas et al., 1997; Leng et al., 1997). Cyclin E remains both rate limiting and essential for the G1-S transition in both RB +/− and RB +/+ cells. It implies that cyclin E and/or its associated kinase(s) target a critical substrate, other than pRB to overcome RB mediated G1-S block (Ohtani et al., 1995).
FIG. 1. The current model suggests that the retinoblastoma protein (pRB) controls G1-S transition and entry into the cell cycle. When pRB binds to E2F1 at G1 phase, expression of the target genes are suppressed. At late G1, pRB is phosphorylated by cyclin D-Cdk4/6 and then by cyclin E-Cdk2. This leads to the release of pRB from E2F and the expression of the target genes. Subsequently, cyclin A-Cdk2 binds E2F and phosphorylates DP, resulting in the release of E2F and DP heterodimers from DNA, and the expression of the target genes are suppressed again. (reproduced from: Taya, 1997).

Besides phosphorylation, pRB is also acetylated in a cell cycle dependent manner by p300. Acetylation of pRB prevents its phosphorylation. Acetylated pRB binds more strongly to MDM2, an E3 ubiquitin ligase (Buschmann et al., 2000). It is also been reported that acetylation regulates the differentiation-specific functions of pRB (Nguyen et al., 2004). MDM2 is also the main mediator between p53 and pRB. Several viral proteins like E1A of Adenovirus target p300 to prevent acetylation of pRB upon infection. This prevents the infected cells to undergo apoptosis. It also makes pRB more amenable to phosphorylation and induces the host cell to undergo replication, so that the viral genome can be replicated using the host replication machinery (Chan et al., 2001). Besides acetylation and phosphorylation, members of pocket proteins, including pRB, undergoes transglutaminase-dependent post-translational modification that plays an important role during apoptosis (Oliverio et al., 1997).

The retinoblastoma gene harbors at least one (the exact number depends on the organism) functional E2F binding site in its promoter, just like the E2F1 promoter itself. It enables such proteins to control their own expression through a feedback loop. Methylation of CpGs in or around the E2F binding sites of the RB promoter abrogate the ability of E2F to bind there and causes certain cancers (Di Fiore et al., 1999).

Repression of several promoters, including that of cyclin E, is accomplished by pRB through its interaction with members of the histone deacetylase (HDAC) protein family (Morrison et al., 2002). [For review on histone acetylation and gene regulation: Eberharter et al., 2002]. Histone deacetylase1 (HDAC1) has been shown to physically interact with pRB through the LXCXE motif (Magnaghi-Jaulin et al., 1998). Inhibition of histone deacetylase activity by trichostatin A (TSA) also inhibits pRB-mediated repression of several E2F-regulated promoters (Brehm et al., 1998). Deacetylation of histones by HDAC1 and subsequent change in chromatin structure helps to repress
E2F mediated transcription, as shown in figure-2. pRB also interacts with several histone methyltransferases (viz. Suv39H1 that specifically methylates K9 of histone H3) and HP1 that bind specifically to the histone H3 methylated at K9. Such interaction leads to the repression of several promoters, including that of the cyclin E gene (Vandel et al., 2001; Nielsen et al., 2001).

Can RB affect chromatin structure by other mechanisms? Chromatin structure appears to be different in RB+/+ and RB−/− MEFs. Chromatin in RB−/− cells is more accessible to micrococcal nuclease digestion, indicative of an open conformation. An alternate model on how RB might affect chromatin structure has been proposed, as briefly described in figure-3.

FIG. 2. A proposed model for HDAC and RB effect. Phosphorylation of pRB disrupts its binding with HDAC. Once HDAC is released, core histones are acetylated resulting in more open chromatin structure. This facilitates E2F-DP mediated transcription of their target genes (reproduced from: Brehm et al., 1999).

FIG. 3. The modified model on how pRB affects chromatin structure. pRB binds factors that are catalytic components of multisubunit complexes that alter chromatin structure by changing the acetylation status of histones and chromatin remodeling (reproduced from: Brehm et al., 1999).
However, biochemical studies have suggested that E2F is only one of many pRB-targets and, to date, at least 110 cellular proteins have been reported to associate with pRB (Morris et al., 2001). So far 97 genes have been identified as physiological targets of the pRB pathway. 74 among them are reported to be repressed by pRB and induced by E2F (Vernell et al., 2003). Data indicates that at least some of the remaining 23 genes are induced by pRB. In addition to the set of classic "E2F target genes", a novel group of "E2F regulated genes" have been identified. This novel group of E2F regulated genes are repressed by E2F/RB complexes in proliferating cells but induced in developmentally regulated and gender specific manner, at least in Drosophila (Dimova et al., 2003).

A consensus caspase cleavage site, Asp(D)-Glu(E)-Ala(A)-Asp(D)-Gly(G), popularly known as "DEAD box", is located in the C terminus of pRB. Cleavage at this site generates RB-ΔI. RB-ΔI is unstable and is degraded rapidly, resulting in the loss of pRB in cells undergoing apoptosis (Tan et al., 1998). Mice fibroblasts expressing RB1 protein mutated at this caspase cleavage site (RB-MI) are protected from apoptosis induced by the tumor necrosis factor-α type-1 receptor (TNFRI), but remain sensitive to cell death by DNA damage (Chau et al., 2002). DNA damage, on the other hand, induces apoptosis via p53. This cellular response via p53 makes use of signals from RB/E2F pathway (Lin et al., 2001). It is known that the E2F1 protein is acetylated and stabilized in response to DNA damage. Acetylated E2F1 has been shown to upregulate the transcription of a subset of its pro-apoptotic target genes e.g NK4α/ARF, Apaf-1, caspase 7 and p73. pRB mediated acetylation of histone H4 plays a major role in the transcription activation of these pro-apoptotic genes (Pediconi et al., 2003). pRB is also directly involved in the DNA damage mediated, p53 independent, apoptotic response. It can activate transcription of one of the survival genes BCL-2 via the transcription factor AP2 in a cell type specific manner. Through this interaction pRB directly inhibits apoptosis and promotes survival (Decary et al., 2002). Mutation of other genes such as e2f1 or e2f3 in rb-deficient animals prevents cell death that occurs in the central nervous system (Dyson, 2003).

In short, the retinoblastoma protein (pRB) has been implicated in many cellular and physiological processes including the G1-S phase transition during cell cycle (Weinberg, 1995; Kundsen et al., 2002), development/differentiation (Lee et al., 1994; Sage et al., 2000; MacPherson et al., 2003), both p53 dependent and p53 independent apoptosis (Hsieh et al., 1999; Decary et al., 2002; Hickman et al., 2002), endoreduplication (Jiang et al., 2000), RNA polymerase-III mediated transcription (rRNA synthesis) (Hirsch et al., 2000; Ciarmatori et al., 2001), maintenance of telomere length (Garcia-Cao et al., 2002) and chromatin structure (Brehm et al., 1999; Williams et al., 2000). It is becoming very important to address questions such as, which cell type needs the RB protein and why it becomes so important at certain times/stages of development (Dyson, 2003).

Among plants, an RBR-related gene (RBR) was first identified in maize (Grafi et al., 1996; Ach et al., 1997; Gutierrez, 1998). Subsequently it was found in many other higher plants viz. Arabidopsis, tobacco, pea, chenopodium, rice, coconut, populus (for review: de Jager et al., 1999; Durfee et al., 2000) and even in algae, Chlamydomonas reinhardtii (Umen et al., 2001). All the known retinoblastoma related proteins from different organisms show a great degree of conservation of their structure, as shown in figure-4, and biochemical properties. Almost all the known RB homologues, including those from plants, contain at least one nuclear localization signal (NLS),
FIG. 4. Retinoblastoma and its homologues are highly conserved among different organisms (reproduced from: Claudio et al., 2002)

several phosphorylation sites and one highly conserved cystine residue in the B domain. RBR loses almost all of its biochemical properties if this cystine residue is mutated (Ach et al., 2000). Figure-5 shows a schematic representation of the modular structure of human pRB and maize RBR1 protein. Most of the plants possess more than one RB homologue but Arabidopsis has only one, RBR1 (Durfee et al., 2000). It provides an added advantage to use Arabidopsis to study the role of this important protein in plants. Gordon-Kamm and colleagues (2002) showed that overexpression of maize RB homologue (ZmRBR1) arrests cell division in tobacco BY2 cells. The role of plant retinoblastoma related proteins with relation to viral infection, mainly by geminiviruses, have been studied more extensively (for review: Gutierrez, 2000). Geminiviruses replicate their small, single-
stranded DNA genome through double-stranded DNA intermediates using the host replication machinery (Settlage et al., 2001). As mature plant cells are not competent for DNA replication, a likely early step in geminivirus infection is virus-induced synthesis of host DNA replication enzymes (Hanley-Bowdoin et al., 1999). This idea was strongly supported by the accumulation of proliferating cell nuclear antigen (PCNA), the processivity factor of DNA polymerase δ, in differentiated cells of TGMV-infected plants (Nagar et al., 1995; Kong et al. 2000). Geminiviruses target and eventually inactivate the host RBR proteins to induce mature host cells to re-enter the cell cycle and undergo DNA replication. Geminiviruses fall into three subgroups—the begomoviruses, curtoviruses and mastreviruses—based on their genome structure and insect vectors (Kong et al., 2000). Different geminiviruses seem to have evolved two different mechanisms to interact with plant RBR proteins. One is dependent on well known LXCXE motif present in viral proteins, such as RepA (encoded by members of the Mastrevirus genus). Another group of viral Rep proteins interact with the host RBR protein in an LXCXE independent way, as they do not have an LXCXE motif. Such viral proteins are encoded by members of the Begomoviruses (e.g. AL1 in TGMV) and perhaps the Curtoviruses as well (Gutierrez, 2000). It is becoming clearer that RBR proteins are very potent targets for at least some plant pathogens to establish infection.

Plant homologues of the E2F and DP family of proteins have been identified in several plants including wheat (Ramirez-Parra et al., 1999; 2000), tobacco (Sekine et al., 1999), carrot (Albani et al., 2000), rice (Kosugi et al., 2002) and Arabidopsis (Mariconti et al., 2002; Kosugi et al., 2002). Arabidopsis has three E2F homologues, E2Fa, E2Fb and E2Fc with 46% overall sequence homology (Vandepoele et al., 2002). Two DP homologues (AtDPa and AtDPb) have also been cloned from Arabidopsis (Shen et al., 2002, Vandepoele et al., 2002). Besides E2F and DP, there are three "DP-E2F Like" (AtDEL1-AtDEL3) genes in Arabidopsis. Each contains two DNA binding domains

![Diagram of DNA-binding properties of Arabidopsis E2F-family proteins](image)

**FIG. 6.** Structural organization and DNA-binding properties of the *Arabidopsis* E2F-family proteins. The Rb-binding domain and the potential cyclin-dependent-kinase-cyclin-A (CDK–CycA)-binding domain and CDK–cyclin phosphorylation site are also indicated. Based on the conservation of different domains, the eight *Arabidopsis* proteins are classified into E2F, DP and DEL groups (reproduced from: Shen et al., 2002).
with high homology to E2F and DP but lack other conserved regions (Shen et al., 2002). This unique group of proteins has not been found in animals. Figure-6 shows the conservation of different domains among different homologues of E2F, DP and DP-E2F like (DEL) proteins in Arabidopsis.

DPα overexpressing (OE) plants have no obvious phenotype while E2Fa OE plants had enlarged cotyledons. Ectopic expression of E2Fa induced sustained cell proliferation in normally differentiated cotyledon and hypocotyl cells in Arabidopsis (De Veylder et al., 2002). The size of cells in the cotyledons of E2Fa OE plants were less than half but they contained about 3 fold more cells compared to the control plants. The phenotype was enhanced strongly by co-expression of E2Fa with its dimerization partner, DPα. Transgenic plants overexpressing both E2Fa and DPα were arrested early in development with enhanced endoreduplication (De Veylder et al., 2002).

Besides E2Fs and DPs, several other RBR1 interacting proteins have been identified in plants. Maize histone deacetylase, Rpd3-type, (ZmRpd3I) was shown to physically interact with the maize RB homologue, ZmRBRI. When mutated, ZmRpd3I affects endosperm development (Rossi et al., 2003). ZmRbpA1, a maize member of the MSI/RBAP family, facilitates this protein interaction. Tobacco protoplasts overexpressing both ZmRBRI and ZmRpd3I showed that the two proteins cooperate in repressing transcription. These findings represented the first indication that in plants too, the RB related protein, ZmRBRI, can recruit a histone deacetylase, ZmRpd3I, to control transcription (Rossi et al., 2002; 2003). The MSI homologue from tomato (LeMSH1) can also physically interact with maize RBR1. Three members of the MSI family have also been reported from Arabidopsis (Ach et al., 1997). At least one member of this protein family (AtMSH1) plays a role during reproductive growth (Hennig et al., 2003) and seed development (Kohler et al., 2003).

Although many of the core regulators of the cell cycle and other cellular processes are similar in both plants and animals, there are many differences. A major striking difference concerns programmed cell death (PCD) or apoptosis. Many key protein regulators (viz. p53, MDM2, caspases etc.) that play a vital role during PCD in animals are not found in plants so far, even in the fully sequenced Arabidopsis genome. We know that programmed cell death plays a major role in plant development and defense against pathogens (Greenberg, 1996; Lam et al., 2000; Liang et al., 2002). Either those proteins are simply not present or they do not have any sequence homologue in plants, including Arabidopsis. We have yet to understand these players.

As previously stated, ectopic expression of cyclin E can override G1 arrest imposed by pRB. Such experimental data, along with the fact that RB−/− mice can survive till mid-gestation, indicates that RB is not exclusively essential for cell cycle control. On the contrary, there is not a single instance showing RB deficient metazoans can ever overcome the developmental abnormalities associated with this mutation. Such a hypothesis that pRB is more important for development and differentiation compared to cell cycle control becomes attractive when we consider the proteins which interact with pRB. There are many interacting proteins that are not involved in the cell cycle but in development and/or differentiation (Taya, 1997). pRB and its homologues offer us the opportunity to understand how the different cellular processes such as the cell cycle, cellular differentiation and programmed cell death are connected to each other and how such cellular processes affect overall development in an organism. It might give us plausible clues on how such developmental events are
connected with different internal and external signals. Plant development is mechanistically different from that of animals. Plants do have a unique developmental program mediated by a unique set of proteins, as discussed later in this chapter. In contrast to development, core cell cycle machineries are more conserved between plants and animals. In the next section I discussed salient points of the plant cell cycle.

**Plant Cell Cycle:**

In all multicellular organisms cells are part of different organs and have defined spatial and temporal relationships with their neighboring cells in creating higher order structures. The term "cell cycle" implies the ever rolling cycle of events which is true for exponentially growing cell suspensions but might not be so relevant in vivo in the context of the whole organism. Cells must sense when they are required to divide and when division must cease or be modified, to allow differentiation into specialized organs to form and function (Francis, 1998). In other words, after every round of replication each daughter cell in a multicellular organism needs to take a fundamental decision whether to undergo another round of replication or exit the cell cycle and undergo differentiation or activate the programmed cell death pathway (Durfee et al., 2000).

Since the last decade of the 20th century a good amount of information on the plant cell cycle and genes that regulate it has been published (for review, Vandepoele et al., 2002; Dewitte et al., 2003; De Veylder et al., 2003). The discovery of retinoblastoma related proteins (RBR) and many of its interacting partners, viz. E2F, DP, histone deacetylase (HDAC) etc. in plants helped in establishing the fact that the core cell cycle machinery is conserved in higher eukaryotes, from human to plants. But very little is known about the role of the plant RB homologues even from the cell cycle point of view (for review, De Jager et al., 1999 and Durfee et al., 2000). Traditionally the major emphasis in plant cell cycle research concentrated on cyclins and their interacting partners, Cyclin Dependent Kinases (CDKs) (for a review on plant cyclins and CDKs: Stals et al., 2000; Joubes et al., 2000).

Plant cyclins can be classified into 9 groups: Cyclin A1, A2, A3, B1, B2, D1, D2, D3 and D4 (Renaudin et al., 1996). Most of these cyclins have multiple members, e.g in Arabidopsis, cyclin A1 has two members (CYCA1.1, CYCA1.2), A2 has 4 (CYCA2.1, CYCA2.2, CYCA2.3, CYCA2.3) and A3 has 4 (CYCA3.1, CYCA3.2, CYCA3.3, CYCA3.4) (Yu et al., 2003) while cyclin D3 has 3 members in Arabidopsis, viz. CYCD3a, D3b and D3c (Meijer et al., 2000). Most of the cyclins have characteristic expression as well as degradation patterns during the cell cycle. The time of expression and degradation plays a major role in determining at which phase of cell cycle they can form a complex with and thus activate the corresponding CDKs (Stals et al., 2000). The regulation of cyclins and CDKs plays a crucial role in determining the activity of pRB by altering its phosphorylation status and thereby influencing its binding with many of its partners such as E2F and DP. Among all the plant cyclins, CYCLIN D seems to play a crucial role in phosphorylating the RB homologue. In animals, CYCLIN D is expressed during G1-S phase and is a very good candidate to integrate different internal and external signals into the cell cycle (Sherr, 1996).
Among plants, Cyclin D group includes three genes, of which CYCD3.1 is the best studied. In cell cultures, CYCD3.1 mRNA level does not depend strongly on the position of cells in cell cycle (Menges et al., 2002). Rather CYCD3.1 expression depends on the availability of sucrose and plant hormones (Riou-Khamlichi et al., 2000). Addition of sucrose to sucrose deprived cell cultures results in the induction of CYCD3.1 in late G_1 phase (Menges et al., 2002), with the mRNA subsequently maintained at a relatively constant level in cycling cells (Ito, 2000; Dewitte et al., 2003). Other mitogenic plant hormones like auxin and gibberelins (Oakenfull et al., 2002), cytokinins (Riou-Khamlichi et al., 2000) and brassinosteroids (Hu et al., 2000; Boniotti et al., 2002; Sasse, 2003) have the ability to induce CYCD3. The expression of CYCLIND3.1 is restricted within the proliferating cells and can not be detected in the fully differentiated cells of Arabidopsis shoot apex (Dewitte et al., 2003). In Arabidopsis, CYCD2 and CYCD4 had been shown to respond to the sugar availability (Riou-Khamlichi et al., 1999; Healy et al., 2001). Tobacco CYCLIN D3 (NtCYCD3.1) has been shown to physically interact with the RB homologue in tobacco, NtRb1 (renamed as NtRBR1). In-vitro experiments have also demonstrated that NtCYCD3.1 can phosphorylate NtRBR1 with the help of CDC2 (Nakagami et al., 1999; Boniotti et al., 2001). This kinase activity fluctuates in a cell cycle dependent manner (Boniotti et al., 2001) while RBR1 is expressed not only in proliferating tissues but also, at higher levels, in differentiated tissues (Xie et al., 1996; Ach et al., 1997; Huntley et al., 1998).

CYCLIN A is also expressed during the G_1-S phase transition. Different A-type cyclins are expressed sequentially at different cell cycle stages, from late G_1/early S phase until mid M phase in synchronized BY2 tobacco cells (Reichheld et al., 1996). Local and transient induction of NtCYCA3.2 induces cell division in both the shoot apical meristem and leaf primordia (Wyrzykowska et al., 2002). Antisense expression of NtCYCA3.2 induces defects in embryo formation and impairs callus induction from leaf explants from tobacco. Arabidopsis plants that overexpress a GFP-NtCYCA3.2 fusion protein showed reduced cell differentiation and endoreduplication. Calli regenerated from leaf explants from these transgenic plants were defective in shoot and root regeneration. Expression of this fusion protein also dramatically modified plant morphology (Yu et al., 2003). Plants do not have all the homologues of cyclins and CDKs found in animals (based on sequence conservation). One striking difference is the absence of E type cyclins and presence of more than one type of CYCLIN A in the plant genomes (animals have only one type of Cyclin A), including the Arabidopsis genome (Yu et al., 2003). Though it is not yet been shown that CYCLIN A along with Cyclin A-dependent-kinase(s) can phosphorylate the RB homologue in plants, it has been proposed that NtCYCA3.2 is the functional homologue of animal cyclin E (Yu et al., 2003).

Two major classes of plant CDKs, known as A-type and B-type CDKs, have been studied in more detail. The A-type CDKs regulate both G_1-S and G_2-M transitions, whereas the B-type CDKs seem to control the G_2-M checkpoint only (Magyar et al., 1997; Porceddu et al., 2001). In addition, the presences of C-type CDKs and CDK-activating kinases (CAKs) have been reported (Magyar et al., 1997; Umeda et al., 1998; Joubès et al., 2001). The function of the C-type CDKs remains unknown while CAKs were shown to regulate the activity of the A-type CDKs. Eight CDKs (one A type, four B
type, two C type, and one E type) and four CAKs (three D type and one F type) have been reported
by homology-based annotation of the *Arabidopsis* genome (Vandepoele *et al.*, 2002).

In contrast to the cell cycle, plant developmental programs differ significantly from those of
animals. In the next section I discussed some aspects of plant development in more detail.

**Plant development and the Shoot Apical Meristem (SAM):**

Plants and animals have independently evolved multicellular development. Members of
each kingdom are composed of different types of cells, implying that the two kingdoms diverged
when their common eukaryotic ancestor was unicellular and that each lineage had a considerable
unicellular history after they separated. This indicates that each kingdom separately evolved its
mechanisms of cellular differentiation and cell-cell communications. Figure-7 shows different
milestones for plant and animal evolution. It appears that there is a gradient of difference between

![Diagram showing the evolution of multicellular plants and animals](image)

**FIG. 7.** Simplified diagram showing events in the evolution of multicellular plants and
animals from a unicellular, eukaryotic common ancestor. Blue shows prokaryotes; brown, the
eukaryotes ancestral to plants and animals; red, animals; and green, plants. Abbreviation:
ybp- years before present. (reproduced from: Meyerowitz, 1999)

plants and animals, from quite similar in housekeeping processes in nucleus and cytoplasm, to
logically similar but biochemically distinct regulatory networks of development and the control of
cellular differentiation, to utterly distinct means of communicating from each cell to its neighbors and
to the outside world (Meyerowitz, 1997). Eukaryotic photosynthesis evolved some 2,000 million
years ago in the oceans. When plants invaded the land, they found the supply and distribution of
water, minerals and light to be much more variable than in oceans (Gilroy *et al.*, 2001). As plants
usually have little choices over their immediate growth environments, they have developed an
enormous variability to increase their fitness. Phenotypic plasticity — that is, the capability of a single
genotype to generate many phenotypes — is a pronounced and unusual characteristic of plant
development (Trewavas, 1986). A specialty of plants is that they can modulate their body plan after embryogenesis, an ability that helps them to deal with the environmental changes that affect their growth (Carles et al., 2003). So, plants are able to respond to different and diverse internal and external signals successfully, as summarized in figure-8.

Two meristematic cell populations arise during embryogenesis and grow in opposite polar directions throughout plant life: the shoot apical meristem (SAM) generates the aerial parts of the plant, whereas the root apical meristem (RAM) generates the underground parts (Jürgens, 2001; Lasufs et al., 1998). For correct growth, plants must maintain a constant flow of cells through the meristem, where the input of dividing pluripotent stem cells offsets the output of differentiating cells. This flow depends on extracellular signaling within the SAM, governed by a spatial regulatory feedback loop that maintains a reservoir of stem cells, and on factors that prevent meristem cells from differentiating prematurely (Carles et al., 2003). The SAM continuously produces cells that will become incorporated into stem tissue, lateral organs (leaves and flowers) and axillary meristems (Long et al., 2000). The program for establishing the SAM differs from dicot to monocot, from gymnosperms to angiosperms, but broadly follows the same logic. It is believed that in dicots, like Arabidopsis, the SAM is established from the torpedo stage onwards and then maintained all through development. Figure-9 shows the establishment of the SAM during embryo development in Arabidopsis (Wolpert et al., 2002). Shoot growth then follows a modular pattern and gives rise to the whole arial part of the plant (Carles et al., 2003). One level of organization established during embryo development is the arrangement of SAM cells into tunica and corpus layers. In Arabidopsis and most other dicots, the tunica consists of an overlying L1 layer and a sub-epidermal L2 layer, each a single cell thick and remain clonally distinct by continuous anticlinal cell division (i.e divides in

FIG. 8. Different internal and external signals that plants need to respond to (adopted from: Gilroy et al., 2001).
a plane perpendicular to the layer). The cells in the L2 layer divide mostly anticlinally but sometimes divide both anticlinally and periclinally, particularly around the site of organ initiation (Vernoux et al., 2000). The corpus or the L3 layer lies below the tunica and consists of cells that divide in all planes (Brand et al., 2001). Such clonal layers of SAM organization are shown in figure-10B. L1 layer derivatives give rise to the epidermis of shoots, leaves and flowers while the L2 layer provides the mesodermal tissue and germ cells, and the L3 layer provides the vascular tissues and pith (Fletcher et al., 2000). Morphological and histological studies indicate that the SAM can also be divided into distinct domains or zones (histological zones)—a central zone (CZ), a rib zone (RZ) and a peripheral zone (PZ), as shown in figure-10A (Fletcher et al., 2000). The central zone contains relatively inactive, slowly dividing cells. The peripheral zone consists of more rapidly dividing cells that become incorporated into organ primordia. The rib zone lies internal to the other two zones and produces the

FIG. 9. The few cells (in red) give rise to SAM during torpedo stage of embryo development and are maintained all through the life of the plant. (adopted from: Wolpert et al., 2002)

cells that form the bulk of the stem. Cells in the central zone of the tunica appear to be symplastically isolated from those in the peripheral zone and they may define gradient fields in which small diffusible morphogen molecules can provide information to cells within local regions of the SAM (Rinne et al., 1998). The CZ is assumed to be 5-6 cells high with weak cytoplasmic staining (Gross-Hardt et al., 2003). All appendages including not only leaves but also new meristems that will give rise to flowers, are formed exclusively from cells in the PZ, and are inhibited from developing from the CZ (Brand et al., 2001). At the organ boundaries, organ separation is characterized by reduced growth and cell division. In developing organs, mitotic activity gradually ceases after the basic morphological domains have been established. Depending on the species, this change in proliferative activity occurs in basipetal direction. It also varies with cell type. In leaves, for example, cell division often continues longer in the layers that will give the palisade tissue (Vernoux et al., 2000). Localized signaling between different cells and regions of the SAM is a critical component of SAM function (Fletcher et al., 2000). Plasmodesmata connect different cells within tunica layers (Gisel et al., 1999) and also among neighboring cells of tunica and corpus (van der Schoot et al., 1999). This intercellular communication could involve transfer of "informational molecules" (mainly proteins) that establish a cytoplasmic continuity among neighboring cells. Thus the cells can act as a syncytium with continuous cytoplasm, called symplasm (Brand et al., 2001).

There is an additional level of SAM organization, determined by the expression patterns of the genes that control the establishment and maintenance of the SAM. These complex gene expression patterns develop stepwise during embryogenesis and are fully established in seedlings. Once they are set up during embryogenesis, these expression domains appear to be static as they can be found throughout all developmental stages (Brand et al., 2001). Figure-11 shows a representative domain structure within the SAM, depending on the expression patterns of some important genes such as **SHOOT MERISTEMLESS (STM), WUSCHEL (WUS), CLAVATA1 (CLV1), CLAVATA3 (CLV3) & APETALA1 (AP1)**. The brief characteristics of these genes are mentioned in the table-1 below. STM is required for SAM initiation and maintenance (Barton et al., 1993; Clark et al., 1996; Long et al., 2000). It encodes a homeodomain-containing protein of the KNOTTED class.

**FIG. 11.** **SHOOT MERISTEMLESS (STM)** is expressed all through the SAM but not in the primordial or flanking meristem. **WUSCHEL (WUS)** is expressed in the cells of the so called "organizing centre" while **CLAVATA3 (CLV3)** expresses in the few cells, known to be the stem cells. The **CLAVATA1 (CLV1)** expression domain is in between that of **WUS** and **CLV3**. **APETALA1 (AP1)** expression is restricted to the L1 layer of SAM in later stages of the floral meristem (adopted from: Brand et al., 2001).
Review of literature

Table-1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type of protein encoded</th>
<th>Function of the protein</th>
<th>mRNA expression pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLV1</td>
<td>Receptor kinase</td>
<td>Promotes differentiation</td>
<td>Underlying domain of L3 in SAM</td>
</tr>
<tr>
<td>CLV3</td>
<td>Receptor kinase</td>
<td>Promotes differentiation</td>
<td>L1 and L2 layer of the central zone of SAM</td>
</tr>
<tr>
<td>STM</td>
<td>Homeodomain transcription factor</td>
<td>Meristem identity and organ separation</td>
<td>Throughout the SAM. Its expression starts after that of WUS</td>
</tr>
<tr>
<td>WUS</td>
<td>Homeodomain Transcription factor</td>
<td>Establish and maintain stem cells in SAM</td>
<td>mRNA expression starts from 16-cell embryo stage. Later its expression is confined to a few cells in L3 layer of SAM</td>
</tr>
<tr>
<td>AP1</td>
<td>MADS domain Transcription factor</td>
<td>Transition from inflorescence shoot to floral meristem and development of sepals and petals</td>
<td>Strictly in the L1 layer of young floral primordia</td>
</tr>
</tbody>
</table>

(Long et al., 1998). Null mutations of STM block the establishment of the SAM, whereas weaker mutations lead to the depletion of undetermined cells in the SAM (Veit, 2004). STM expression first appears in a single, off-centre cell in the late globular stage and extends to a broad domain between the developing cotyledons in heart stage. Later, the STM domain extends throughout the meristem with the exception of primordia (Long et al., 1998; Aida et al., 1999).

The SAM contains approximately six to nine longterm stem cells that are arranged in three tiers. These stem cells can not be distinguished histologically but are thought to be located at the very tip of the CZ (Lenhardt et al., 2003). This position roughly coincides with the expression of CLAVATA3 (CLV3) gene, which has been used as a stem cell marker (Schoof et al., 2000; Haecker et al., 2001). The expression patterns of the WUS and CLAVATA gene family has been studied in great detail in different tissues, starting from SAM to the floral meristem to developing ovule, even in different ecotypes of Arabidopsis (Clark et al., 1997; Fletcher et al., 1999; Trotchaud et al., 1999; Schoof et al., 2000; Lenhard et al., 2001; Brand et al., 2001; Lohmann et al., 2001; Ishiguro et al., 2002). WUS is not expressed within the stem cells but in an underlying group of cells, termed the "organizing centre (OC)". WUS expression is established in subapical cells of 16-cell-stage embryos and becomes restricted to a small number of cells in the very centre of the cortex at later stages (Mayer et al., 1998). Unlike WUS and CLV3, CLV1 is not expressed in the OC or L1 or flanking meristems but its expression is detected above and around the WUS expressing OC (Clark et al., 1997; Clark, SE., 2001; Brand et al., 2001), as shown in figure-11. Sequential expression of the WUS and CLV3 genes is very important during embryogenesis in Arabidopsis, as shown in figure-12. WUS is necessary and sufficient to induce stem cell identity (Haecker et al., 2001). Loss-of-function mutants of wus result in premature termination of both the...
FIG. 12. Initiation of shoot meristem stem cells in the Arabidopsis embryo. An “organizing centre” (OC) precursor cell lineage expressing WUS (red) is established in the four subepidermal cells of the 16-cell embryo. As judged from CLV3 expression (blue), stem cells are induced in heart stage embryos. The thick line indicates the clonal boundary between apical and basal derivatives of the 8-cell embryo. (reproduced from: Gross-Hardt et al., 2003)

SAM and floral meristems after formation of a few organs. Ectopic expression of WUS can abolish organ formation and instead induce stem cell identity, even in the root meristem, based on the expression of the stem cell marker CLAVATA3 (CLV3) (Schof et al., 2000; Gallois et al., 2004). WUS has also been shown to be expressed in the nucellus and genetic analysis showed that WUS is not only required but sufficient for integument formation from the chalaza during ovule development (Gross-Hardt et al., 2002). Repression of WUS by AGAMOUS (AG) (a MADS domain transcription factor) is essential to terminate stem cell maintenance in flowers (Lenhard et al., 2001). CLAVATA 3 also acts as a repressor of WUS, loss of function of CLV3 results in an enlarged WUS expression domain and an increase in stem cell number (Lenhard et al., 2003). Mutations in any of the three CLAVATA genes (CLV1, CLV2 and CLV3) lead to progressive enlargement of the stem cell population. Genetic data suggest that they all act in the same WUS-CLAVATA pathway. CLV1 encodes a putative receptor kinase an with extracellular leucine-rich repeat receptor domain and an intracellular serine-threonine kinase domain. CLV2 encodes a protein with an extracellular domain similar to that of CLV1 but lacks the kinase domain and probably forms a membrane associated complex with CLV1 (Jeong et al., 1999; Waites et al., 2000). CLV3 functions as an extracellular ligand that binds to CLV1-CLV2 complex at the cell surface. Expression of the WUS and CLV genes in their strictly restricted domains are crucial in maintaining the stem cells in relation to the OC in the

FIG. 13. Signaling between stem cells and the organizing center (OC). (A) WUS expression in the OC promotes a yet unidentified signal that specifies the overlying cells as stem cells. The stem cells signal back via CLV3 and restrict the size of the OC. (B) Model for the protection of the OC from CLV3 signaling. Binding of CLV3 (red circles) to the CLV1 complex (blue crescents) results in repression of WUS in L3. An excess of CLV1 receptor complex prevents CLV3 from entering the underlying OC cells, allowing WUS to be expressed there. (reproduced from: Haecker et al. 2001)
SAM, as summarized in figure-13. The recessive clv mutants (clv1, clv2 and clv3) of Arabidopsis accumulate too many cells in the central zone of the SAM and floral meristems, resulting in fasciation of the shoot and initiation of supernumerous floral organs. Initially it was suggested that CLV1 and CLV3 are needed to limit cell divisions within SAM (Clark et al., 1995; 1996). Later it was shown that the increase in size of the CZ in clv mutants is due to an increase in the size of the cells rather than an increase in cell number (Laufs et al., 1998). It also has been proposed that the CLV3 gene is necessary for the transition of cells from the central to the peripheral zone (Laufs et al., 1998).

Floral meristems arise on the flanks of the SAM and are organized in a similar fashion. Floral meristems are not equivalent to the SAM because they generate different types of organs in different spatial arrangements and because they ultimately terminate in the formation of the female reproductive organs (Fletcher, 2002). Their separate identity is conferred by floral identity genes such as LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL) etc which is transcribed in initiating floral meristem primordia (Mandel et al., 1992; Gustafson-Brown et al., 1994; Kempin et al., 1995; Blazquez et al., 1997; Ferrandiz et al., 2000). The floral homeotic genes specify the identity of four types of floral organs from the outer whorls to the inner whorls, according to the well characterized ABC model, briefly described in figure-14 (Fletcher, 2002; Wolpert et al., 2002).

AP1 regulates the transition from inflorescence shoot to floral meristem and the development of sepals and petals (Irish et al., 1990; Mandel et al., 1992; Hempel et al., 1997; Yalovsky et al., 2000). Data indicate that AP1 alone can convert inflorescence shoot meristems into floral meristems, and ectopic AP1 expression can dramatically reduce the time to flowering (Mandel et al., 1995; Weigel et al., 1995). Constitutive expression of the Arabidopsis AP1 gene shortens the juvenile phase and promotes precocious flower initiation, which results in normal and fertile flowers that set fruits and seeds in citrange, a hybrid Citrus (Citrus sinensis L. Osbeck X Poncirus trifoliata L. Raf.) (Pena et al., 2001). AP1 is initially expressed uniformly throughout floral

![Expression of wild-type gene functions](image)

**FIG. 14.** ABC Model for flower development in brief: In the wild type flower, it is assumed that 3 regulatory functions, a, b, c are expressed in whorls 1 and 2, 2 and 3 and 3 and 4 respectively. "a" alone specifies sepal, "a" and "b" together specify petals, "b" and "c" stamens and "c" alone carpels. Mutations alter the regions within the meristem where these functions are expressed. (reproduced from: Wolpert et al., 2002)
meristem while no expression can be detected in the inflorescence meristem (Mandel et al., 1992; Gustafson-Brown et al., 1994; Hempel et al., 1997; Parcy et al., 1998; Liljegren et al., 1999; Wolpert et al., 2002). During later stages of flower development, its expression declines in the centre of floral meristem, and gradually AP1 transcript becomes restricted to the cells of the L1 layer in floral primordia, that eventually give rise to the outer two whorls of flowers. Thereafter AP1 expression is restricted to whorls 1 and 2, i.e. in sepals and petals (Gustafson-Brown et al., 1994; Wolpert et al., 2002). AP1 RNA also accumulates in the pedicle (floral stem), axis of cauline leaves and at low levels in the stem, where the rosette leaves originate (Gustafson-Brown et al., 1994).

It is conceivable that developmental programs in plants are well connected with cell division and internal and external signals. However, there has no direct connection between cell cycle and such internal and external signals with plant development yet been established, particularly with any of the genes that influence plant developmental programs described till date. RBR1 is a potential candidate that might correlate different internal and external signals and cell cycle with plant development.

**RNA interference (RNAi):**

RNA silencing is a conserved mechanism that occurs in various eukaryotic organisms and leads to targeted degradation of homologous RNA sequences or their translational inhibition. The potency of double stranded RNA (dsRNA) in activating RNA silencing was first demonstrated in 1998 by Fire and Waterhouse along with their colleagues (Fire et al., 1998; Waterhouse et al., 1998). Waterhouse and his colleagues demonstrated that transforming plants with virus or reporter gene constructs that produce RNAs capable of duplex formation confer virus immunity or gene silencing on the plants (Waterhouse et al., 1998). Fire and his colleagues found that the injection of double-stranded (ds) RNA into Caenorhabditis elegans led to an efficient sequence specific gene silencing, which was referred to as RNA interference (RNAi) (Fire et al., 1998). This RNA silencing phenomenon is observed in many other organisms including fission yeast, Schizosaccharomyces pombe (Schramke et al., 2003), Drosophila (Bernstein et al. 2001), mammalian cells including those from humans (Paddison et al., 2002; 2003). In animals it is known as RNAi, while it was termed post-transcriptional gene silencing (PTGS) in plants and quelling in fungus, when they were discovered in the early 1990's (Voinnet, 2002). Now the term "RNA interference" (RNAi) has been used to describe the same RNA silencing phenomenon in all the organisms (for review; Dykxhoorn et al., 2003). RNA silencing seem to play an important biological role in protecting the genome against instability caused by the accumulation of transposons (Sijen et al., 2003); repetitive sequences (Zamore et al., 2000), and as an adaptive defense against viral attack in plants (Waterhouse et al., 2001; Eckard, 2003; Wang et al., 2003, Lu et al., 2003). It also plays important roles during development of plants (Aukerman et al., 2003; Palatnik et al., 2003, Kasschau et al., 2003), C. elegans (Ketting et al., 2001), fission yeast (Verdel et al., 2004) and Drosophila (Pal-Bhadra et al., 2004). The RNAi phenomena can be experimentally activated by ds-RNA. Ds-RNA is a much more potent silencing trigger than either strand alone (Denli et al., 2003). The mechanism by which ds-RNA
silences gene activity is not completely understood. Growing evidence suggests that it involves cleavage of both the ds-RNA and the corresponding endogenous mRNA into 21-24 nucleotide long fragments (Hamilton et al., 1999; Dudley et al., 2002). These small degraded RNA products, also known as small interfering RNAs (siRNA), have symmetric 2-3 nt 3' overhangs, 5'-phosphate and 3'-hydroxyl termini. The structure of siRNA is characteristic of an RNase-III type enzymatic cleavage that led to the identification of the highly conserved DICER family of RNase-III enzymes as the mediators of the ds-RNA cleavage (Bernstein et al., 2001). DICER family members are large, multidomain proteins that contain a putative RNA helicase domain, two tandem ribonuclease III (RNase III) domains, and one or two dsRNA-binding domains. The tandem RNase III domains are believed to mediate endonucleolytic cleavage of dsRNA into small interfering RNAs (siRNAs). DICER, siRNAs and one or more of the ARGONAUTE family proteins form a protein-RNA complex.

FIG. 15. The RNA interference pathway: (A) Short interfering (si)RNAs. Molecular hallmarks of an siRNA include 5' phosphorylated ends, a 19-nucleotide (nt) duplexed region and 2-nt unpaired and unphosphorylated 3' ends. (B) The siRNA pathway. Long double-stranded (ds)RNA is cleaved by the RNase III family member, Dicer, into siRNAs in an ATP-dependent reaction. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA. (C) The micro (mi)RNA pathway. Drosha processes the long RNA into ~70 nt miRNA precursor inside nucleus and then transported out to cytoplasm. Then Dicer processes the miRNA precursor to produce ~22-nt miRNA. Unlike siRNAs, the miRNAs are single stranded and are incorporated into a miRNA–protein complex (miRNP). miRNAs pair with partial sequence complementarity to target mRNA leading to translational repression. (adopted from: Dykxhoorn et al., 2003; Mallory et al., 2004)
the RNA-Induced Silencing Complex (RISC), in Drosophila and mammals. siRNAs need to be 5' phosphorylated before they can be incorporated into RISC (Schwarz et al., 2002). The short duplex siRNAs are then unwound by RNA helicase activity. The antisense strand then guides the RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved at a single site at the centre between the guide siRNA and the target mRNA i.e 10 nt from the 5' end of the siRNA (Elbashir et al., 2001). Several studies have shown that this process is restricted to the cytoplasm (Hutvagner et al., 2002 and Kawasaki et al., 2003). Figure-15 briefly describes the different steps in RNA silencing.

It has been observed that mismatches >1-2 bp within the 21- to 24- nt siRNA effectively disrupt proper degradation of the target mRNA (Dillin, 2003). Later this mechanism was discovered and the term micro RNA was coined to describe such small RNA molecules that do not degrade target RNA but disrupt their translation or do both. Over 200 genomically encoded miRNA have been identified in several organisms including plants (for review, Reinhart et al., 2002; Kinder et al., 2003). DICER in animals and DICER LIKE1 (DCL1) [previously known as CARPEL FACTORY (CAF)], a DICER homolog in plants, generate microRNAs (miRNAs) as well (Schauer et al., 2002). These are 21-24 nt long, single-stranded noncoding RNAs thought to regulate endogenous mRNA expression (Lee et al., 1993; Reinhart et al., 2000, 2002; Grishok et al. 2001; Hutvágner et al., 2001; Ketting et al., 2001; Mourelatos et al., 2002; Park et al., 2002; Vazquez et al., 2004). miRNAs can reside on either the 5’ or 3’ side of the double-stranded stem (Lee et al., 1993; Pasquinelli et al., 2000; Lee et al., 2000). In humans, pre-miRNAs are transcribed as longer primary transcripts that are processed by RNaseIII DROSHA into ~70 nt long fragments with compact, folded structures (pre-miRNAs) in the nucleus (Lee et al., 2003; Schmittgen et al., 2004; Mallory et al., 2004). Then they are exported to the cytoplasm, where they are cleaved by DICER to yield mature miRNAs of 21-24 nt (Lee et al., 2002; Lee et al., 2003). Exportin-5 (EXP5) mediates this efficient nuclear export of the short miRNA precursors (pre miRNAs) to the cytoplasm (Lund et al., 2004). Once fully formed, the mature 21 nt RNAs alter gene expression by binding to multiple copies of partially complementary sites within the 3’ untranslated region (UTR) of their target mRNAs and repress translation (Mallory et al., 2004). Animal miRNAs are only partially complementary to their target mRNAs. This partial complementarity has been proposed to cause miRNAs to repress translation of their targets, rather than direct target cleavage as in siRNA pathway, described in figure-15C (for review, Ruvkun, 2001; Hutvágner et al., 2000). Plant miRNAs have far greater complementarity to cellular mRNAs and also have been proposed to mediate target RNA cleavage via a siRNA-like mechanism (Llave et al., 2002; Rhoades et al., 2002). Like animal miRNAs, many plants miRNAs do not accumulate ubiquitously but instead show preferential accumulation in specific tissue types. This suggests the existence of transcriptional and/or post-transcriptional mechanisms that partially and temporally regulate miRNA expression (Mallory et al., 2004). Intriguingly, a large fraction of plant miRNA targets are either known to be transcription factors that are involved in cell fate determination or are homologous to such transcription factors (Rhoades et al., 2002). This strongly suggests that the miRNA pathway is important for proper plant
development (Palatnik et al., 2003). This is strongly supported by the plant mutants that are impaired in miRNA accumulation (Mallory et al., 2004).

A key step in RNA interference is the assembly of the RISC, the protein-RNA complex that mediates the target RNA cleavage. It has been shown that the two strands of siRNA duplexes are not equally eligible for assembly into RISC. Rather, both absolute and relative stabilities of the base pairs at the 5' ends of the two siRNA strands determine the degree to which each strand participates in the RNAi pathway. siRNA duplexes can be functionally asymmetric, with only one of the two strands being able to trigger RNAi. It has been suggested that single-stranded miRNAs are initially generated as siRNA-like duplexes whose structures predestine one strand to enter the RISC and the other strand to be destroyed (Schwarz et al., 2003). Hence, the thermodynamic properties of small RNAs play a critical role in determining the molecule's function and longevity, possibly biasing the steps involved in duplex unwinding and strand retention by RISC.

Temperature also has an affect on the efficacy of RNAi. Low temperature inhibits RNA silencing-mediated defense by the control of siRNA generation. Szittya and his colleagues have shown that at low temperature both virus and transgene-triggered RNA silencing are inhibited. Therefore, in the cold, plants become more susceptible to viruses, and RNA silencing-based phenotypes of transgenic plants are lost. In contrast, RNA silencing was activated and the amount of siRNAs gradually increased with rising temperature. However, temperature does not influence the accumulation of micro (mi) RNAs which play a role in development. This indicates that although the two classes of small (si and mi) RNAs are mechanistically similar, they are generated by different nuclease complexes (Szittya et al., 2003).

Besides si/mi RNA pathways, dsRNA can also induce gene silencing through a different mechanism, more commonly known as "transcriptional gene silencing (TGS)". This is mainly achieved through methylation of homologous DNA sequences (Vaistij et al., 2002; Meng et al., 2003). dsRNA induced TGS provides another efficient tool to specifically downregulate gene(s) in plants (Sijen et al., 2003). This can potentially overcome an important disadvantage of RNAi or PTGS that can not be used to inactivate promoter sequences or to target nuclear RNAs or introns (Scherer et al., 2003). It is beyond the scope of this thesis to discuss more about TGS (for review; Vaucheret et al., 2001).

The proteins that participate in RNAi pathways are quite conserved among different organisms (Hutvagner et al., 2002). Besides DCL1, a member of DICER family of proteins, plants do contain an RNA directed RNA polymerase SDE1/SGS2 (Dalmay et al., 2000) which may amplify dsRNA used as a trigger for silencing; the RNA helicase, SDE3 (Dalmay et al., 2001; Reinhart et al., 2002) and the ARGONAUTE family proteins. The exact biochemical functions of the ARGONAUTE family proteins have not yet been discovered but they were shown to influence plant development and stem cell fate determination, besides its role in gene silencing. Arabidopsis has 10 members of the ARGONAUTE family. Two of them, AGO1 and ZWILLE, are more extensively studied. AGO1 is very important for RNAi while ZWILLE is not at all involved in PTGS or RNAi (Carmell et al., 2002). Recently several other proteins are identified that take part in RNA interference in plants, viz. DICER LIKE1 (DCL1), HUA ENHANCER 1 (HEN1) and HY Ponastic
LEAVES 1 (HYL1) have also been identified and partially characterized. All the three proteins (DCL1, HEN1 and HYL1) have a nuclear localizing signal (NLS) and dsRNA binding domain and when mutated reduce miRNA accumulation as well (Vazquez et al., 2004). dcl1 null alleles are embryo lethal (Schauer et al., 2002). The phenotype for the partial loss-of-function mutants, dcl1-7, dcl1-8 and dcl1-9 are viable but all are sterile, confirming that DCL1 is needed not only for miRNA metabolism but also for reproduction in plants (Schauer et al., 2002; Vazquez et al., 2004). HEN1 seem to control AGAMOUS (AG) expression (Chen et al., 2002; Park et al., 2002). hen1 mutants are viable but they produce very few seeds and have many developmental defects overlapping with that of some of the dcl1 partial loss-of-function mutants (Park et al., 2002; Vazquez et al., 2004). Several HYL1 target mRNAs, including meristem and auxin related genes, have been identified. PTGS does occur in hyl1 mutants, suggesting HYL1 has a specialized function in the plant miRNA pathway (Vazquez et al., 2004).

We know that RNA can move intercellularly in plants and can act as a local signaling molecule (Wu et al., 2002). It has been reported that RNA can function as a long distance, systemic signaling molecule transported through phloem in plants (Kim et al., 2001). It has also been reported that the RNAi signal in the form of small RNAs can systematically spread throughout the plant via phloem (Van Houdt et al., 2003). Most viruses and long-distance post-transcriptional gene silencing (PTGS) signals are excluded from the shoot apex. This revealed the presence of an RNA signal surveillance system that allows selective entry of RNA into shoot apex (Foster et al., 2002). We do not yet know if the small RNA molecules (si and mi RNAs) can spread within the cells of SAM via cell-cell or cell-to-cell movement.

RNAi seems to provide a wonderful tool to biologists to substantially downregulate the expression of any gene(s) of interest. (For review: Scherer et al., 2003). There are some conflicting reports about the efficacy of RNAi to downregulate its target gene in animals. Gene expression profiling analysis in human cells reveals that siRNA can affect non-target gene expression that has as few as eleven contiguous nucleotide similarities to the siRNA sequences (Jackson et al., 2003). Another research group suggested that siRNA-induced gene silencing is highly gene-specific and does not produce secondary effects detectable by genome-wide expression profiling (Sijen et al., 2001). Although siRNAs appear to be highly sequence-specific, the extension of RNAi-mediated silencing to sequences 5' upstream to the mRNA sequence complementary to the siRNA could generate secondary siRNAs that could potentially target other mRNAs with sequence similarity. Such a phenomenon, termed "transitive RNAi" has been shown to occur in C. elegans (Sijen et al., 2001) and Drosophila (Zamore et al., 2000) but not in human cells (Chi et al., 2003). Such a phenomenon has not yet been reported in plants either. Later, Semizarov and his colleagues reasoned that if siRNA caused non-specific effects, then comparison of three different siRNA experiments targeting three different genes should share some commonality within their transcriptional profiles. They tested that hypothesis and could not find any "significant commonality". They found out that though that high does of siRNAs did indeed induce many non-specific genes which are known to be involved in apoptosis and the stress response. Lower doses did not have such unspecific effects and ultimately they concluded that "siRNA is a highly specific..."
tool for targeted gene knockdown and a reliable approach for large-scale screening of gene function" (Semizarov et al., 2003). Later, it has been reported that dsRNAs induce the interferon (IFN) response in mammals (Moss et al., 2003; Sledz et al., 2003). It was then proposed that dsRNAs induce the IFN response in mammals, thereby shutting down translation, inducing RNaseL and then apoptosis. Such mammals (human, mice) do not show "transitive RNAi" effect, unlike worms (Dillin, 2003). It should be mentioned here that, unlike animals, there is no systemic study, reported so far, to ascertain the off-target effects or side effects (e.g induction of defense/immune pathways) of RNAi in plants. Numerous reports have been published using this new technology in plants to downregulate gene expression (Chuang et al., 2000; Escobar et al., 2001; Guo et al., 2003; Harmon et al., 2003; Zhao et al., 2003; Berg et al., 2003; Kurata et al., 2003; Stevens et al., 2004; Ketelaar et al., 2004). Published data indicate that RNAi is very specific in plants and does not alter the expression of even closely related genes, at least in Arabidopsis (Zhao et al., 2003; Berg et al., 2003). This is a welcome relief while using the RNA interference technique by plant biologists, particularly those using Arabidopsis, as we still cannot generate recombination based gene specific knock-outs, unlike our animal colleagues. RNAi is thus a very important tool to study the function of an important gene, such as RBR1, whose loss of function possibly causes lethality and for which finding a T-DNA or transposon tagged mutant lines might be very difficult.
Materials and Methods

Chapter 3

Materials and Methods

1. Materials:

1.1: Plant material: Wild type Arabidopsis, Columbia-0 ecotype, plants was used in all the experiments mentioned in this thesis.

1.2: Bacterial Strains:

1. E. Coli: DH5a (GIBCO, Basel, Switzerland)
XL-1-Blue (Stratagene, La Jolla, California, USA)


1.3: Plasmids:

- pBluescript SK (+) and KS (+): Amp' (Stratagene, La Jolla, California, USA)
- pCAMBIA1303: (Kan'Hyg') (CSIRO Plant Industry, Canberra, Australia)
- pCAMBIA1303B [modified pCAMBIA1303]
- pCATRBR563AS [35S:RBR1 (Antisense)]
- pC2.5 (vector control)
- PCARB3473-S [modified pCAMBIA1303B with full length RBR1 cDNA]
- pCAR3134SG
- pCATRBR563Ri [35S:RBR1(RNAi)]
- pCARi-323 [vector containing RBR1(RNAi)]
- pAP1Ri323 [RBR1(RNAi) construct driven by AP1 promoter]
- pCLV1Ri323 [RBR1(RNAi) construct driven by CLV1 promoter]
- pCLV3Ri323 [RBR1(RNAi) construct driven by CLV3 promoter]
- pSTM Ri323 [RBR1(RNAi) construct driven by STM promoter]
- pWUSRi323 [RBR1(RNAi) construct driven by WUS promoter]
- pAP1R3473S [AP1 promoter driving full length RBR1 cDNA]
- pCLV1 R3473S [CLV1 promoter driving full length RBR1 cDNA]
- pCLV3 R3473S [CLV3 promoter driving full length RBR1 cDNA]
- pSTM R3473S [AP1 promoter driving full length RBR1 cDNA]
- pWUS R3473S [WUS promoter driving full length RBR1 cDNA]
- pC1303BG [modified pCAMBIA1303B for expressing eGFP]
- pAP1C1303BG [AP1 promoter driving eGFP expression]
- pCLV1C1303BG [CLV1 promoter driving eGFP expression]
Materials and Methods

- pCLV3C1303BG [CLV3 promoter driving eGFP expression]
- pSTM C1303BG [STM promoter driving eGFP expression]
- pWUSC1303BG [WUS promoter driving eGFP expression]
- pCGUS [modified pCAMBIA1303B for expressing GUS, driven by different promoters]
- pAP1CGUS [AP1 promoter driving GUS expression]
- pCLVICGUS [CLV1 promoter driving GUS expression]
- pCLV3CGUS [CLV3 promoter driving GUS expression]
- pSTMCGUS [STM promoter driving GUS expression]
- pWUSCGUS [WUS promoter driving GUS expression]

1.4: Enzymes:
- Restriction enzymes: New England Biolabs (NEB), Allschwil, Switzerland
- Asp718 (restriction enzyme): Roche Diagnostics (Schweiz) AG; Rotkreuz, Switzerland
- T4 DNA ligase, T4 polynucleotide Kinase, T4 DNA polymerase, RNaseH, RNasel: Exonucleaselll (DNase): New England Biolabs (NEB), Allschwil, Switzerland
- Shrimp Alkaline phosphatase: Roche, Rotkreuz, Switzerland
- Pfu polymerase: Amersham Biosciences Europe GmbH, Otelfingen, Switzerland.
- Taq polymerase: Eurobio, Basel, Switzerland
- Superscript II™ Reverse-transcriptase: Invitrogen, Basel, Switzerland

1.5: Kits:
- Promega™ Wizard SV A34 Plasmid DNA miniprep isolation kit: Catalys Serva (Promega), Wallisellen, Switzerland.
- Qiagen Plasmid Midi kit: Qiagen, Basel, Switzerland.
- QIAquick Gel Extraction Kit: Qiagen, Basel, Switzerland.
- QIAquick PCR Purification Kit: Qiagen, Basel, Switzerland.
- PCR DIG probe synthesis kit: Roche Diagnostics GmbH. (Mannheim, Germany).
- Advantage RT for PCR (cDNA synthesis) kit: Becton Dickinson (BD), Allschwil, Switzerland.
- Nucleon Phytopure Plant DNA extraction kit: Amersham Biosciences Europe GmbH, Otelfingen, Switzerland.
- MicroPoly(A) pure (Plant mRNA isolation) kit: Ambion (Europe) Ltd, Cambridge, United Kingdom.
- SYBR green PCR Master Mix (for real time PCR): Applied Biosystems, Rotkreuz, Switzerland.
- ECL Western Blotting analysis system (for detecting HRP conjugate secondary antibody): Amersham Pharmacia Biotech AB, Uppsala, Sweden.
1.6: Oligonucleotides:

All oligonucleotides were synthesized at Microsynth Gmbh (Balgach, Switzerland) in the desalted grade at "genomic" scale.

List of Oligos/primers (5'→3'):

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### Materials and Methods

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**AtActin2-1252R1**
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ACCACGAACCAGATAAGACAAGACA
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**AtActin2-1F1**
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TAGTGTAGCTGCTGCCGCTGTTG
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### 1.7: Chemicals/Reagents: All the reagents that are not mentioned below are obtained from Fluka Chemie AG (Buchs, Switzerland).

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<td>Formaldehyde Solution 37%</td>
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<td>4',6-Diamidino-2-Phenyl Indole (DAPI)</td>
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<tr>
<td>Gelrite (Gelrite agar)</td>
<td>Duchefa, Haarlem, The Netherlands</td>
</tr>
<tr>
<td>Glycerol (Molecular biology grade, RNase free)</td>
<td>Sigma, Buchs, Switzerland</td>
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</table>
## Materials and Methods

<table>
<thead>
<tr>
<th>Material/Equipment</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybond N+ nylon membrane</td>
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<td>Hygromycin B solution</td>
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<td>Kanamycin sulfate</td>
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<td>100 mM dNTP set</td>
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<td>MS Medium, Micro and Macro incl. Vitamins</td>
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<td>Schleicher &amp; Schuell, Dassel, Germany</td>
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<td>Nylon membrane, positively charged</td>
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<td>Phenol</td>
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<td>Protease Inhibitor cocktail for plant cell and tissue culture</td>
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<td>Rotiphorese Gel 40 (Acrylamid/bis 40%)</td>
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<td>Spectinomycin sulphate</td>
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<td>Lehle Seeds, Round Rock, TX 78680-2 , P.O. Box 2366, USA</td>
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<tr>
<td>Technovit 7100</td>
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<td>Toluidine blue</td>
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<td>Trizol™</td>
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<td>Trypan Blue</td>
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<td>Water saturated Phenol</td>
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<td>X-Gluc (5-Bromo-4-chloro-3-indolyl-b-D-glucuronic acid-cyclohexylammonium salt)</td>
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</tr>
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</table>

### 1.8: List of specialized technical equipments and softwares:

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<th>Equipment</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cameras</td>
<td>Photo Camera, Contax 167MT Kyocera, Kyoto, Japan</td>
</tr>
<tr>
<td></td>
<td>Digital still camera, Sony-DSC-F505V Sony, Tokyo, Japan</td>
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</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCD camera</td>
<td>Zeiss, Jena, Germany</td>
</tr>
<tr>
<td>Axiocam HRC</td>
<td></td>
</tr>
<tr>
<td>Microscopes</td>
<td></td>
</tr>
<tr>
<td>Hitachi S-900 Scanning Electron Microscope</td>
<td>Hitachi, Tokyo, Japan</td>
</tr>
<tr>
<td>Leica DM-IRBE (Confocal Laser Scanning Microscope)</td>
<td>Leica Microsystems, Wetzlar, Germany</td>
</tr>
<tr>
<td>Axioplan-2</td>
<td>Carl Zeiss, Gottingen, Germany</td>
</tr>
<tr>
<td>M²BIO</td>
<td>Carl Zeiss, Gottingen, Germany</td>
</tr>
<tr>
<td>Stemi SV11</td>
<td>Carl Zeiss, Gottingen, Germany</td>
</tr>
<tr>
<td>Wild M3C (Low magnification stereo Microscope)</td>
<td>Wild, Heerbrugg, Switzerland</td>
</tr>
<tr>
<td>Microtome</td>
<td>Leica Microsystems, Nussloch, Germany</td>
</tr>
<tr>
<td>Leica RM 2155 (rotary microtome)</td>
<td></td>
</tr>
<tr>
<td>Thermal cycler (PCR machine)</td>
<td>MJ Research Inc. Watertown, Massachusetts, USA</td>
</tr>
<tr>
<td>PTC-200 Peltier Thermal Cycler</td>
<td></td>
</tr>
<tr>
<td>Real Time PCR</td>
<td>Applied Biosystems, California, USA</td>
</tr>
<tr>
<td>ABI Prism® 7700 Sequence Detection system</td>
<td></td>
</tr>
<tr>
<td>Software for picture processing</td>
<td>Adobe Photoshop 7 Adobe Systems, San Jose, USA</td>
</tr>
<tr>
<td>Software for nucleotide and protein sequence analysis</td>
<td>Discovery Studio Gene (DS Gene): Version 1.5 Accelrys Inc. San Diego, USA</td>
</tr>
<tr>
<td>WinGene and WinPep</td>
<td>Lars Hennig, ETH, Zurich, Switzerland</td>
</tr>
<tr>
<td>Software for Real-Time PCR data analysis</td>
<td>sequence detection software for ABI Prism® 7700 Applied Biosystems, California, USA</td>
</tr>
<tr>
<td>Software for PCR primer design</td>
<td>Primer3 online software <a href="http://frodo.wi.mit.edu/cgi-bin/primer3/primer3">http://frodo.wi.mit.edu/cgi-bin/primer3/primer3</a> <a href="http://www.cgi">www.cgi</a></td>
</tr>
</tbody>
</table>

2. Methods:

The common molecular biology techniques were followed as discussed in Sambrook et al (2001). The modified portion of the protocols as used is mentioned below.
Materials and Methods

2.1: Seed treatment and plant growth conditions: The Arabidopsis seeds were treated with 70% ethanol for 5 min and followed by 7 min with 5% sodium hypochlorite solution with (0.01%) Triton-X-100. Then the seeds were washed 3 times with sterile distilled water and were sown either on MS (MS with vitamins+1% Sucrose+0.3% Gelrite) or MS with hygromycin (MS with vitamin+1% Sucrose+50µg/ml hygromycin+0.3% Gelrite) or on autoclaved soil (“Einheitserde”, H. Gilgen optima-Werke, Munchenstein, Switzerland). After 2 days of stratification at 4°C under dark conditions, the seeds were put in the smaller growth chamber (Weiss Technik AG, Kilchberg, Switzerland) under long day condition, with 16 hours light (mixed cold fluorescent and incandescent light, 100-200 µM/m²/s) and 8 hours darkness with 80% relative humidity and 23 (±2)°C temperature. The plants/seedlings on soil were put under the same long day conditions in the bigger growth chamber (Conviron, IG Instrumenten-Gesellschaft AG, Zurich, Switzerland). Age of the seedlings is referred to as number of days after germination, i.e (unless stated otherwise) 3 days more after sowing. For example, 3 days old seedlings mean 3 days after germination and 6 days after sowing. Some T3 WUS:RBR1(RNAi) plants were grown in presence of 5’Azacitidine (10µM) along with hygromycin and (1%) sucrose for 8 days and then transferred to soil for further growth.

2.2: Preparation of competent cells for Agrobacterium: Agrobacterium strain (LBA4404) were grown at 28°C with constant shaking at 250 rpm in LB with appropriate antibiotics until the culture attains OD600 of about 0.75. The 250 ml culture was then incubated on ice for about 30 min, centrifuged at 3000g for 5 min at 4°C. The supernatant was discarded and the pellet was washed with 0.5% NaCl. After a brief centrifugation at 3000g, the pellet was dissolved in 1ml of ice-cold 20 mM CaCl2 solution. It was dispensed in 100µl aliquots into pre-chilled eppendorf tubes and immediately dipped into liquid nitrogen. These frozen aliquots were stored at -80°C for future use.

2.3: Transformation of agrobacteria by freeze-thaw method: 0.5 – 1 µg of plasmid DNA was added into the thawed competent agrobacteria, mixed and was incubated on ice for about 30 min. Then it was frozen in liquid nitrozen for 1 min and thawed at 37°C for 5 min. 600µl of LB was added and incubated at 28°C for about 4 hours with shaking at 250 rpm. Aliquots were then plated on LB agar plates containing kanamycin (50µg/ml), rifampicin (25µg/ml) and streptomycin (50µg/ml).

2.4: Transformation of Arabidopsis plants by vacuum infiltration: This protocol was adopted and modified from floral dip method of transformation (Zupan et al. 2000; Clough et al. 1998). Agrobacterium cells containing the binary and the helper plasmids were grown in LB in presence of kanamycin (50µg/ml), rifampicin (25µg/ml) and streptomycin (50µg/ml). The overnight grown culture was used to inoculate fresh medium (at 1:10 ratio) with same antibiotics. In the mean time all the matured flowers and siliques of the Arabidopsis plants are clipped off. Once the OD600 of the culture reaches about 0.75, the cells were centrifuged at 4400g at room temperature. The pellet was washed with 5% sucrose solution and then resuspended in MS medium with vitamins and 5% sucrose. 150µl of Silwet/L of sucrose solution was added. Plants were dipped into the Agrobacteria solution upside down and kept under vacuum for about 45 sec. These plants were then put horizontally on a plastic
tray and kept under total darkness. After 2 days, they were put straight and watered till the plant was matured or siliques turned from green to yellow. Then the plants were allowed to dry and seeds were harvested.

2.5: Segregation analysis: Wild type Arabidopsis seeds do germinate on MS media containing hygromycin. But they can not grow further and die within 4-5 days after germination. Many of the transformants seem to die at that very young age. Such deaths seem not due to the lack of hygromycin resistance gene but most probably due to the expression of the RNAi transgene. This created a problem for segregation analysis for knowing the number of transgene(s) loci integrated. So, to maintain uniformity and consistency, I considered those seedlings as "resistant" that were able to germinate and then survive till 7 days after sowing (DAS) while those were considered as "susceptible" that germinated but died within 7 DAS. The seeds that could not germinate at all were considered "non-viable" and were not considered while calculating the "resistant" versus "susceptible" ratio. All the "resistant" versus "susceptible" ratios were statistically validated by the chi square ($X^2$) test at 5% level ($P_{0.05} = 3.841$, df=1) to check the fitness of different transgenic lines for 3:1 segregation pattern. All the lines that had an $X^2$ value less than 3.841 were accepted to have single locus transgene(s) integration.

2.6: Cloning binary vectors: First 563 bp fragment of RBR1 cDNA was PCR amplified using Pfu polymerase, pBluescript SK+ (p4A1.1) having RBR1 cDNA as templates and Xho1-Di-AtRB, Cla1-Di-AtRB and AtRBR563XbaI, BamH1A/RBR1 primer combinations. These two 563 long fragments were then ligated with the 300bp long myc-tag obtained from pART6M (after digesting with BamHI and XhoI). This 1.4 kb long fragment was then cloned at ClaI and XbaI sites in pART6M (map given in Appendix-1) to get pART6MidRB. It was then digested with NotI, end filled with T4 DNA polymerase and then digested with SpeI. This resulting 3.6 Kbp fragment was then cloned in pCAMBIA1303 at Smal and XbaI sites to get pCATRBR563Ri [35S: RBR1 (RNAi)] construct. Two clones were sequenced at the junctions and were confirmed to be the right ones.

To get pCATRBR563AS [35S: RBR1 (Antisense)], first N-terminal 563 bp fragment of RBR1 cDNA was PCR amplified using Pfu polymerase, p4A1.1 as template and Nhe1AsAtRb and Cla3AtRB primers. This fragment was cloned in pART6M at XbaI and ClaI sites to get pART6MRB563as. It was then digested with NotI. This linearised plasmid was end filled with T4 DNA polymerase and then digested with SpeI. The resulting 2.7 Kbp fragment was cloned in pCAMBIA1303 at Smal and XbaI sites to get pCATRBR563AS.

For cloning the vector control (VC) plasmid, pART6M was digested with NotI. The linearised plasmid was end filled and then digested with SpeI. This 2.5 Kbp fragment was cloned in pCAMBIA1303 at Smal and XbaI sites to get p2.5 (Vector Control). Figure-16 shows a schematic diagram of pCATRBR563Ri, pCATRBR563AS and p2.5 (will now be referred to as 35SRi, 35SAS and VC respectively). For cloning RBR1 (cDNA), RBR1(RNAi) and GFP/GUS under the control of
FIG. 16. Schematic diagram of 35SRI, 35SAS and VC vector constructs.

Five different promoters, I first made pC1303B, a derivative of pCAMBIA1303 in following way. Two oligos, MCS-1 and MCS-2 are mixed in equimolar amount and incubated for 30 mins at 94°C. It was digested with MfeI and BstEII. It was then cloned in pCAMBIA1303 at EcoRI and BstEII sites to give rise to pC1303A. A second oligo pair (1303B-Oligo1 and 1303B-Oligo2) was cloned in pC1303A at Apal and BamHI sites to get pC1303B.

p4A1.1 was digested with Smal and Xhol to take out the 3743 bp long RBRI cDNA fragment. This fragment was cloned in pC1303B at SpeI and SalI site to get pCARB3473-S (fig-17A). This was the basic construct into which all the five different promoters (AP1, CLV1, CLV3, 

FIG. 17. Representative diagram with important restriction sites of different vector constructs (see text for detail).
STM and WUS) were dropped in for the expression of full length RBR1 cDNA under the control of those promoters. GFP containing pRTL2 (map given in Appendix-1) was digested with Ncol and BamHI. The resulting 720 bp fragment was cloned in pCATRBR3473-S at Ncol and BamHI sites to get pCAR3473SG, where RBR1 full length cDNA was fused with GFP to get pCAR3134SG (fig-17B). HindIII sites were present in the RBR1 cDNA sequence at position 323, 1266 and 1676. pCAR3473SG was digested with HindIII and Ncol. The larger fragment of the vector was eluted; end filled and then ligated back to get pCAR323SG (fig-17C). The first 326 bp of RBR1 cDNA was PCR amplified using Pfu polymerase, BstEII-AR-F1 and BamHI-AR323 primers. This 326 bp long fragment was cloned in pCAR3473SG at BstEII and BamHI sites to get pCAR1-323 (fig-17D). eGFP containing pCL60 (map given in Appendix-1) was digested with NotI, end filled and redigested with XbaI. The resulting 720 bp long eGFP fragment was cloned in pC1303B at SpeI and Hpal sites to get pC1303BG (fig-17F). GUS containing pHRintG plasmid was digested with BamHI and HindIII. The resulting 1.8 Kbp fragment was cloned in pCAMBIA1303B at BamHI and HindIII sites to get pCGUS (fig-17E). All the promoter regions for the selected genes, viz. AP1, CLV1, CLV3, STM and WUS were PCR amplified using Pfu polymerase, forward primers having Kpnl and the reverse primers having Apal sites and Arabidopsis genomic DNA as templates. These PCR amplified upstream promoter regions were then cloned at Kpnl and Apal sites of different vectors, viz. pCARB3473-S, pCARi-323, pC1303BG and pCGUS. The resulting "promoter" RBR1, RBR1(RNAi), GFP and GUS constructs are shown in fig-18 below. For STM, AP1 and CLV1, about 1.5 Kbp upstream from the translational start ATG was cloned while for CLV3 and WUS, it was about 1.1 Kbp and 1.7 Kbp respectively. All the following 23 binary vectors were used to transform Columbia-0 ecotype Arabidopsis plants; pCATRBR563Ri, pCATRBR563AS, p2.5 (Vector Control); pAP1Ri323, pCLV1Ri323, pCLV3Ri323, pSTMRI323, pWUSRi323, pAP1R3473S, pCLV1R3473S, pCLV3R3473S, pSTM3R3473S, pWUSR3473S, pAP1C1303BG, pCLV1C1303BG, pCLV3C1303BG, pSTM1C1303BG, pWUSC1303BG, pAP1CGUS, pCLV1CGUS, pCLV3CGUS, pSTMCGUS and pWUSCGUS.

2.7: GUS staining: Fresh tissue sample was incubated at 37°C in GUS staining buffer [0.1M Sodium/potassium phosphate, pH 7.0; 10mM sodium EDTA; 0.1% Triton X-100; 5mM potassium
Materials and Methods

ferrocyanide; 5mM potassium ferricyanide; 0.1% X-Gluc (5-Bromo-4-chloro-3-indolyl-b-D-glucuronic acid-cyclohexyl ammonium salt)]. The tissues were then incubated in 70% ethanol overnight before proceeding for subsequent steps.

2.8: Histological analysis: The protocol for histological analysis was adopted from several sources (Wyrzykowska et al., 2002; Schoof et al., 2000; Siddiqi et al., 2000; technical notes for low viscosity Spurr’s resin kit, Ted Pella Inc., Redding, California, USA). Tissue samples or seedlings were fixed in 2% gluteraldehyde and 2% paraformaldehyde in 0.05M PIPES solution (pH 7.2). Then 3 times of 5 min vacuum infiltration was performed (releasing vacuum every minute) before incubating in fresh fixative for overnight at 4°C. The samples were washed thrice with the PIPES solution. Then step wise dehydration was performed at 4°C, in: 30%, 50%, 75%, 90% and 100% for about 2 hour in each step. The samples were then incubated for 24 hours in absolute ethanol at 4°C with at least twice exchanging EtOH with fresh ones. The samples were embaded in Technovit-7100 resin as per manufacturer’s instructions with slight modifications as mentioned below. The incubation time with solution-A was increased to overnight instead of 1 hour. Thin sections of either 7μm or 2μm were cut and then put on conventional SuperFrost® glass slides (Menzel Glaser, Braunschweig, Germany). Those sections were stained with toluidine blue solution (0.002% toluidine blue in 1% aqueous solution of sodium tetraborate) for about 10-15 min. Rinsed thoroughly at least 3 times with distilled water to get rid of excess stains. The sections were dried thoroughly before covering them with DePex and then coverslips.

2.9: Clearing ovules, seeds and other plant tissues for DIC analysis: The seeds and ovules were cleared following the protocol of (Yadegari et al. 1994) with slight modifications. In short, the siliques were opened with very fine Becton Dickinson 1cc insulin syringe (code: U-100 28G1/2; order no. 9410; Becton Dickinson, Dublin, Ireland) over a glass slides. It was then put in fixing solution (Ethanol:Acetic acid: 9:1) for overnight at 4°C. Then washed first with 90% EtOH for 30 mins and then stored in 70% EtOH. Once ready for mounting on slide for microscopic observation, it was put in the clearing solution (66.7g Chloralhydrate and 8.3g glycerol dissolved in 25ml ddH2O. Once dissolved, volume was adjusted to 100ml with ddH2O) for overnight incubation at room temperature. The cleared samples were observed under Differential Interference Contrast (DIC) optics of Zeiss Axioplan-2 microscope.

Whole plant tissue or seedlings were cleared with Herr’s solution. First the fresh tissue or the seedling was fixed with freshly prepared 4% formaldehyde for 1 hour at room temperature. It was then gradually dehydrated with different strength of EtOH (30%, 60%, 85% and 100%), 10 min in each step. The sample was incubated overnight in absolute EtOH. The dehydrated tissue/seedling then incubated in Herr’s solution (Lactic acid 85%-2 part, Chloral hydrate-2 part, water saturated phenol-2 part, Methyl salisylate-2 part, Xylene-1 part) for 24 hours before observing under microscope with DIC optics.
2.10: Microscopy: Light microscopy and photomicrographs were taken by Zeiss Axioplan-2 or M²BIO or Stemi SV11 microscopes. Axioplan-2, or M²BIO was fitted with Zeiss Axiocam HRC digital CCD while Stemi SV11 was attached with a Contax 167MT analog film camera. Scanning electron microscopy (SEM) was performed on Arabidopsis SAM according to Fleming et al., (1999). Confocal Laser Scanning Microscopy was performed with GFP filters as per manufacturer’s instructions.

2.11: Molecular analysis:
2.11.1: Plasmid isolation: All miniprep plasmid isolations were done with Promega™ Wizard SV A34 Plasmid DNA miniprep isolation kit while Qiagen Plasmid Midi kit was used for higher volume of culture and yield.

2.11.2: Extraction of genomic DNA, total RNA and Poly-A RNA: Plant genomic DNA was extracted by Nucleon Phytopure® Plant DNA extraction kit (Amersham Biosciences Europe GmbH, Otelfingen, Switzerland) while total RNA was isolated by TRIZOL®, reagent as per manufacturer’s instructions. Poly-A RNA from plant samples were isolated by MicroPoly(A) pure® (Plant mRNA isolation) kit [Ambion (Europe) Ltd, Cambridge, UK]. Unless specifically stated, the entire DNA, RNA and protein isolation was done with rosette leaves.

2.11.3: DNA and RNA blot analysis: All the DNA and RNA blot analysis was performed by DIG labeled, PCR amplified probes using Roche kit, as per manufacturer’s instructions (DIG application manual for filter hybridization). For DNA blots, 5-10μg of genomic DNA was digested with appropriate restriction enzyme(s) overnight before proceeding towards the subsequent steps. For RNA blots to detect RBR1 transcripts, about 1ug of poly-A RNA was used.

2.10.4: Real Time PCR and RT-PCR: For real-time PCR, 1-3μg total RNA was used to prepare cDNA using Advantage RT for PCR (cDNA synthesis) kit (Becton Dickinson, Allschwil, Switzerland) in 20μl reaction volume. Out of that, 1μl was used as a template for real-time PCR using SYBR green [cDNA template-1μl, SYBR green PCR master mix-12.5μl, 10μM forward primer-2μl, 10μM reverse primer-2μl, ddH₂O-7.5μl]. Primers were designed as per recommendations of ABI Prism® 7700 users’ manual and Primer3 online software (http://frodo.wi.mit.edu/cgi-bin/primer3/ primer3_web.cgi). All the primers used for real time PCR had a Tₘ not less than 60°C, had an amplicon below 150 bp and were designed around the intron-exon junctions from cDNA sequences. The data was analyzed using “sequence detection software”. After each experiment, the quality of the PCR amplification, i.e unwanted amplifications, was checked using “melting curve analysis” procedure and also by running the PCR products on 2% agarose gels. This helped to check the quality of the real-time PCR data. Each sample was run in triplicate. Average Cᵥ values was calculated and normalized to Cᵥ values for either GAPDH or ACTIN2, as indicated. The
following primer combinations were used for real-time PCR for detecting GAPDH, AtGAPDH433F and AtGAPDH553R; for ACTIN2, AtActin2-1146F1 and AtActin2-1252R1; for RBR1, SgRB-F2 and SgRB-R2; RB2819F1 and RB2970R1; RB524F1, RB641-R1 and RB700R1.

For semi quantitative RT-PCR, cDNA was prepared from 5μg of total RNA using Superscript II™ Reverse-transcriptase, as per manufacturer’s instructions, in 25μl final reaction volume. Out of which 1μl was used as a template for each PCR reaction. For DNA POLYMERASE5, CYCLINB1.1, CYCLIND3 and GAPDH the following primer combinations were used for RT-PCR (At-POL-D-108F and At-POL-D-1150R1), (At-CycB1-273F1 and AtCycB1-1161R1), (At-CycD3-74F1 and At-CycD3-925R1) and (AtGAPDH433F and AtGAPDH-1218R1) respectively.

2.10.5: Protein immuno blot: Total soluble protein was isolated using extraction buffer2 (Urea 7M, Thiourea 2M, DTT 20mM, CHAPS 2%, Brij35 0.5%, Tris base 40mM, PMSF 2mM, Trypsin inhibitor 0.1mg/ml, Sigma plant protease inhibitors cocktail 20μl/ml, Sodium fluoride 2mM). 3% acrylamide gel was used as stacking (upper) gel while 8% acrylamide gel was used as separating (lower) gel. Polyclonal antibody against 3 different peptides from RBR1 protein was raised in rabbit (Eurogentec Inc., Belgium). The sequence of the peptides in context of RBR1 protein is shown in figure-19. The primary anti-RBR1 peptide antibody was used at a dilution of 1:1000 while the chicken anti-goat IgG secondary antibody (Santa Cruz Biotechnology, California, USA) used as per manufacturer’s instructions. Protein concentration was determined by amido black method. 5-10μl of total soluble protein was mixed with ddH2O to a final volume of 200μl. 800μl of coloring solution (10% acetic acid, 90% methanol, 0.05% Amido black w/v) was added to that. It was centrifuged for 10 min at 15,000 rpm. Supernatant was discarded and the pellet was washed with decoloring solution (10% Acetic acid, 90% Methanol) and centrifuged again for 10 min at 15,000 rpm. The pellet was dissolved in 0.2N NaOH and absorbance was measured at 605 nm. The protein concentration was determined by the formula: Abs₆₀⁵=0.0258 X protein concentration (μg/ml).

Unless stated otherwise, 30μg of protein was loaded for each lane. Some salient features and domain structure of the RBR1 protein is mentioned in figure-19, in the next page.
FIG. 19. Some salient features and domain structure of RBR1 protein. The sequences in red indicate the peptides used to raise polyclonal antibodies against RBR1 in rabbit.
Chapter 4

Results

1: Low transformation efficiency with all the \textit{RBR1(RNAi)} constructs: After completing the cloning of the different constructs, I started transforming wild type \textit{Arabidopsis} (Columbia-0 ecotype). A significant amount of effort was invested to transform wild type \textit{Arabidopsis} (Columbia-0 ecotype) with different RNAi constructs. Different variations of flower dip and vacuum infiltration method were used to increase the transformation efficiency, particularly for the 35S::\textit{RBR1(RNAi)}. It was observed that the vacuum infiltration with 45 sec vacuum was giving the best result (detail in materials and methods section). Subsequently all the transformations were performed with that procedure. Transformation with different RNAi constructs resulted in a very low transformation efficiency, about 0.1% while it was about 2-3% in case of other, non-RNAi constructs. The transformation efficiency was even lower for 35S::\textit{RBR1(RNAi)} [for simplicity it will now be referred to as 35SRi]. The efficiency varied widely and the highest efficiency was 0.03%.

2: "\textit{Promoter}"::\textit{RBR1} lines had no apparent phenotype: None of the \textit{RBR1} over-expressing lines under the control of different "promoters" had any obvious phenotype. The number of transgenes integrated was confirmed by segregation and DNA blot analysis in \textit{WUS::RBR1} lines. Table-2 shows the segregation analysis for \textit{WUS::RBR1} to determine lines with a single transgene insertion. The segregation analysis indicates that line 11-1 was having a single copy insert. This was confirmed by DNA blot analysis of the next generation (T2) plants (fig-20). Genomic DNA was digested with Ncol and the first 326 bp PCR amplified DIG labeled \textit{RBR1} cDNA (\textit{RBR1}^\textit{e}) was used as a probe. Apart from line 11-1; line 7-2, 8-1 and 8-4 also have single copy transgene integration. Full length integration of the transgene was confirmed with another DNA blot analysis by using Asp718 and BamHI to digest genomic DNA and the same DIG labeled \textit{RBR1}^\textit{e} as probe.

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of seeds sown</th>
<th>Germinated &amp; grown (resistant)</th>
<th>Germinated but did not grow (susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>(X^2) (P_{0.05}=3.841) (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>30</td>
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<td>5</td>
<td>0.6:1</td>
<td>57.75</td>
</tr>
<tr>
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<td>51</td>
<td>22</td>
<td>12</td>
<td>2.3:1</td>
<td>1.03</td>
</tr>
<tr>
<td>8-1</td>
<td>97</td>
<td>74</td>
<td>18</td>
<td>5</td>
<td>4.1:1</td>
<td>1.45</td>
</tr>
<tr>
<td>8-2</td>
<td>95</td>
<td>66</td>
<td>27</td>
<td>2</td>
<td>2.4:1</td>
<td>0.81</td>
</tr>
<tr>
<td>8-3</td>
<td>75</td>
<td>38</td>
<td>20</td>
<td>17</td>
<td>1.9:1</td>
<td>2.78</td>
</tr>
<tr>
<td>8-4</td>
<td>84</td>
<td>56</td>
<td>28</td>
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<td>2:1</td>
<td>3.11</td>
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<td>8-5</td>
<td>87</td>
<td>0</td>
<td>0</td>
<td>87</td>
<td>!!!</td>
<td>!!!</td>
</tr>
<tr>
<td>8-6</td>
<td>95</td>
<td>18</td>
<td>14</td>
<td>63</td>
<td>1.2:1</td>
<td>6.00</td>
</tr>
<tr>
<td>10-1</td>
<td>99</td>
<td>93</td>
<td>3</td>
<td>3</td>
<td>31:1</td>
<td>24.5</td>
</tr>
<tr>
<td>10-2</td>
<td>74</td>
<td>72</td>
<td>2</td>
<td>0</td>
<td>36:1</td>
<td>19.62</td>
</tr>
<tr>
<td>11-1</td>
<td>101</td>
<td>72</td>
<td>25</td>
<td>4</td>
<td>2.9:1</td>
<td>0.03</td>
</tr>
</tbody>
</table>
Asp718 is an isoschizomer of KpnI. Both these DNA blots indicate that line 7-2, 8-1, 8-4 and 11-1 had full length and single copy insertion. Line 11-1 was selected for future use.

FIG. 20. DNA blot analysis of WUS::RBR1 T2 lines using NcoI and RBR1−326 probe. All the lines tested here had single copy transgene insertion. The lower bands indicate endogenous RBR1.

FIG. 21. DNA blot of WUS::RBR1 T2 lines using Asp718 and BamHI, with RBR1−326 probe. All the lines tested had full length transgene integration.

3: “Promoter”::RBR1(RNAi): I transformed Arabidopsis with different constructs using SAM specific “promoters” to express RBR1(RNAi) to understand the role of RBR1 in different domains and layers in the SAM. Four of the five different “promoter”::RBR1(RNAi) lines had specific and reproducible phenotypes, except CLV1::RBR1(RNAi). These are discussed in detail below.

3.1: CLV1::RBR1(RNAi): None of the 15 primary T1 transformants had any obvious phenotype. So these lines were not pursued any further.

3.2: CLV3::RBR1(RNAi): Most of the independent primary transformants (T1) of CLV3::RBR1 (RNAi) had slow growth and smaller leaves. Three out of eight (37.5%) T1 transformants had terminal flowers. Figure-22A shows two 34 days old T1 line 22-1 and 18-1. Most of the T1 plants, including lines 22-1 and 18-1 had smaller leaves (fig-22B) and produced very few seeds. Data for the segregation analysis for six T1 lines has shown in table-3 below. Subsequent generations that grew from the seeds of these primary transformants (T1) did not have any obvious phenotype, unlike their parents, and grew normally.
Table 3. Segregation analysis for CLV3::RBR1(RNAi) T1 lines:

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of seeds sown</th>
<th>Germinated but did not grow (susceptible)</th>
<th>Germinated &amp; grown (resistant)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>$P_{0.05}=3.841$ (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-1</td>
<td>115</td>
<td>24</td>
<td>91</td>
<td>0</td>
<td>3.8:1</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>13-1</td>
<td>33</td>
<td>12</td>
<td>14</td>
<td>7</td>
<td>1:1</td>
<td>6.21</td>
<td></td>
</tr>
<tr>
<td>13-2</td>
<td>109</td>
<td>29</td>
<td>69</td>
<td>11</td>
<td>2.4:1</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td>13-3</td>
<td>85</td>
<td>27</td>
<td>45</td>
<td>13</td>
<td>1.7:1</td>
<td>6.00</td>
<td></td>
</tr>
<tr>
<td>18-1</td>
<td>112</td>
<td>19</td>
<td>90</td>
<td>3</td>
<td>4.7:1</td>
<td>3.33</td>
<td></td>
</tr>
<tr>
<td>22-1</td>
<td>97</td>
<td>1</td>
<td>94</td>
<td>2</td>
<td>94:1</td>
<td>29.06</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 22. (A) Two 34 days old CLV3::RBR1(RNAi) primary transformants. They were slow growing and had terminal flowers. (B) The primary leaves of line 21-1 and 18-1 are of smaller in size as compared to wild type Columbia plants.

FIG. 23. 33% of the AP1::RBR1(RNAi) T1 transformants showed altered petal development (inset) while about 80% of the T1 plants were sterile.
3.3: **AP1::RBR1(RNAi):** All independent transformants of AP1::RBR1(RNAi) had normal vegetative growth but about 80% (7 out of 9) of them were sterile. Three of the nine (33.3%) T₁ lines had terminal flowers (line 1-1, 1-4, 5-2). Flowers of another 33.3% of the T₁ transformants were showing *apetala-1* (*ap1*) like phenotype (line 1-1, 1-4, 5-3), i.e the development of the petals were affected, as shown in figure-23. None of these three T₁ plants produced any seed. Line 5-2 also did not produce any seed either. Among all the nine T₁ independent transformants only two, line 5-1 and 1-3, produced seeds.

3.4: **STM::RBR1(RNAi):** The primary transformants (T₁) of STM::RBR1(RNAi) had a wide range of phenotypes. Four among the 25 T₁ transformants (17.4%) seem to have multiple SAMs (fig-24A) and these lines did not grow much further. Four different T₁ lines had normal looking flowers but did not produce any seed. Three other T₁ lines (12%) had proper cotyledons but had altered phylotaxy (fig-24B). Such plants had severely altered phylotaxy, yet managed to produce seeds. A few T₀ seeds germinated on the selection media containing hygromycin but grew very slowly. They were maintained on MS (with sucrose) but without having hygromycin. Later a callus like cell mass was observed around their leaves and shoot apex (figure-24C). Ultimately they died around 70 days after sowing. Although most of the T₁ lines managed to produce at least a few seeds unfortunately subsequent generations of these lines did not show any obvious phenotype but grew normally like wildtype plants.

3.5: **WUS::RBR1(RNAi):** All the T₁ seedlings of WUS::RBR1(RNAi) lines had a slower initial growth as compared to control (WUS::GFP) seedlings. Later they recovered and grew normally. Nine out of eleven (81.8%) T₁ lines had a fasciated primary inflorescence or stem. Figure-25 shows

![Image](image-url)
FIG. 25. Most of the WUS::RBR1(RNAi) lines showed stem fasciation. (A) Six of the independent T1 transformants. All, shown in the picture had fascinated stems. (B) Close up view of the fasciated stem of line 1-7 and (C) line 1-3. All plants are 24 days old.

some of the 24 days old WUS::RBR1(RNAi) T1 plants. The segregation analysis indicated the number of transgene(s) inserted (table-3). Three lines (line 1-3, 1-8 and 1-11) were thought to have single copy insert and selected for DNA blot analysis. DNA blot analysis using those three T2 lines was performed using PstI and a 708 bp long DIG labeled, PCR amplified GFP probe (fig-26). Line 1-3 and 1-8 had a single insert. Hence progenies of line 1-3 and 1-8 were used for later experiments.

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of seeds sown</th>
<th>Germinated &amp; grown (resistant)</th>
<th>Germinated but did not grow (susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>$P_{0.05} = 3.841$ (df=1)</th>
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</thead>
<tbody>
<tr>
<td>1-1</td>
<td>77</td>
<td>59</td>
<td>17</td>
<td>1</td>
<td>3.5:1</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>90</td>
<td>59</td>
<td>29</td>
<td>2</td>
<td>2.0:1</td>
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<td></td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<td>65</td>
<td>28</td>
<td>2</td>
<td>2.3:1</td>
<td>1.29</td>
<td></td>
</tr>
<tr>
<td>1-8</td>
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<td>76</td>
<td>22</td>
<td>2</td>
<td>3.5:1</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
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<td>3</td>
<td>2.3:1</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>1-10</td>
<td>78</td>
<td>49</td>
<td>25</td>
<td>4</td>
<td>2.0:1</td>
<td>3.05</td>
<td></td>
</tr>
<tr>
<td>1-11</td>
<td>99</td>
<td>75</td>
<td>24</td>
<td>0</td>
<td>3.1:1</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>1-12</td>
<td>111</td>
<td>90</td>
<td>19</td>
<td>2</td>
<td>4.7:1</td>
<td>3.33</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 26. DNA blot analysis for \textit{WUS::RBR1(RNAi)} T\textsubscript{2} lines. All the progenies from lines 1-3 and 1-8 had single insert.

The specific phenotype of \textit{WUS::RBR1(RNAi)} primary transformants was absent in subsequent generations and they grew normally without any obvious phenotype. I investigated the reason for such absence of T\textsubscript{1} phenotype in subsequent generations. It was plausible that the \textit{WUS} promoter driving the \textit{RBR1(RNAi)} transgene was silenced due to methylation in subsequent generations. To check this possibility, I did DNA blot analysis using \textit{MspI} and \textit{Hpall}. \textit{MspI} and \textit{Hpall} are isoschizomers. \textit{Hpall} cannot digest its target sequence (CCGG) when either of the cytosine is methylated, while \textit{MspI} is sensitive only to methylation of the external cytosine (Promega\textsuperscript{TM} catalog, technical reference). Fortunately there is only one \textit{Hpall/MspI} site in the \textit{WUS} promoter sequence and four sites within the \textit{RBR1(RNAi)} transgene sequence that makes it easier to monitor. Genomic DNA from two different T\textsubscript{2} lines (1-3 and 1-8) of \textit{WUS::GFP} plants and untransformed wild type plants were used as controls. DIG labeled \textit{RBR1\textsubscript{p326}} cDNA sequence was used as a probe. No difference was observed between \textit{MspI} and \textit{Hpall} digested lanes (fig-27). This indicates that

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig26}
\caption{DNA blot analysis for \textit{WUS::RBR1(RNAi)} T\textsubscript{2} lines. All the progenies from lines 1-3 and 1-8 had single insert.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig27}
\caption{DNA blot analysis for methylation of the transgene in \textit{WUS::RBR1(RNAi)} T\textsubscript{2} lines. No methylation of the \textit{Hpall/MspI} sequence within the transgene, including the \textit{WUS} promoter sequence, could be detected.}
\end{figure}
CpG methylation within the promoter or the transgene was not responsible for the disappearance of the T₁ phenotype in subsequent generations. This line of reasoning was further strengthened by germinating some seeds of the T₂ plants (line 1-3-2, 1-3-3, 1-8-4, 1-8-5 and 1-8-6) and control plants, in presence of 5'Azacitidine (10μM) for 8 days and then transferring them to grow further on soil. These T₃ WUS::RBR1(RNAi) plants too did not show any of the characteristic phenotype, like fasciation, that was observed in their predecessor T₁ plants.

4: "Promoter"::GFP: Initially all the "promoters" were used to express GFP to monitor the expression pattern controlled by those "promoters". These lines were also used as control lines for respective RNAi and RBR1 overexpressing lines. Segregation and DNA blot analysis was done with WUS::GFP T₂ lines to determine the number of transgene(s). The lines having single copy insertion were used as a control for experiments using WUS::RBR1(RNAi) and WUS::RBR1 lines. Although we could detect GFP expression in leaves and flowers of 22 days old seedlings, we could not differentiate GFP expression from the background fluorescence in and around the SAM region of young Arabidopsis seedlings by confocal laser scanning microscopy. I experienced the same problem with AP1::GFP lines as well. So I stopped using these lines to monitor the expression patterns of the "promoters" and developed "promoter"::GUS lines for the same purpose.

5: "Promoter"::GUS:

5.1: AP1::GUS: All the 17 independent transformants had no obvious phenotype. Table-4 shows segregation analysis for some AP1::GUS Lines. Line 7-1 was chosen for subsequent experiments. GUS expression in the 9 days old seedlings of several lines, including line 7-1, showed similar patterns, though the level of expression differs from line to line as expected. Expression of GUS in line 7-1 after 1 hour staining at 37°C is shown in figure-28. No GUS expression could be detected around the SAM of 6 days old seedlings, either by hand section or by thin sections even after 2 hours of staining, though GUS expression was evident outside the SAM region (data not shown).

FIG. 28. Expression of GUS in 9 days old AP1::GUS seedlings. (A) GUS was expressed almost constitutively throughout the seedling. Expressed highly and uniformly in cotyledons. (B) Expression within leaves was mainly confined to the upper half. (C) Expression in roots was mainly restricted to vasculature. Root apical meristem and root cap region seemed to be free from GUS expression. (Bar: A=1mm, B=500μm, C=200μm)
Table-4: Segregation analysis for AP1::GUS lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>$P_{0.05}=3.841$ (df=1)</th>
</tr>
</thead>
<tbody>
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<td>1-1</td>
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<td>110</td>
<td>6</td>
<td>4</td>
<td>18.33:1</td>
<td>24.32</td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td>5.31:1</td>
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<td>21</td>
<td>7</td>
<td>4.61:1</td>
<td>3.27</td>
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</tr>
</tbody>
</table>

5.2: CLV3::GUS: Segregation analysis of 17 independent T₁ transformants of CLV3::GUS, having no obvious phenotype, was performed (table-5). Lines 4-5 and 10-1 were used as representative lines for CLV3::GUS for future experiments. Different lines showed different intensity and sometimes a different expression pattern, too, for GUS expression among 5 days old seedlings. GUS expression was always observed in petioles of cotyledons and leaves, the tips of the young leaves and throughout roots except for the root cap region, as shown in figure-29. As I was getting a specific phenotype with CLV3::RBR1(RNAi) lines and that phenotype was a bit different than that of 35S::RBR1(RNAi) lines, I wanted to know the expression pattern of the CLV3 promoter within SAM region. Hand sections indicated that there might be GUS expression in the tunica (L1-L2) layer of 6 days old seedlings of line 4-5-2 (fig-30A) but thin sections (10μm) could not reveal any GUS staining within the SAM of the 6 days old CLV3::GUS seedlings (fig-30B).

Table-5: Segregation analysis of CLV3::GUS lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>$P_{0.05}=3.841$ (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-3</td>
<td>50</td>
<td>39</td>
<td>11</td>
<td>0</td>
<td>3.5:1</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>4-5</td>
<td>67</td>
<td>50</td>
<td>15</td>
<td>2</td>
<td>3.3:1</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>7-1</td>
<td>63</td>
<td>60</td>
<td>3</td>
<td>0</td>
<td>20:1</td>
<td>13.76</td>
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</tr>
<tr>
<td>7-2</td>
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<td>59</td>
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<td>0</td>
<td>5.9:1</td>
<td>4.06</td>
<td></td>
</tr>
<tr>
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<td>77</td>
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<td>0</td>
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<td>21.27</td>
<td></td>
</tr>
<tr>
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<td>46</td>
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<td>6.6:1</td>
<td>3.93</td>
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</tr>
<tr>
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<td>0</td>
<td>2.9:1</td>
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<td>55</td>
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<td>1</td>
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</tr>
<tr>
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<td>43</td>
<td>12</td>
<td>0</td>
<td>3.6:1</td>
<td>0.30</td>
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</tr>
<tr>
<td>11-3</td>
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</tr>
<tr>
<td>11-4</td>
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<td>56</td>
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<td>1</td>
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</tr>
<tr>
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<td>2</td>
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</table>
FIG. 29. Different independent CLV3::GUS lines showed variable intensity and sometimes pattern too, for GUS expression. A and B represent two different seedlings from two different T1 lines (A= line 10-1-1 and B= line 4-5-2) showing 3:1 segregation ratio. C and D are from line 4-5-2. (Bar: A, B=1mm; C=500μm, D=100μm)

FIG. 30. GUS expression was suspected within the SAM area of 6 days old CLV3::GUS seedlings by hand section (A). But it could not be confirmed by thin sections (B). (Bar: 20μm)

5.3: STM::GUS: Segregation analyses of some of the independent T1 transformants, having no obvious phenotype, were performed (table-6). Line 1-1 and 2-2 were used for subsequent experiments. GUS expression pattern among 9 days old STM::GUS seedlings is summarized in figure-31 below. The STM promoter, I used, did not show the expected expression pattern reported for STM gene. GUS expression was almost uniform in cotyledons and leaves (fig-31A) but seemed

Table-6: Segregation analysis for some of the STM::GUS T1 lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>X²</th>
<th>P₀.₀₅=3.₈₄₁ (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>118</td>
<td>83</td>
<td>19</td>
<td>16</td>
<td>4.3:1</td>
<td>2.20</td>
<td></td>
</tr>
<tr>
<td>2-2</td>
<td>137</td>
<td>104</td>
<td>18</td>
<td>15</td>
<td>5.7:1</td>
<td>6.83</td>
<td></td>
</tr>
<tr>
<td>3-3</td>
<td>117</td>
<td>92</td>
<td>12</td>
<td>13</td>
<td>7.6:1</td>
<td>10.05</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>118</td>
<td>86</td>
<td>16</td>
<td>16</td>
<td>5.4:1</td>
<td>4.72</td>
<td></td>
</tr>
<tr>
<td>5-5</td>
<td>96</td>
<td>69</td>
<td>16</td>
<td>11</td>
<td>4.3:1</td>
<td>1.73</td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td>113</td>
<td>81</td>
<td>18</td>
<td>14</td>
<td>4.5</td>
<td>2.45</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 31. GUS expression in 9 days old STM::GUS seedlings. (A) GUS was expressed almost uniformly throughout leaves and cotyledons. (B) No expression was detected in the stem but GUS was expressed around the junctions of root and shoot and (C) primary and secondary roots. (D) Very low GUS expression was detected within vascular bundle of the roots and could not be detected around the root meristem and root cap. (Bar: A and B-1mm, C-500um, D-100um)

to be absent in stems (fig-31B), petioles, root meristem and root cap (fig-31D). GUS expression was very weak in roots and was mainly confined to the junctions of primary and secondary roots and vascular bundles (fig-31C). The expression pattern within the SAM region was checked. Expression of GUS could be detected within 5 mins of staining and became stable within 25 mins. Hand sections of 8 min and 25 min stained SAMs indicated the possibility of GUS expression at the bottom of corpus layer of the SAM, as shown in figure-32 (A and B). But no GUS expression could be detected in the thin sections of the SAM of 7 day old seedlings after 1 hour (fig-32C) or even overnight staining.

FIG. 32. Hand sections of SAM of 7 days old seedlings of STM::GUS, stained with GUS buffer for (A) 8 min and (B) 25 min. Thin (10 μm) sections of the seedlings could not detect any GUS expression around SAM region even after 1 hour of staining (C). (Bar: A, B=50μm; C=20μm)

5.4: WUS::GUS: Segregation analysis of 16 T₁ independent transformants of WUS::GUS was performed (table-7). Line 5-1 was chosen for future experiments as it was showing a 3:1 segregation pattern and was representative for GUS expression observed in most of the other
Table-7: Segregation analysis for some of the *WUS::GUS* T₁ lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>$P_{0.05}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>54</td>
<td>51</td>
<td>3</td>
<td>0</td>
<td>17:0:1</td>
<td>10.892</td>
<td></td>
</tr>
<tr>
<td>2-1</td>
<td>55</td>
<td>41</td>
<td>13</td>
<td>1</td>
<td>3.15:1</td>
<td>0.024</td>
<td></td>
</tr>
<tr>
<td>2-2</td>
<td>73</td>
<td>50</td>
<td>23</td>
<td>0</td>
<td>2.17:1</td>
<td>1.642</td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>69</td>
<td>51</td>
<td>18</td>
<td>0</td>
<td>2.83:1</td>
<td>0.045</td>
<td></td>
</tr>
<tr>
<td>2-4</td>
<td>73</td>
<td>49</td>
<td>22</td>
<td>2</td>
<td>2.23:1</td>
<td>1.353</td>
<td></td>
</tr>
<tr>
<td>3-2</td>
<td>64</td>
<td>47</td>
<td>17</td>
<td>0</td>
<td>2.77:1</td>
<td>0.081</td>
<td></td>
</tr>
<tr>
<td>3-3</td>
<td>72</td>
<td>53</td>
<td>17</td>
<td>2</td>
<td>3.11:1</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>3-4</td>
<td>73</td>
<td>53</td>
<td>17</td>
<td>1</td>
<td>3.11:1</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>4-1</td>
<td>63</td>
<td>31</td>
<td>29</td>
<td>3</td>
<td>1.07:1</td>
<td>17.422</td>
<td></td>
</tr>
<tr>
<td>5-1</td>
<td>71</td>
<td>52</td>
<td>17</td>
<td>2</td>
<td>3.06:1</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>5-3</td>
<td>72</td>
<td>53</td>
<td>17</td>
<td>2</td>
<td>3.11:1</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>5-4</td>
<td>79</td>
<td>54</td>
<td>23</td>
<td>2</td>
<td>2.35:1</td>
<td>0.974</td>
<td></td>
</tr>
<tr>
<td>6-1</td>
<td>85</td>
<td>63</td>
<td>19</td>
<td>3</td>
<td>3.31:1</td>
<td>0.146</td>
<td></td>
</tr>
<tr>
<td>6-2</td>
<td>58</td>
<td>46</td>
<td>10</td>
<td>2</td>
<td>4.6:1</td>
<td>1.523</td>
<td></td>
</tr>
<tr>
<td>6-3</td>
<td>66</td>
<td>51</td>
<td>15</td>
<td>0</td>
<td>3.4:1</td>
<td>0.181</td>
<td></td>
</tr>
<tr>
<td>6-4</td>
<td>81</td>
<td>59</td>
<td>22</td>
<td>0</td>
<td>2.68:1</td>
<td>0.201</td>
<td></td>
</tr>
</tbody>
</table>

Different lines of *WUS::GUS* had the same pattern of expression though with different intensity, as expected. Seedlings from three different independent lines are shown in figure-33A, B and C. GUS expression was more uniform in the roots of different lines and was restricted to the vascular bundle (fig-33D) but not expressed in the root meristem and root cap area (figure-33E). Cotyledons were uniformly expressing GUS while its expression was stronger in the upper part of...
FIG. 34. GUS expression around SAM of 6 days old WUS::GUS seedlings (line 5-1-1). Hand sections indicated expression around corpus (A) and (B). Thin sections confirmed that (C) and (D). (Bar: A= 100µm; B=50µm; C and D=20µm)

the leaves. Hand sections of SAM of 7 day old seedling of WUS::GUS, line 5-1-1, showed a strong GUS expression around the corpus of the SAM (fig-34A and B). Thin sections (7µm) confirmed it (fig-34C, D). GUS was not expressed in the L1 or L2 layers but expressed in cells including the so called “organizing centre” where the WUS gene is reported to be expressed.

6: Vector Control (VC): Most of the promoters I used did not show a specific expression pattern within SAM, thus making the interpretation of the result more difficult. Hence, I concentrated on the 35S::RBR1(RNAi) and 35S::RBR1(antisense) lines [for simplicity 35S::RBR1(RNAi) and 35S::RBR1(antisense) will be referred to as 35SRi and 35SAS respectively] and VC lines were used as control for all the experiments using 35SAS and 35SRi lines. Segregation analysis was done with some of the Ti lines as shown in table-8. Lines 1-1, 3-2, 4-1 and 4-4 showing around 3:1 ratio of segregation were used to confirm number of transgene(s) inserted by DNA blot analysis, using Xhol and OCS sequence as a probe (fig-35). Progenies from vector control (VC) line 4-1-2 were used as a control for all the experiments involving 35SRi and 35SAS.

<table>
<thead>
<tr>
<th>Line</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but did not Grow (susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Total</th>
<th>Ratio (Resistant: susceptible)</th>
<th>P&lt;sub&gt;0.05&lt;/sub&gt;=3.841 (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>94</td>
<td>32</td>
<td>0</td>
<td>126</td>
<td>2.9:1</td>
<td>0.015</td>
</tr>
<tr>
<td>1-2</td>
<td>68</td>
<td>29</td>
<td>0</td>
<td>97</td>
<td>2.3:1</td>
<td>1.240</td>
</tr>
<tr>
<td>2-1</td>
<td>91</td>
<td>28</td>
<td>1</td>
<td>120</td>
<td>3.2:1</td>
<td>0.137</td>
</tr>
<tr>
<td>3-2</td>
<td>95</td>
<td>32</td>
<td>1</td>
<td>128</td>
<td>3:1</td>
<td>0.002</td>
</tr>
<tr>
<td>3-3</td>
<td>110</td>
<td>11</td>
<td>1</td>
<td>122</td>
<td>10:1</td>
<td>16.333</td>
</tr>
<tr>
<td>4-1</td>
<td>94</td>
<td>30</td>
<td>1</td>
<td>125</td>
<td>3.1:1</td>
<td>0.043</td>
</tr>
<tr>
<td>4-3</td>
<td>95</td>
<td>27</td>
<td>0</td>
<td>122</td>
<td>3.5:1</td>
<td>0.535</td>
</tr>
<tr>
<td>4-4</td>
<td>89</td>
<td>30</td>
<td>0</td>
<td>119</td>
<td>3:1</td>
<td>0.003</td>
</tr>
<tr>
<td>5-1</td>
<td>86</td>
<td>33</td>
<td>0</td>
<td>119</td>
<td>2.6:1</td>
<td>0.473</td>
</tr>
<tr>
<td>5-2</td>
<td>77</td>
<td>10</td>
<td>10</td>
<td>97</td>
<td>7.7:1</td>
<td>8.464</td>
</tr>
<tr>
<td>5-3</td>
<td>89</td>
<td>18</td>
<td>4</td>
<td>111</td>
<td>4.9:1</td>
<td>3.816</td>
</tr>
</tbody>
</table>
7: 35S::RBR1(antisense): Initially we expected to get moderate to mild phenotype in the 35SAS lines. But out of the 17 independent 35SAS transformants, none had any obvious phenotype. The number of transgene(s) inserted in some of the lines was determined by segregation analysis followed by DNA blot analysis. Segregation analysis was performed as summarized in table-9. DNA blot analysis using Xhol and OCS sequence as a probe was done as shown in figure-36 (A and B). Several lines had a single transgene insert while some of them had double inserts as well. Line 16-3 (having double inserts) and 17-2 (having single insert) were selected for subsequent experiments. Initial experiments using trypan blue staining indicated enhanced cell death among spongy mesophyll tissue of the young leaves of 35SAS lines. But after extensive investigation it could not be confirmed beyond doubt and so was not pursued further (data not shown).

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
<th>P_{0.05}=3.841 (df=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-1</td>
<td>115</td>
<td>104</td>
<td>11</td>
<td>0</td>
<td>9.5:1</td>
<td>14.611</td>
<td></td>
</tr>
<tr>
<td>16-1</td>
<td>118</td>
<td>105</td>
<td>13</td>
<td>0</td>
<td>8.1:1</td>
<td>12.305</td>
<td></td>
</tr>
<tr>
<td>16-3</td>
<td>139</td>
<td>105</td>
<td>33</td>
<td>1</td>
<td>3.2:1</td>
<td>0.087</td>
<td></td>
</tr>
<tr>
<td>16-4</td>
<td>110</td>
<td>86</td>
<td>23</td>
<td>1</td>
<td>3.7:1</td>
<td>0.884</td>
<td></td>
</tr>
</tbody>
</table>

FIG. 35. DNA blot analysis of VC T2 lines using Xhol and OCS sequence as probe. All the lines tested, except progenies from 4-4, were having single copy of the transgene.

FIG. 36. DNA blot analysis of 35SAS lines using Xhol and OCS sequence as probe. Several lines showed single transgene insert. (A) T2 progenies from lines 16-3, 17-2 and 19-2. (B) T2 progenies from lines 16-4, 17-1, 19-1 and 19-3.

Table-9: Segregation analysis of 35SAS lines.
8: **35S::RBR1(RNAi):** I mainly concentrated my efforts on the 35SRi lines and studied these in more detail, particularly after I noticed that the characteristic expression pattern of the AP1, CLV1, CLV3, STM and WUS were absent in the GUS expressing lines controlled by the upstream (promoter) sequence I used.

8.1: **Phenotypic description:** Among all the RBR1(RNAi) lines, the 35SRi construct showed the lowest transformation efficiency. Total number of T₀ plants transformed with 35SRi construct was 112 and the number of independent transformants obtained was only 125. The transformation efficiency was very low, around 0.03% as compared to 0.1% in case of other RNAi constructs and 2-3% in the case of non-RNAi constructs. Different independent transformants (T₁) showed highly variable phenotype. Severity of phenotypes also varied among different T₁ lines. For simplicity, I divided all the T₁ lines in five different categories (A to E) as shown in figure-37. About 8.8% of the T₁ transformants were in category-A. Such seedlings did not have any visible root but managed to survive on MS media supplemented with 1% sucrose for a long time. Some of them were even able to produce normal looking flowers but could not produce any seed (fig-37A). About 32.8% of the T₁ transformants were in category-B. These plants could manage to produce short roots but did not grow enough to be transplanted to soil (32.8%) (B). Category-C transformants were able to grow further and could be transplanted to soil (25.6%). But after transplantation they did not grow much further and subsequently died (C). Category-D transformants were able to produce flowers. These flowers were having normal floral organs but could not have seeds (9.6%) (D). Transformants belonging to category-E could overcome all the problems and managed to produce seeds (23.2%) (E).

![Figure 37](image-url)
FIG. 38. Some extreme phenotype of a few 35SRi primary transformants. (A) A 3 days old seedling could not develop and produce any shoot or root structure. Instead grew like a stick and remained in that phase before dying around 30 days after germination. (B) Another 3 days old seedling that could produce only the two cotyledons and some callus like transparent cell mass below it. (C) A 24 days old seedling having only the two cotyledons, no visible root and produced callus like cell mass throughout its epidermal layer. (D) This 24 days old seedling had two cotyledons and one terminal leaf with very short root. (E), (F), (G) are the roots of three different 24 days old seedlings. (E) had no primary root but full of secondary roots. (F) Most of the epidermal layer of these roots was covered with callus like cell mass. (G) The primary root was almost fully covered with root hair. Even the root cap area was also having root hair.

Transformants belonged to category-B. These seedlings were able to produce a very short root and could not develop further but survived on MS+1% sucrose for about 2-3 months (fig-37B). Category-C transformants (25.6%) were able to overcome these initial problems and were able to grow further. These seedlings were transplanted to soil. But they did not grow much after transplantation to soil and died before producing any flower (fig-37C). Some of the transplanted seedlings were able to produce flowers. These flowers were had a normal shape, size and number of floral organs but could not produce seeds. This category of sterile plants, category-D, comprises about 9.6% of all the T₃ transformants (fig-37D). About 23.2% of the T₁ transformants were able to overcome all the foresaid problems during their development and were able to produce fertile seeds. These are the category-E plants (fig-37E). Figure-38 shows some independent T₁ transformants having severe shoot as well as root phenotypes. Subsequent generations of all the primary transformants that were able to produce seeds lost all the characteristics phenotypes of the T₁ generation. Hence, all the data presented below involving 35SRi lines were essentially obtained from primary transformants.

8.2: Scanning Electron Microscopy (SEM) of the SAM: Initial observations indicated that some of the 35SRi lines had multiple SAMs. I tried to check this possibility by scanning electron microscopy (SEM). It was a bit difficult to prepare and then mount the fresh and naked SAM of
FIG. 39. Ectopic differentiation of the cells in L1 layer of SAM in 35SRi line 15-2. (A) SAM of a 10 days old wild type seedling, (B-I) 35 days old seedling of 35SRi line 15-2. (B-II to B-IV) L1 layer of SAM the 35SRi seedling (Bar: AI=70µm, All=10µm, BI=500µm, BII=20µm, BIII=20µm, BIV=10µm)

young 35SRi seedlings, as most of the young tiny SAMs were covered with very small young leaves and/or leaf primordia. Ultimately I was successful in mounting the SAM of line 15-2. The 35 day old seedling was maintained on MS+1% sucrose media. It did not have any visible flower or flower bud (fig-39B I). The wild type control plant was 10 days old to match the developmental stage of the 35SRi seedling and was in a vegetative growth phase. The cells in the L1 layer of the wild type SAM were fairly uniform in size and shape (fig-39A), while the cells in the L1 layer of SAM of the 35SRi seedling were not uniform in size and shape and had ectopic differentiation (fig-39B II, III). There were many bigger sized cells with a "cobblestone-like" structure that were randomly distributed all over the L1 layer (fig-39B IV). Such cells are typical pattern of differentiation, usually found in the outer epidermal cells of some floral organs, like sepals.

8.3: Histology of the SAM: Encouraged by the SEM of the SAM of 35SRi seedlings, I started checking the histology of the SAM using technovit. Most of the 35SRi seedlings had a SAM without any effect in their histology as observed in line 34-2 (fig-40B). Organization of the cells in the SAM of some of the 35SRi lines were affected, as in line 36-1 (fig-40C). The cells there seem to divide periclinally in all the clonal layers, including L1 and L2, and were arranged one over another. During taking thin sections, I could detect several SAMs in the shoot apex of some of the 35SRi seedlings, as in line 36-1. Unfortunately I was not successful in mounting and thus observing such 35SRi seedlings with multiple SAMs by scanning electron microscopy (SEM) as those were covered with tiny leaves and/or leaf primordia.
8.4: **Histology of the leaves:** In most of the cases the leaves of the 35SRi T1 plants were smaller as compared to the control plants. Cross-sections around the middle of technovit embedded leaves were taken for histological analysis. Histology of primary leaves of several 35SRi lines indicates that the expansion of the cells is affected. Histology of primary leaves of 18 days old plants of different 35SRi lines was performed. Line 67-1 and 36-2 represent the more commonly occurring phenotype while line 36-1 represents a more severe and rare phenotype among the 35SRi T1 lines (fig-41A). It is also interesting that the two primary leaves of the same seedling (line 36-1) had different histology. Primary leaf-1 (PL1) was smaller than PL2 in this T1 seedling. The cells in PL1 were more compact, having much less intracellular space, smaller in size and not properly differentiated (fig-41C) as compared to that of PL2 of the same seedling (fig-41D). Both the primary leaves of line 67-1 were comparable in size (fig-41B) and histology (fig-41E). Cells of any of the primary leaves of line 67-1 are larger and also had larger intercellular space than that of 36-1. I compared number of cells per unit area (100X100 sq. μm) among the thin sections of primary leaves of 36-1 and 36-2. PL1 of line 36-1 had 164 ± 77.4 cells while PL2 had about 71 ± 36.4 cells per 10,000 μm² area (fig-42A). This count excluded the epidermal cells as they are more irregular in shape and size and thin sections cannot trace them properly. This difference in cell density was clearly evident from their histology too. I suspected that cells in the primary leaves of 35SRi lines were unable to develop after attaining a particular stage. So I checked the histology of leaves of VC plants having comparable size (referred to as “same size” leaves) with those of primary leaves of 35SRi lines (fig-41B). These young leaves had smaller cells with less inter...
FIG. 41. Histology of primary and "same size" leaves from 18 days old seedlings. (A) Seedlings of VC, 35SRI/ line 67-1 and 36-1. (B) Primary leaves (PL) from VC and 35SRI/ line 67-1 and "same size" leaf from VC. (Bar: 10mm). (C) Histology of the primary leaf 1 (PL-1) of line 36-1 (Bar: 50μm both) and (D) PL2 (Bar: 50μm both). (E) Histology of the PL1 (left) and PL2 (right) from line 67-1 (Bar: 100μm). (F) PL1 (left) and PL-2 (right) from VC (Bar: 100μm). (G) Histology of the "same size" leaf from VC (Bar: 100μm).
cellular space (fig-41G) and as expected, had a high number of cells per unit area as compared to the primary leaves of the same VC plant. Primary leaves of 24 day old VC plants had only 4.2 (±0.8) cells (n=6) cells per 10,000 μm² while the most commonly occurring 35SRi lines (as line 36-2 and 67-1) had 8.7 cells (±2.2) (n=6), and the “same size” leaves of the VC plants had 21 cells (±1.6) (n=4) in that same area of 10,000μm² (fig-42B). These data indicate that development of primary leaves of 35SRi lines is affected and they could not develop beyond a certain stage and this stage varied from line to line.

Younger primary leaves had smaller cells with less intercellular space but had a higher number of cells per unit area as compared to older primary leaves of control (VC) plants. Primary leaves of 18 day old VC plants had 6.2 (±2.3) cells while 24 day old plants had 4.2 (±0.8) cells per 10,000 μm² leaf area [fig-42A (VC) and 42B (VC-PL)]. It seems that the primary leaves of 35SRi plants are unable to develop beyond a certain stage.

8.5: Effect on gametophyte development causes sterility: As stated earlier, 9.6% of the all the independent 35SRi transformants were sterile. Line 72-1 was one such sterile line (fig-43A). The mature plant did produce flowers having floral organs of normal shape, size and number. It was able to produce very small siliques of only 3-4 mm long (fig-43B) as compared to 2-3 cm in the case of control plants. These small siliques were full of pale white unfertilized ovules (fig-43C).
FIG. 43. (A) 47 days old 35SRi line 72-1. (B) The siliques were very small, only about 3mm (Bar: 1mm) and (C) full of unfertilized ovules (Bar: 500μm). (D) A properly developed silique from a 47 days old VC plant (Bar: 1mm).

FIG. 44. Development of ovules in flowers of different stages of 35SRi (line 72-1) and control (VC) lines. (A) The polar nuclei (PN) were yet to fuse to give rise the secondary endosperm nucleus (SEN) in the ovule isolated from a flower bud (FB) of the control line (VC). (B) SEN and egg cell nucleus (EN) was properly developed in the ovules from FB of 35SRi line 72-1. (C) Properly developed ovules from more mature "open flower" (OF) of VC. (D) About 50% of the ovules from (OF) of 35SRi line had normal looking SEN and EN while (E) the rest 50% did not have any visible SEN and EN and seemed to have degenerated embryo sac. Ovules from more matured flowers or early silique (ES) from VC produced globular embryo (F), but those from 35SRi line showed symptoms of senescence (G). (Bar: 20μm)
No seed abortion, as characterized by deformed brown seeds, could be found in any of these siliques. Both male and female gametophyte development, in line 72-1, was checked more closely through clearing and then by DIC microscopy. It seems that female gametophyte or ovule development was not much affected during initial stages. Meiosis was not affected and so secondary endosperm nucleus (SEN) and egg cell nucleus (EN) could be clearly identified in the ovules, isolated from very young flower buds (fig-44B). Ovule development seems to be affected in more matured open flowers (OF). In such cases about 50% of the ovules had normal SEN and EN (fig-44D) while the other 50% did not have those characteristic features of megagametophyte development (fig-44E). Almost all the ovules isolated from dried out flowers with early siliques, were found to have symptoms of senescence (fig-44G) while ovules from comparable stages of early siliques from VC lines had healthy globular embryos (fig-44F).

I checked morphology of the pollen. The pollen from line 72-1 seem to be affected as well. About 50% (not statistically validated) of the pollen grains were non-viable as evident from their morphology (fig-45A, B). This was true irrespective of the stage or age of flower. I could not get any viable seed when I performed reciprocal crosses using wild type plants and line 72-1. Pollens from wild type plants could not fertilize ovules of line 72-1 and pollen from line 72-1 also could not fertilize wild type ovules to produce viable seeds. Upon close observation it appeared that anthers from line 72-1 could not have proper dehiscence. No pollen could be found on the stigmas of the wild type flowers during pollination.

**Table-10: Segregation analysis for some of the 35SRi lines.**

<table>
<thead>
<tr>
<th>Line</th>
<th>Total</th>
<th>Germinated and grown (Resistant)</th>
<th>Germinated but not grown (Susceptible)</th>
<th>Not germinated (non-viable)</th>
<th>Ratio (Resistant: susceptible)</th>
<th>$X^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-2</td>
<td>103</td>
<td>72</td>
<td>25</td>
<td>6</td>
<td>2.9:1</td>
<td>0.031</td>
</tr>
<tr>
<td>5-4</td>
<td>104</td>
<td>77</td>
<td>21</td>
<td>6</td>
<td>3.6:1</td>
<td>0.666</td>
</tr>
<tr>
<td>5-5</td>
<td>101</td>
<td>63</td>
<td>20</td>
<td>18</td>
<td>3.1:1</td>
<td>0.0361</td>
</tr>
<tr>
<td>7-1</td>
<td>101</td>
<td>65</td>
<td>36</td>
<td>0</td>
<td>1.8:1</td>
<td>6.102</td>
</tr>
<tr>
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<td>104</td>
<td>73</td>
<td>29</td>
<td>2</td>
<td>2.5:1</td>
<td>0.641</td>
</tr>
<tr>
<td>7-4</td>
<td>91</td>
<td>64</td>
<td>25</td>
<td>2</td>
<td>2.5:1</td>
<td>0.453</td>
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<tr>
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<td>89</td>
<td>68</td>
<td>17</td>
<td>4</td>
<td>4:1</td>
<td>1.133</td>
</tr>
</tbody>
</table>
8.6: Segregation and DNA blot analysis: To check the number of transgene integrated, I did the segregation analysis for some of the T<sub>1</sub> lines as shown in table-10 followed by DNA blot analysis using Ncol and OCS as probe (fig-46). This indicated that some of the 35SRi lines showing 3:1 segregation ratio actually had multiple copies of the transgene. Most of the lines also showed rearrangement of the integrated transgene.

FIG. 46. (A) DNA blot analysis using Ncol and OCS sequence as a probe. Most of these lines had more than one copy of the transgene but line 7-4-2 had single insert. (B) Location of the Ncol sites in the transgene.

8.7: Effect on RBR1 transcript level: The transcript level of RBR1 in the 35SRi lines was measured by real time PCR. Initially I used both forward (SgRB-F2) and reverse primers (SgRB-R2) away from the RNAi homology region in accordance with RBR1 cDNA sequence (fig-47B). Surprisingly both 35SRi and 35SAS showed stronger signals for RBR1 transcript as

FIG. 47. Real time PCR to measure relative expression level of RBR1. (A) Relative expression level of RBR1 was much higher in 35SRi lines (n=3) as compared to both 35SAS (AS) (n=6) and vector control (VC) lines (n=3). Figures within Bars indicate the mean value while that in parenthesis indicates SD. RBR1 expression level was normalized to GAPDH. (B) Position of the primers used, with respect to RBR1 cDNA sequence and 35SRi construct, is indicated by the arrows. Ri=35SRi; AS=35SAS, VC=Vector Control.
FIG. 48. (A) Relative expression of RBR1 using two different set of primers for real time PCR (for detail see text). RBR1 expression level was normalized to GAPDH. (B) Position of the primers used, with respect to RBR1 cDNA sequence and 35SRI construct, are indicated by the arrows. Figures within bars indicate the mean value while that in parenthesis indicates SD.

FIG. 49. RNA blot analysis with (A) RBR1 probe and then the same blot was re-probed with (B) GAPDH. (C) Poly-A RNA was isolated from these 35SRI plants for the RNA blot analysis (not according to the same scale).

compared to VC plants, expression of RBR1 was normalized to GAPDH (fig-47A). The level of upregulation was higher among 35SRI lines than in 35SAS lines. It is known that truncated transcripts beyond the 3' downstream of RNAi homology region can be accumulated while RNAi
homology and the 5' upstream sequence of the target RNA is degraded (Zamore et al., 2000). To check the possibility if partial RBR1 transcripts were being accumulated in the 35SRi lines, I did real-time PCR with two different set of primers. The forward primer (RB-524F1) was within the RNAi homology region while both the reverse primers (RB-641R1 and RB-700R1) were outside the RNAi homology region, as shown in figure-48B. Again the relative expression of RBR1 was stronger in both RNAi and antisense lines, as summarized in figure-48. Here too the relative expression was normalized to GAPDH. I was getting consistently stronger signals for RBR1 transcript in both 35SRi and 35SAS lines by real time PCR, normalized to GAPDH using different primer combinations. RNA blot analysis revealed that the level of RBR1 transcript was not significantly changed in any of the 35SRi T1 lines and 35SAS lines tested so far. I also could not detect accumulation of any partial RBR1 transcript in any of the lines (fig-49A). The surprise came when I reprobed the same RNA blot with GAPDH probe (fig-49B). Expression of the so called house keeping gene, GAPDH, was deregulated in both the 35SAS lines and 35SRi line 72-1, a fully sterile line. The RNA blot indicated that GAPDH is not a suitable control in this case. I then performed real-time PCR again with six 35SRi lines (67-1, 69-1, 69-2, 72-1, 80-4, and 85-2) and two 35SAS lines (1722 and 1633) (fig-50). This time the relative expression level was normalized to ACTIN2. For RBR1, I used RB524F1 and RB641R1 while for ACTIN2, I used At-Actin2-1146F1 and At-Actin2-1252R1 primer combinations. The real-time PCR data now corroborated the RNA blot data to indicate that there was no significant change of RBR1 expression among 35SAS and 35SRi lines.

**FIG. 50.** Relative expression of RBR1 (normalized to ACTIN2). Figures within bars indicate mean values while that in parenthesis indicates SD.

8.8: Effect on RBR1 protein level: RBR1 protein level (~113KDa) was not changed in the case of 35SAS plants. Protein-immuno blot data indicates that the RBR1 level was up-regulated in many of the 35SRi lines while there was no significant change in RBR1 level in many other lines. The degree of upregulation was roughly correlated with the severity of phenotype as shown in figure-51. Despite my best effort, I could not manage a decent blot with category-A (fig-36A) 35SRi seedlings. It was at best like a smear as shown in figure-51A for lane 80-6 and 80-12. RUBISCO large subunit (RBCL) around 53 kDa that was used as a (rough) loading control, was low in those two lanes (fig-51A, lower panel). Those two seedlings seem to have very high protease activity that could not be neutralized by using even 4 times higher concentration of the protease inhibitors.
FIG. 51. (A) Protein immuno blot for different categories of 35SRi plants. The more severe the phenotype, the higher is the RBR1 level. Bands around 113.9 kDa indicate RBR1. Bottom panel is for the ponceau stained blot for mainly RBCL, as a loading control. (B) Total soluble protein was isolated from several 35SRi plants of different categories. Line 80-12 and 80-6 belong to category-A, line 96-1 to category-B, 75-2 and 80-13 to category-D and 99-1 and 99-3 belong to category-E.

8.9: Upregulation of several cell cycle marker genes: Expression of several cell cycle marker genes was checked by semi-quantitative RT-PCR. Expression level of CYCLINB1.1, a G2-M marker, was upregulated in both the 35SRi lines (lines 7-1 and 14-2) (fig-52A). Expression of two G1-S marker genes, viz DNA POLYMERASE-δ and CYCLIN D3.1 was also upregulated in those two 35SRi lines (fig-52B and C).

FIG. 52. Semi quantitative RT-PCR showed upregulation of (A) CYCLIN B1.1, (B) DNA POLYMERASE-δ, (C) CYCLIN D3.1. GAPDH was used as control (D). Lane 1 = 35SRi line 7-1; 2= 35SRi line 14-2; 3= 35SAS, line 1633; 4= 35SAS, line 1722; 5= VC, 6= Wild type Col-0
8.10: CYCLIN B1.1 promoter was deregulated in presence of 35S::RBR1(RNAi): It was a bit surprising to notice the upregulation of the G2-M marker gene CYCLINB1.1 by RT-PCR. CYCLINB1.1 expresses only in the meristematic cells undergoing active cell division. GUS expression in 9 days old seedling of CYCLINB1.1::GUS is restricted to the young leaves and root meristems (fig-53A). Its expression becomes more restricted in an 18 days old seedling as shown in fig-53B. CYCLINB1.1::GUS line (kindly provided by Prof. Andrew Fleming) was transformed with 35SRi construct. GUS expression in an 18 days old seedling of CYCLINB1.1::GUS ::35S::RBR1(RNAi) is shown in fig-53C. Expression of GUS was totally deregulated in all the six independent T1 lines of CYCLINB1.1::GUS::35S::RBR1(RNAi), tested so far. GUS was constitutively expressed in these lines and was found to be comparable to 35S::GUS lines. This result was consistent with my earlier RT-PCR data showing CYCLIN B1.1 transcript was upregulated in the 35SRi lines. The T2 generation of the CYCLINB1.1::GUS::35S::RBR1(RNAi) lines did not show the deregulation of the CYCLINB1.1 promoter, unlike their T1, predecessor. They rather had the typical GUS expression pattern as observed in the CYCLINB1.1::GUS line.

FIG. 53. (A) Expression of GUS in a 9 days old CYCB1.1::GUS seedling. (I) whole seedling [Bar; 1mm], (II) in the young leaves [Bar: 500μm], (III) during secondary root initiation [Bar: 100μm], (IV) around meristemic region of the primary root [Bar: 100μm]. (B) Expression of GUS in an 18 days old CYCB1.1::GUS seedling. GUS expression was found only in the young leaves (II), and root meristem (III) [Bar: 100μm] but not in older leaves (I). (C) GUS expression in an 18 days old CYCLINB1.1::GUS::35S::RBR1(RNAi) seedling. (I) whole seedling [Bar: 1mm], (II) roots [Bar: 1mm], (III) root apex. (Bar: 100μm)
Chapter 5

Discussion

1. Low transformation efficiency with RBR1(RNAi) constructs: The best reported efficiency by agrobacterium-mediated transformation is below 2% (Clough et al., 1998). Though it has been reported that use of vacuum is not necessary to improve transformation efficiency (Clough et al., 1998), it seems that the use of moderate vacuum improved the efficiency to 2-3% in my case. All the RBR1(RNAi) constructs driven by different promoters showed lower transformation frequencies (~0.1%) as compared to 2-3% for other non-RBR1(RNAi) constructs. This low frequency became even lower (~0.03%) when I used 35S CaMV promoter to drive the RBR1(RNAi) construct.

It can be mentioned here that RB null mice are embryo lethal and die during mid gestation (Lee et al., 1992; Jacks et al., 1992; Sage et al., 2000). Unpublished data from our lab have shown that T-DNA insertion lines in the RBR1 locus are gametophytic lethal.

It is been reported that 35S promoter is not active during early stages of embryogenesis (Boisnard-Lorig et al., 2001; Sunilkumar et al., 2002). 35S promoter starts expressing during late embryo development and continues to express at varying levels in different cells and tissues during seed germination (Sunilkumar et al., 2002). As a result the embryos would have started accumulating defects that might lead to its death in most of the cases while a few could overcome that. Essentially I was selecting the survivors as very severe phenotypes were plausibly being eliminated during embryo development and germination. It should be noted here that hygromycin, the antibiotic used for selection, does not inhibit germination of non-resistant or wild type seeds. Susceptible seeds can germinate in the presence of hygromycin but die within 2-3 days after germination. However, if the seedlings died, within a few days after germination, due to deregulation of RBR1 activity, it was not possible to separately identify them from hygromycin susceptible ones. This might have affected the transformation frequency as well.

2. Promoter::RBR1 lines had no apparent phenotype: Overexpression of retinoblastoma gene resulted in dwarf mice and the degree of dwarfism correlated roughly with the level of pRB protein (Bignon et al., 1993). But the effect of constitutive expression of the retinoblastoma related gene in plants seems to be different. Earlier results from our group showed that 35S::RBR1 overexpressing lines had no obvious phenotype. Molecular analysis revealed higher level of RBR1 transcripts but the protein level was not significantly changed (WG unpublished data). It appears that the Arabidopsis RBR1 gene has a very tight control over its expression at the post-transcriptional level as well. The correlation between transcript level and that of protein is not always linear. As these Promoter::RBR1 lines did not have any obvious phenotype, they were not pursued further.

3. CLV3::RBR1(RNAi) and STM::RBR1(RNAi) lines showed similar but not identical phenotype with 35SRi lines: Though CLV3::RBR1(RNAi) and STM::RBR1(RNAi) had some
specific phenotypes, yet they can be compared to that of plants displaying a less strong phenotype of 35SRI. As I did not have a large number of independent transformants from CLV3::RBR1(RNAi) and STM::RBR1(RNAi) constructs, the phenotypic variability was less as compared to that of 35SRI. No specific expression was observed within the SAM from either of the CLV3 or STM promoters, as observed in CLV3::GUS and STM::GUS lines. It was also a bit surprising to notice that even the 3S promoter was not expressed within the SAM of 5-7 days old 35S::GUS seedlings. It is plausible that the phenotype we got for CLV3::RBR1(RNAi), STM::RBR1(RNAi) and 35SRI lines was mainly due to the expression of RBR1(RNAi) outside the SAM region.

4. Most of the AP1::RBR1(RNAi) transformants were sterile: The AP1 promoter I used had a unique expression profile which was different from that of CLV3 and STM promoters. It expressed uniformly throughout the cotyledons and only in the top half of the leaves. No GUS expression was observed in the petiole and primary inflorescence (stem). More importantly GUS was not expressed within the SAM of the 6 days old seedlings of AP1::GUS lines. This might be the reason for the relatively normal vegetative growth for most of the AP1::RBR1(RNAi) lines. However, the occurrence of high sterility among the AP1::RBR1(RNAi) lines fits well with the typical AP1 expression pattern. The AP1 promoter control expression at high level in the L1 layer of the SAM during transition from the vegetative to the reproductive phase. Deregulation of RBR1 activity within the AP1 expression domain plausibly disrupted flower development and caused sterility.

5. WUS::RBR1(RNAi) lines had fasciated stems: Stem fasciation has been attributed to defects in the SAM (Kaya et al., 2001). It could be a result of: (i) continuous fragmentation of the existing SAM, or (ii) ectopic formation of new meristems, as observed in mgoun3 (mgo3) mutants (Guyomarc'h et al., 2004); or (iii) enlargement of the central meristem as observed in clavata1 (clv1) and fasciata (fas1 and fas2) mutants (Leyser et al., 1992; Clark et al., 1993, 1995; Kaya et al., 2001). In all the cases, fasciation has been correlated with an increased size of the SAM. Differential increase in cell number within the SAM results in fasciation, as observed in clv1 mutants. Moderate clv1 mutants, clv1-4 and clv1-1, showed "strap like" fasciation, while weaker mutants like clv1-5 rarely showed any. More severe clv1 (clv1-6 and clv1-7) mutants did not show it at all (Clark et al., 1993). Cell accumulation within a meristem is controlled mainly by ULTRAPETALA (ult) via the CLAVATA pathway (Fletcher, 2001). ult1 and ult2 mutants have wider primary inflorescence meristems due to more cells within the SAM. Yet these lines do not show fasciation (Fletcher, 2001). These data indicate that there is a threshold level for the increase in cell number of the SAM to cause fasciation. Neither mild nor severe but moderate change in cell number of the SAM causes it. Besides an increase in cell number, an increase in cell size within the SAM has also been attributed to the increase of the SAM size that causes stem fasciation, as in mgo3 lines. The type of fasciation varies from different mutant lines. clavata1 and fasciata mutant lines show fasciation resembling "straps" (Leyser et al., 1992; Clark et al., 1993) while that of mgo3 resembles a "power cord" (Guyomarc'h et al., 2004). Fasciation observed in WUS::RBR1(RNAi) lines resembles that of mgo3 lines.
Stem fasciation in WUS::RBR1(RNAi) lines correlates with the expression pattern controlled by the WUS promoter. GUS expression in the WUS::GUS lines included the cells of the so called organizing centre (OC) within the SAM, where WUS is reported to be expressed. Downregulation of RBR1 activity through RNAi potentially can result in over proliferation of cells around OC which ultimately resulted stem fasciation. It is yet to be shown if the WUS::RBR1(RNAi) plants do possess enlarged SAM with either a higher number of cells or larger cells.

6. Leaf development of several 35SRi lines was affected: The shape of any organ is a result of a coordination of cell division and cell expansion (Kessler et al., 2004). Although the control of leaf shape and size is not fully understood, it seems to be more complex than in other organs. The role of cell behavior in the morphogenesis of a determinate organ such as leaves is also not clear (Tsukaya, 2003). During leaf morphogenesis, the control of cell proliferation seems to be related to the control of cell size (Meijer et al., 2001; Tsukaya, 2002). In addition, cooperation at both the cell-cell level and the whole-plant level is clearly a factor in regulating leaf size. Moreover, leaf development is more complex in dicotyledonous plants, in which cell division and cell elongation occur simultaneously in the same region throughout leaf expansion (Poethig et al., 1985). The cell cycle related to leaf morphogenesis, tissue-specific patterns of cell proliferation and cell differentiation occurs concurrently and unique regulatory pathways are thought to be operating at each level (Donnelly et al., 1999).

Early events of leaf development can be divided into three main processes; i) Initiation of leaf primordium, ii) establishment of dorsoventrality and iii) development of marginal meristem (Tsukaya, 2002). These early stages of leaf development were successfully completed among the primary leaves of the 35SRi lines. Different cells within a tissue of a leaf exit the cell cycle at a particular size limit and then continue expansion to a certain limit. These limits are different in different tissue layers; e.g palisade mesophyll cells cease cycling when they are about 200μm² in area and then continue to expand 4-5 fold, while dermal pavement cells exit mitosis when the mean cell size is about 400μm² and continue to expand up to 16 fold (Donnelly et al., 1999). It seems that the total number of cells in a leaf is established quite early during leaf development and later it is only expansion of those cells which results in an increase of the leaf area. An increase in cell division does not seem to trigger suppression of expansion of cells in Arabidopsis leaves (Tsukaya, 2002). There are several mutants available in Arabidopsis where the cell size in reduced without affecting cell number in a leaf (Tsuge et al., 1996; Hanson et al., 2001; Cho et al., 2000; Tsukaya, 2003). My data regarding leaf histology of 35SRi lines clearly showed that expansion of the cells was affected; as a result cell density per unit area was increased. The total number of cells was not significantly changed, at least within cross sections of the primary leaves. These data support the "cell theory" of leaf development (Tsukaya, 2002) and demonstrate that proliferation and differentiation of these cells are controlled by different pathways. It was difficult to ascertain if the rate of cell division among the primary leaves of category-C 35SRi lines (e.g line 36-1) was higher during the early stage of leaf development. Clearly the developmental stage and also the age of "same size" leaves were not similar to that of the primary leaves of line 36-1. Probably
checking the histology of leaves belonging to even earlier developmental stages might give us some clue.

7. **Ectopic differentiation of SAM of several RNAi lines**: The L1 layer of the SAM of 35SRI line 15-2 showed ectopic differentiation (fig-39). Several cells in the L1 layer of that 35 days old seedling showed a "cobblestone-like" appearance with prominent cuticular thickenings. Such a typical pattern of differentiation is observed in the outer surfaces of several floral organs viz sepal, petal, anther and style (Bowman, 1994) and considered as typical of the morphology of the cells on the outer surface of a sepal (Meyerowitz et al., 1994). It seems that several cells within the L1 layer of the SAM of 35SRI (line 15-2) was ectopically differentiated to sepaloid cells while the plant was unable to switch from the vegetative to the reproductive phase even after 35 days of germination. Such ectopic differentiation of the L1 layer of the SAM has never been reported so far and can not be explained by the classical ABC model of flower development.

RNA molecules can be transported both locally and systematically via plasmodesmata and phloem transport respectively (Opraka et al., 1999; Kim et al., 2001; Wu et al., 2002; Heinlein, 2002). Recently it has been reported that primary siRNAs from locally initiated silencing can move to 10-15 cells and activate RNA silencing (Himber et al., 2003). It was also reported that the RNAi signal too, in form of small RNA molecules can systematically spread throughout the plant via the phloem (Wu et al., 2002; Lakatos et al., 2004). We now know that phloem development starts after the torpedo stage of embryo development (Bonke et al., 2003) and its development is restricted below the shoot apex (Imlau et al., 1999). Most virus particles and long-distance post-transcriptional gene silencing (PTGS) signals are also excluded from the shoot apex (Foster et al., 2002). Phloem-mobile endogenous RNA is allowed selectively to enter into the shoot apex. The presence of an RNA signal surveillance system that allows selective entry of RNA molecules into the shoot apex is now an established fact (Haywood et al., 2002; Foster et al., 2002) and briefly described in figure-54.

![FIG. 54. Model depicting the selective trafficking of macromolecules within the shoot apex. A surveillance field monitors the exit of macromolecules from the protophloem. Such macromolecules involved in developmental regulation, e.g. CmNACP and PFP-LeT6 transcripts, are permitted to pass through the surveillance field and traffic through the cells of the apex, accumulating within the SAM proper and developing lateral organs. Aberrant or inappropriately delivered macromolecules (e.g., viral RNPs or systemic PTGS signals) detected by the surveillance system are degraded. (reproduced from: Haywood et al., 2002)](image-url)
Intercellular exchange of different signals can occur by two different routes; cell-cell, via the apoplasm, and cell-to-cell, through plasmodesmata (PD), as shown in figure-55 (Haywood et al., 2002). Positional information can be transmitted through a combination of both the pathways, jointly referred to as the "non-cell-autonomous" pathway (Clark, 2001; Haywood et al., 2002). Plasmodesmata are an excellent and the best studied mode through which cell-to-cell connectivity is established (McLean et al., 1997). We now know that the PD is very dynamic and allows only selective cell-to-cell trafficking of various molecules ((McLean et al., 1997; Ghoshroy et al., 1997; Haywood et al., 2002; Heinlein, 2002). Though there is evidence that proteins can migrate between the cells within the SAM (Clark, 2001; Haywood et al., 2002; Heinlein, 2002; Lenhard et al., 2003), still there is no proof showing RNA molecules can do the same within the SAM. Unspecific RNA molecules are selectively degraded by the so called surveillance mechanism before entering the SAM (Lucas et al., 2001). It indicates that such unspecific small RNA molecules probably are not able to pass through and are unable to enter the SAM.

Meristem development is known to depend on signals from other parts of the plant. Excised SAMs of a variety of plants have the ability to develop into a shoot, complete with leaves, if grown on a proper media supplemented with auxin and cytokinin. Removal of the whole meristem leads to formation of a new meristem at a new site (Wolpert et al., 2002). Though the RNAi signals, in form of siRNAs, are not likely to be allowed to enter the SAM, but it is plausible that other signals, mainly protein molecules, do convey the message that affect the development of the SAM of many of the RBR1(RNAi) transformants. Several lines of STM::RBR1(RNAi) and 35SRI had multiple shoot meristems, terminal leaves and terminal flowers while several other lines of CLV3::RBR1(RNAi) did not have proper meristem activity and terminated their growth after producing terminal flowers. Such a phenotype with multiple SAMs and terminal leaves/flowers was not observed in the case of WUS::RBR1(RNAi) and AP1::RBR1(RNAi) lines. As mentioned before, none of the STM, CLV3 and 35S promoters were expressed within SAM of young seedlings as
observed in STM::GUS, CLV3::GUS and 35S::GUS lines, respectively. The data indicate that the effects on SAM development as observed in STM::RBR1(RNAi), CLV3::RBR1(RNAi) and 35SRI lines are not the primary effects due to RNAi mediated deregulation of RBR1 activity within SAM, rather seem to be secondary effects. Here I should mention that we still do not know the expression level of RBR1 within SAM, neither do we know the effect of RBR1(RNAi) on RBR1 protein level within SAM, mainly due to lack of a good antibody. Even with a good antibody, it will be extremely difficult to show the change (particularly if it is not very high) in RBR1 protein level by in-situ protein-immuno analysis or any other technique we can think of.

8. Gametophytic development was affected in many 35SRI lines: Megagametophyte development in Arabidopsis is a complex process, as briefly described in figure-56 below. In a typical Polygonum type gametophyte development, as exhibited by Arabidopsis, successful completion of meiosis and subsequent early stages of megagametogenesis give rise to the egg nucleus (EN), two polar nuclei (PN) and then secondary endosperm nucleus (SEN). Fusion of polar nuclei occurs after cellularization is completed (Christensen et al., 1998; Drews et al., 2002). Problems in the sterile 35SRI lines seem to start after fusion of polar nuclei and continue through the subsequent steps. About 50% of the ovules from open flowers (stage FG 7-9) did not have any of the characteristic cellularization in the gametophyte while all the ovules harvested from early flower buds (till stage FG6) showed proper megagametophyte development. All the ovules from flowers beyond stages 10 showed definite sign of senescence and no egg cell nuclei (EN) or SEN could be observed in any of the mature ovules tested. It could be due to problems either during the later part of megagametophyte development or a lack of pollination. It had been reported.

![Gametophyte Development Diagram](image)

**FIG. 56.** Reproductive development in Arabidopsis. The process can be divided into two stages: megasporogenesis, during which meiosis occurs, and megagametogenesis, during which the surviving haploid megaspore divides mitotically to form the female gametophyte. Cellularization after the third mitotic division during megagametogenesis generates seven cells: one egg cell and two synergids at the distal end, and three antipodal cells at the proximal end. In the large central cell, two polar nuclei (PN) fuse completely to form the secondary endosperm nucleus (SEN). Bottom panel shows the corresponding stages of female gametophyte development. AC-antipodal cell, SC-synergid cell, EC-egg cell, CC-central cell. (adapted from: Buchanan et al., 2000; Drews et al., 2002)
that synergid cells are the source of the female gametophyte guidance cue for the pollen tube (Higashiyama et al., 2001). Typically the synergids die just before or upon arrival of pollen tubes. In other words, in many species, including Arabidopsis, synergid cell death requires pollination and does not occur if pollination is prevented (Jensen et al., 1983; Huang et al., 1992; Christensen et al., 1997; Drew et al., 2002). In these sterile 35SRI lines, synergid cells along with egg cell and central cell died without any pollen tube being present there.

Over the past few years, the phenotypes of over 50 Arabidopsis female gametophyte mutants have been characterized. These mutants fall into five different categories (Drew et al., 2002). The majority (≥ 90%) of such mutants fall into categories 1 to 4, indicating mutations affecting megagametogenesis occur at high frequency (Drews et al., 2002). My data suggest that the sterile 35SRI mutant lines fall into the rare category 5 where mutants have phenotypically wild type ovules at the terminal developmental stage, at least till stage FG7 and were affected at a later stage. But such sterile 35SRI lines are characteristically different from other known category-5 type mutants, viz. fertilization-independent endosperm (fie) (Chaudhury et al., 1997), medea (mea) (Grossniklaus et al., 1998), and fertilization-independent seed2 (fis2) (Ohad, et al., 1996).

About 50% of the pollen of the sterile 35SRI plants were aborted and non viable. It is plausible that only those pollen were sterile that inherited the 35SRI allele during meiosis. Even then, it was not sufficient to explain why the whole plant was sterile. To address that issue I did reciprocal crosses with wild type plants and sterile 35SRI lines. I could not get any seed irrespective of the source of pollen or ovules. I observed that though the 35SRI lines had proper looking anthers and pollens inside them, they could not fertilize the wild type ovules. No pollen grain was found over the wild type stigma when I tried to pollinate by brushing with 35SRI anthers of opened flowers. It indicated that such 35SRI flowers could not have proper dehiscence to release the pollen from the anthers, although I could detect viable pollen after opening the anthers.

Sterile plants due to non-dehiscent anthers have been reported from Arabidopsis myb26 mutant lines (Steiner-Lange et al., 2003). Downregulation of another member of MYB family in Arabidopsis, AtMYB103, also affect anther development and reduces pollen viability, besides affecting trichome development (Higginson et al., 2003). Members of MYB family are known to physically interact with different members of "pocket protein family" that includes pRB and play an important role, at least during neuroblastoma development in mammals (Sala et al., 1996; Raschella et al., 1997; Raschella et al., 2001). So far there is no report demonstrating a physical interaction of plant RBR proteins with any of the members of the MYB family. More detailed description about the Arabidopsis MYB family is given later in the discussion.

9. Effect of RNAi on RBR1 and GAPDH expression: As in many other organisms, the Arabidopsis retinoblastoma related gene contains two putative E2F binding sites in its upstream promoter sequence. Retinoblastoma related genes are known to regulate their own transcription through these E2F sites which in turn depend on the pRB level (Dyson, 1998). Retinoblastoma protein or its transcript is not known to influence translation, neither RBR1 expression is known to be regulated by micro-RNAs. Though I used RNAi to downregulate RBR1 expression, but there
was no significant change in \textit{RBR1} transcript level among the 35SRi lines (fig-49A and fig-50) yet some of the 35SRi lines were showing a higher level of RBR1 protein (fig-51A). Unpublished data from our group suggest that 35S::\textit{RBR1} overexpressing lines showed upregulation of \textit{RBR1} transcript without any significant change in the protein level. It was puzzling that severe (category A and B) 35SRi lines did not have any significant change in the \textit{RBR1} transcript levels, as observed by RNA blot analysis (fig-49A) and real time PCR (fig-50), yet some of the 35SRi lines had a stronger signal for the RBR1 protein (fig-51A) and the level of up-regulation was roughly correlated with severity of the phenotype. It should be emphasized here that more protein does not necessarily mean more active protein. As discussed in chapter-2, members of pocket proteins, including pRB, undergo extensive post-translational modifications to become functionally active. It is plausible that all the RBR1 protein molecules expressed in these severe category of \textit{RBR1(RNAi)} lines were not properly post-translationally modified and hence were not biologically active. My data showing upregulation of several pRB regulated genes (e.g \textit{CYCD3}, \textit{CYCB1.1} and \textit{DNA POLYMERASE 5}) indicate that RBR1 activity in these 35SRi lines were, in fact, down-regulated.

Deregulation of the so called house keeping gene, glyceraldehyde-3-phosphate dehydrogenases (\textit{GAPDH}) among several 35SRi and 35SAS lines, as observed in RNA blot analysis (fig-49B), is also very puzzling. It became particularly striking in case of 35SAS lines which did not have any obvious phenotype. The 741bp long \textit{GAPDH} probe for RNA blot analysis was made using \textit{Arabidopsis GAPDH A} subunit; \textit{GAPA} (AT3G26650), the length of the cDNA is 1467 bp. The probe had significant sequence homology with \textit{GAPDH B} subunit; \textit{GAPB} (AT1G42970) [cDNA length is 1721bp] and a calreticulin related calcium binding protein (AT1G12900) [cDNA length is 1453bp]. In higher plants, there are two chloroplast GAPDH isozymes, with subunit structures of A\textsubscript{4} and A\textsubscript{2}B\textsubscript{2}. These are the key enzymes in photosynthetic carbon fixation cycle (Cerff, 1982). Light (Chan et al., 2002) and several abiotic stresses (Yang et al., 1993) are known to regulate the expression of these nuclear genes that encode the A and B subunits of chloroplast \textit{GAPDH} (\textit{GAPA} and \textit{GAPB} respectively). It is plausible that some of the 35SRi lines generate signals that normally signify stress conditions which are consistent with their phenotypes. That in turn deregulates the \textit{GAPDH} expression. The deregulation of \textit{GAPDH} expression among the 35SAS lines have opened the possibility of a whole new direction and cannot be explained with our limited knowledge, at present.

10. Several cell cycle regulated genes were deregulated in 35SRi lines: Expression of \textit{CYCLINB1.1}, \textit{DNA POLYMERASE 5} and \textit{CYCLIND3} are broadly correlated with a particular phase of cell cycle. Cyclin D group includes three genes, of which \textit{CYCD3.1} is the best studied. In cell cultures, \textit{CYCD3.1} mRNA level does not depend strongly on the position of cells in cell cycle (Menges et al., 2002). Rather \textit{CYCD3.1} expression depends on the availability of sucrose and plant hormones (Riou-Khamlichi et al., 2000). Addition of sucrose to sucrose deprived cell cultures results in the induction of \textit{CYCD3.1} in late G\textsubscript{i} phase (Menges et al., 2002), with the mRNA subsequently maintained at a relatively constant level in cycling cells (Ito, 2000; Dewitte et al.,
Discussion

2003). Other mitogenic plant hormones like auxin and gibberelin (Oakenfull et al., 2002), cytokinins (Riou-Khamlichi et al., 2000) and brassinosteroids (Hu et al., 2000) have the ability to induce CYCD3.1. Its expression is restricted within the proliferating cells and can not be detected in the fully differentiated cells of the Arabidopsis shoot apex (Dewitte et al., 2003). The CYCD3.1 promoter contains a consensus E2F binding site (de Jager et al., 2001). Hence down-regulation of RBR1 activity can potentially induce the CYCD3.1 promoter, as observed in 35SRi plants. This is consistent with the observation that CYCD3.1 over expressing plants contained very high level of several E2F target genes, including RBR1 and E2F2 (Dewitte et al., 2003). Over expression of CYCD3.1 reduced the proportion of cells in the G1 phase and resulted in hyperproliferation of leaf epidermal cells and differentiation defects in leaf tissues (Dewitte et al., 2003). All these data support that CYCD3 operates in the RB-CYCD pathway, controlling the entry into S phase by phosphorylating pRB and hence resulting in E2F activation (Nakagami et al., 1999).

The catalytic subunit of DNA polymerase δ (p125) can physically interact with pRB through the LXCXE motif in several human cell lines. This association is dependent on the phosphorylation state of pRB and thus dependent upon the cell cycle phase (Krucher et al., 2000). It has been proposed that unphosphorylated pRB enhances DNA polymerase δ activity (Krucher et al., 2000) while hyperphosphorylated pRB, by CDK2/CYCLIN D, strongly stimulates DNA POLYMERASE α, but not DNA POLYMERASE δ, ε or primase activity (Takemura et al., 2001). Expression of DNA POLYMERASE δ is known to be controlled by RB-E2F pathway (Dimova et al., 2003; Stevaux et al., 2002). The promoter region of the DNA polymerase δ in Arabidopsis has a putative E2F site, just a few nucleotides upstream of the translational start ATG (as par BAC clone: accession number AC002561). Down-regulation of active RBR1 in these 35SRi lines plausibly resulted in the over-expression of the DNA POLYMERASE δ gene.

Arabidopsis mitotic CYCLINB1.1 is expressed exclusively during G2-M phase of dividing cells (Ferreira et al., 1994; Colon-Carmona et al., 1999; Donnelly et al., 1999). Transcript level of this crucial G2-M phase marker gene was upregulated in all the 35SRi lines but not in 35SAS and control lines tested. This indicates down-regulation of RBR1 activity in the 35SRi lines. The up-regulation of CYCLINB1.1 was correlated with the in-vivo deregulation of its promoter, as discussed below.

11. Upregulation of CYCLINB1.1 promoter in presence of 35SRi: Human CYCLIN B1 mRNA expression varies through the cell cycle with its peak in G2-M. In cycling mammalian cells, its lowest level is in G1 with a steady increase during S phase until a level, 50-fold greater than that in G1, is reached (Hwang et al., 1995). Both transcriptional and post-transcriptional control plays an important role in determining its expression (Hwang et al., 1998). Expression of CYCLIN B1 is regulated through interactions with the region 90 bases upstream of the translational start ATG codon in human (Hwang et al., 1995) but this is not sufficient (Hwang et al., 1998). To make things a bit more complex, at least in mammalian systems, CYCLIN B1 promoter activation depends on Cdk2 kinase activity associated with cyclin A and also on cyclin D1 and cyclin E (Moro et al., 1997). Plant CYCB1.1 expression was investigated using CYCB1.1 promoter driving GUS containing the
cyclin destruction box (CDB) of CYCB1, so that the CDBGUS protein is degraded at the end of mitosis, similar to a mitotic cyclin (Donnelly et al., 1999; Colon-Carmona et al., 1999). B-type cyclins in higher plants are specifically expressed during G2-M phase (Donnelly et al., 1999; Colon-Carmona et al., 1999; Ito M, 2000) in contrast to the omni present, though at varying levels, human B-type cyclins (Piaggio et al., 1995). Activation of the human CYCLIN B1 promoter is regulated by a complex mechanism involving multiple cis-acting elements, e.g. v-myb site, and none of which are sufficient for its G2-M specific up-regulation (Hwang et al., 1998). In contrast, plant B type cyclins employ a simpler mechanism for cell cycle-regulated promoter activation. Most of the promoters, if not all, that specifically express during G2-M phases, contain a common cis-acting element, called the MSA (M-Specific Activator) element. This MSA element is necessary and sufficient for the phase-specific promoter activation (Ito et al., 2001). Besides CYCLIN B1, MSA-like sequences are also found in the promoters of several G2-M specific genes, e.g. NACK1 (Ito et al., 2001), suggesting that a defined set of G2-M specific genes are co-regulated by a common MSA-mediated mechanism in plants. Thus, the molecular mechanisms regulating B-type cyclin gene expression are evolutionarily divergent, and the MSA-mediated mechanism seems to be specific to plants. The MSA consensus sequence (TCYAAACGGYYA, where Y indicates C or T) closely matches the binding sites of animal transcription factors c-myb (JCCAACNGYC, where J indicates A or C) and v-myb (SYAACGG, where S indicates G or C) (Howe et al., 1991; Grotewold et al., 1994). A novel, putative MYB transcription factor and a MYB-type hypothetical protein (HYP) were identified. These proteins have the ability to bind the myb core sequence (AACGG) present in Arabidopsis CYCB1.1 promoter (Planchais et al., 2002). Over expression of HYP was able to trans-activate the GUS reporter gene downstream from the -202 promoter fragment as well as the endogenous CYCB1.1 gene (Planchais et al., 2002).

Pocket proteins, including pRB, have considerable influence on promoter regulation of *myb* genes, including *b-myb*, in mammals (Raschella et al., 2001). MYB-dependent promotion of cell proliferation and trans-activation might also be specifically repressed by the members of the pocket protein family through direct interaction with *b-myb* (Sala et al., 1996). Neuroblastoma cells can undergo neural differentiation upon treatment with a variety of chemical inducers and growth factors. During this process, many cell cycle-related genes are down-regulated while differentiation-specific genes are activated. The retinoblastoma family of proteins, pRb, p107, and p130, are involved in transcriptional repression of "proliferation genes", mainly through their interaction with the E2F transcription factors. Expression of p130 increased during differentiation of neuroblastoma cells (Raschella et al., 1997). On the other hand, both pRb and p107 decreased and underwent progressive dephosphorylation at late differentiation stages. Expression of *b-myb* and *c-myb*, two targets of the retinoblastoma family proteins, were downregulated in association with the increase of p130, which was detected as the major component of the complex with E2F on the E2F binding site of the *b-myb* promoter in differentiated cells (Raschella et al., 1997). More than 125 members of the MYB family genes have been identified in *Arabidopsis*. This largest transcription activator gene family in *Arabidopsis* is known to play important roles in the regulation of secondary metabolism, cell shape, disease resistance and hormone responses in plants (Jin et al., 1999;
Stracke et al., 2001; Jia et al., 2003). It is plausible that downregulation of RBR1 activity in the 35SRi lines induces myb related gene(s), resulting in upregulation of CYCB1.1 promoter. It should be mentioned here that none of the plant MYB genes have been reported, so far, to interact with any retinoblastoma related protein in plants.

Working with the RBR1(RNAi) lines became more difficult as I could not have any homozygous line, to work with, for any of the RNAi constructs and all the data I have presented here were obtained mainly from the primary T1 transformants. However, it has been shown here that retinoblastoma related gene, RBR1, in Arabidopsis plays an important role in various aspects of plant development, from shoot apical meristem organization and maintenance to gametophyte development. Currently there are more questions than answers regarding the functions of retinoblastoma related proteins, particularly in plants. My work, along with the characterization of T-DNA insertion lines and inducible RBR1 lines, undertaken by our group, can serve as the basis of understanding the role of this very important gene and the protein it encodes in planta. It can shed new lights on several physiological processes like gametophyte development, and coordination of the cell cycle with development and differentiation. Ultimately it would enable us to build an effective integrated model of plant growth and development.
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78
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