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Monitoring cell cycle modulated gene expression in plants by means of a whole-genome-based approach

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Spending five years preparing a PhD thesis was quite an experience. Not only did I learn a lot about performing good science, I am convinced that I also learned plenty about myself as a person. In this period, there were many people who supported me in all possible ways and this text is meant to express my sincere appreciation to them.

I am not only grateful to **Marc Van Montagu** for allowing me to start working here, but I want also mention my admiration for the fact that throughout the years, he was able to build up a well equipped and multicultural laboratory in which working was a real pleasure. In the last two years, **Marc Zabeau** took the responsibility of being my promotor. Marc, I want to thank you for your confidence and the "AFLP" opportunity you gave to me.

My stay in the Lab of Genetics can be divided in two periods. The first years, I spent as a happy member of the *Rhodococcus* group with **Koen Goethals** as my team leader. Dear Koen, it was really nice to be one of your Rhodo-girls. Thanks a lot for sharing your experience and enormous literature knowledge with me. Our many funny conversations I will always remember. No wonder that I give these Rosa Lisa smiles to you! It was always a good feeling to be surrounded by my other group members: **Wim** (mijn baasje) was a dear companion from the first day on and **Danny** was always there to give good advices (or to go to IKEA). I always enjoyed the Dutch touch from **Tita** in our group and I am profoundly grateful for the support she gave to me in finishing the flower work. With **Karen** and **Tania**, I worked closely together in our "white" and "small" labs. How they could bear my continuous noise production is still a miracle! In addition to all the fun, Tania learned me to incorporate some preciseness in my life and with my colleague "serpent" Karen I spent many nice moments in and outside the lab. Moreover, she learned me the pleasures of working on a clean bench! Last but not least, working together with **Filip** was a fine experience. Even though we sometimes both suffered from the difficulties of being a PhD-student, we always managed to encourage each other to keep on trying.

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neighbour, **Rudy** for being “mijn maatje”, **Stijn** for sharing some fine moments and **Enric** for being my unconditional friend.

II consider myself to be a very lucky person, since I am surrounded by a fine circle of friends. By a glass of wine, **Chantal**, **Mieke** and **Katrien** were always willing to listen to my complaints about failing experiments and helped me to relativate things. **Inge** and **Guy** brought order in my head when chaos was everywhere and my writing-retraite with **Karen** was a great experience.

The last part is completely dedicated to the people, which are most close to me. They always say that you can’t choose your family, but in my case this doesn’t matter at all. I’m privileged to have **wonderful parents** that have been supporting me in many ways as long as I can remember and always encouraged me to go for my ideals. I want to thank my mother for her continuous love and understanding and practical help during the last months (cleaning and other domestical activities have never been one of my qualities) and I guess my father is the person, which I respect most in life. I also want to thank my **auntie Julie** to be always there for me (and spoiling me enormously). The last words are for my dear brother **Rein**: From a little pain in the ass (boterham met kaas pappie?), he became an important value in my life. His common sense many times helped to shape order in my chaotic mind. You’re my best friend…

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Part I

Chapter One

A strategy of conserved mechanisms proves to be decisive to complete a successful round of cell division in eukaryotic cells

General Introduction

Living beings are in a constant need to propagate the cells they are consisting of. In essence, cells reproduce in a cyclic mode by duplicating their contents and a subsequent division into two parts. Therefore, it is not exagerated to proclaim this cell division cycle to represent a fundamental aspect of life. In unicellular organisms, such as bacteria and yeast, the propagation procedure leads to the production of an additional entity. For multicellular species, many cells are needed to make up a new individual and even in the adult body, cell division is required to replace the cells that are lost by tear and wear or by more complex reactions such as progammed cell death. For instance, an adult human has to manufacture many millions of new cells every second, simply to maintain the status quo and if this for some reason becomes halted, the individual will die within a few days.

The details of a cell cycle may vary for a certain species, but some requirements are considered to be universal in order to achieve the production of a pair of genetically identical daughter cells. First and foremost, the DNA has to be faithfully replicated and the replicated chromosomes have to become equally segregated into the resulting cells. Of equivalent importance is the problem to coordinate these events during the cell division process. Therefore, the existence of an adequately operating control system is compulsory.

This chapter is concerned with the processes obligatory for proper cell division and the different ways they are coordinated with one another. After an introductory outline of the discrete phases, which make up a complete cell cycle, we will go into detail about the complex organization to control the right follow up of these events as well as the action of different extra cellular signals to direct cell cycle progress. Thorough research taught us that the proteins making up the control system first appeared over a billion years ago and have been well conserved during evolution. Consequently, one can consider the cell cycle to be highly preserved among eukaryotic species. For this reason, the decision was made to integrate the knowledge of yeast genetics together with studies on mammals and plants to draw a general picture of the status in molecular-genetic cell cycle research nowadays.

1.1. For a cycling cell, there are four successive stages to go through in order to produce its offspring

1.1.1. Description of the different phases of an eukaryotic cell cycle

1.1.1.1. General considerations

By microscopical observation, it had already been apparent from the end of the 19th century that a cell division cycle consists of two discrete phases (figure 1). The most dramatic moment is the so-called M-phase, the process in which nuclear division takes place and that ultimately leads up to the moment of cell division itself. The much longer period that elapses between one M-phase and the next one to happen is called the interphase. Under the microscope, this appears as an uneventful interlude in which the cell only seems to grow in size. Implementation of other techniques revealed that this interphase actually is a busy time for the dividing cell. In particular, it is during interphase that the DNA content of the nucleus becomes replicated.

In the early fifties, Howard and Pelc (1953) introduced a modern terminology for the standard eukaryotic cell cycle. They made up a subdivision of the interphase into a G1-, S-, and G2-phase thereby recognizing the fact that the DNA replication happening in the S-phase needs to be separated from the M-phase by two gap-phases (G1 and G2). A complete round of cell division occurs in the sequence G1-S-G2-M and it is this arrangement that will allow a precise control of both the process of DNA replication and the events taking place in the M-phase. The G1- and G2-phases also provide time for additional growth. During G1, the cell monitors its environment and own size and, when conditions are favorable, takes the decision to commit to DNA replication and the completion of the

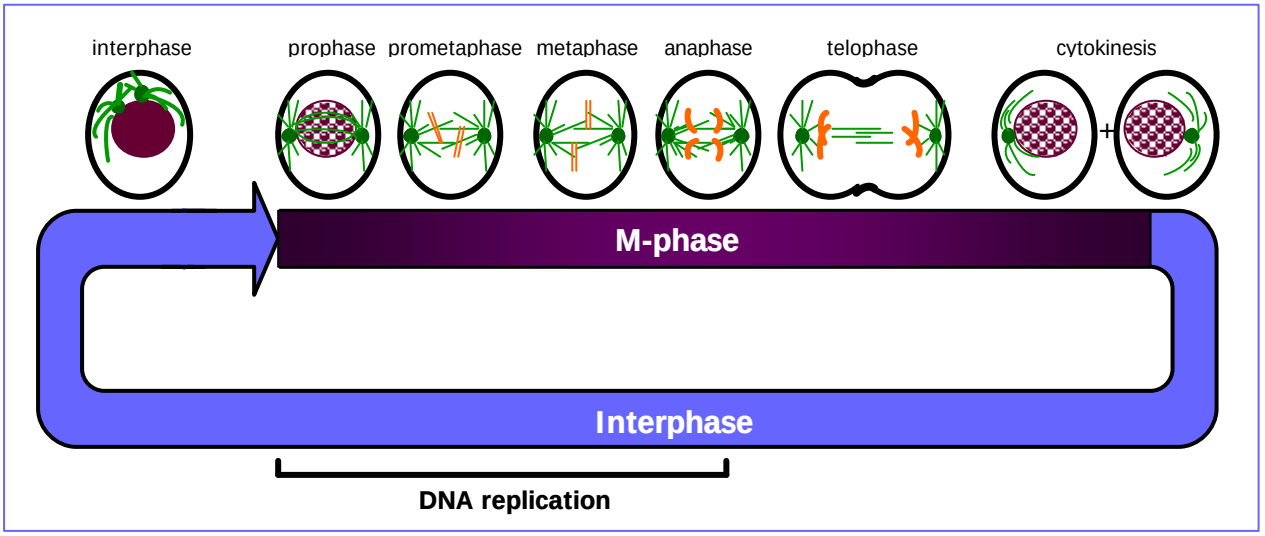


Figure 1: Events of cell division as seen under the microscope

division cycle. The G2 phase can be regarded as a kind of safety gap, allowing cells to ensure that DNA replication has completed before going into mitosis.

1.1.1.2. The M-phase is the culminating event of the cell cycle

The M-phase includes the various stages of nuclear division, called mitosis, and cytoplasmic division, known as cytokinesis. In a comparatively brief period, the contents of the parental cell that have been doubled as a consequence of the biosynthetic activities during the preceding interphase, become segregated into two daughter cells by the action of these two distinct mechanical processes. Traditionally, the M-phase is divided into subsequent stages nominated as prophase, prometaphase, metaphase, anaphase, and telophase. These five stages constitute mitosis (as defined to be the period in which the chromosomes are visibly condensed), whereas an additional sixth stage can be discriminated, overlapping with the end of mitosis, in which cytokinesis occurs.

As judged from microscopical observations, the transition from the G2-phase to the M-phase of the cell cycle cannot be regarded as a sharply defined moment. The chromatin, appearing as diffusely spread material in interphase, will slowly condense into well-defined chromosomes. Each chromosome has been duplicated during the preceding S-phase and consists of two sister chromatids. Toward the end of prophase, the main component of the mitotic apparatus, named the mitotic spindle, begins to form. This is a bipolar structure composed of microtubules and associated proteins (for further discussion, see section 1.1.2.2.). The nucleolus will disperse. Prometaphase starts abruptly with the disruption of the nuclear envelope, breaking into membrane vesicles that are indistinguishable from bits of the endoplasmatic reticulum. The chromosomes become attached to the mitotic spindle by the assembly of specialized protein complexes, called kinetochores that mature on the centromeres and are capable to catch some of the spindle microtubules. Eventually, in metaphase, the tension exerted by the microtubuli with the attached kinetochores leads to the alignment of the chromosomes in one plane halfway between the spindle poles (the metaphase plate). Triggered by a specific signal, anaphase starts abruptly as the paired kinetochores on each chromosome separate and thereby allow each chromatid (from this moment on called a chromosome) to be pulled slowly toward the spindle pole it faces. In telophase, the separated daughter chromosomes arrive at the poles, after which the kinetochores will disappear. A new nuclear envelope re-forms around each group of daughter chromosomes. The nucleolus reappears and the chromosomes will decondense into chromatin again. The mitosis has come to an end. Simultaneously, by the process of cytokinesis the cytoplasm will divide and the cell will be split in two daughter cells.

1.1.2 The significance of the cytoskeleton for a proper execution of cell division

Eukaryotes possess a large, complex network of linear polymers that serves as a roadway and scaffold for the contraction and motility of their cells, as well as the intracellular organization of the cytoplasm, organelle integrity and the movement of cargo within the cell. This set of connections is commonly referred to as the

cytoskeleton and it is, unlike a skeleton made of bone, observed to be a highly dynamic structure. The dynamic features of the cytoskeleton and its essential function in the cell division machinery are best illustrated at the moment when mitosis is impending. At this moment, a dramatic reorganization takes place, resulting in the mitotic spindle. The formation of this structure is of vital importance since it will, upon mitotic progress, take care of the equal partitioning of the DNA from mother to daughter cells. Additionally, as will be discussed in the following sections, specific cytoskeletal arrangements are fundamental to direct cytokinesis in the ultimate stage of M-phase.

1.1.2.1. Constituents of the cytoskeleton

The polymeric network of the cytoskeleton consists of three types of protein filaments (Kreis and Vale, 1993; figure 2). Actin filaments and microtubules are built of actin or a- and ß -tubulin subunits respectively. All eukaryotes are known to have tubulins and actins, so they both must have evolved at the moment that these kinds of cells became established. They are believed to form the core of the cytoskeleton (Baluska *et al*., 2001). The third group of cytoskeletal polymeric structures is called the intermediate filaments. Different classes are known to occur in animal cells and are classified according to the subunits they are consisting of: lamin, keratin, vimentin or neuronal protein filaments (Albers and Fuchs, 1992; Stewart, 1993; Baskin, 2000). Evidence for the occurrence of intermediate filaments in plant species remains inconclusive (Baskin, 2000).

The protein filaments of the cytoskeleton are known to bind a large variety of accessory proteins, which enable the same filament to participate in distinct functions in different regions of the cell (Lee, 1993; Baskin, 2000). They show to be less conserved in eukaryotic evolution. Some of these accessory proteins are responsible for the bundling of cytoskeletal polymers of the same type. Others are involved in the cross-linking of the cytoskeleton to distinct components of the cell such as membranes, biosynthetic enzymes, or signal transduction components (Baskin, 2000). However, the most important group of the accessory proteins are considered to be a collection of mechanicochemical enzymes, colloquially referred to as the motor proteins. Three super families of molecular motors have been identified up till now and have been well characterized in animal systems (Mountain and Compton, 2000; Vale and Milligan, 2000). Myosins move along actin filaments, whereas kinesin and dynein have been shown to be associated with microtubules. They can be considered as true motors, since they are capable of converting the chemical energy released from hydrolysis of a nucleotide triphosphate into kinetic energy for movement along the cytoskeletal tracks. By means of this kind of energy generation it becomes possible to transport cargo in various forms, including vesicles, organelles and chromosomes. Relatively little is known about molecular motors and their possible roles in plant cells (Reddy, 2001). However, in recent years, by application of various techniques, a few molecular motors have been isolated and characterized from plants (belonging to the kinesin and myosin super families). These studies, together with the analysis of the *Arabidopsis* genome sequence, indicate the existence of some motors in plants with novel, plant-specific functions (Reddy, 2001; Reddy and Durner, 2001).

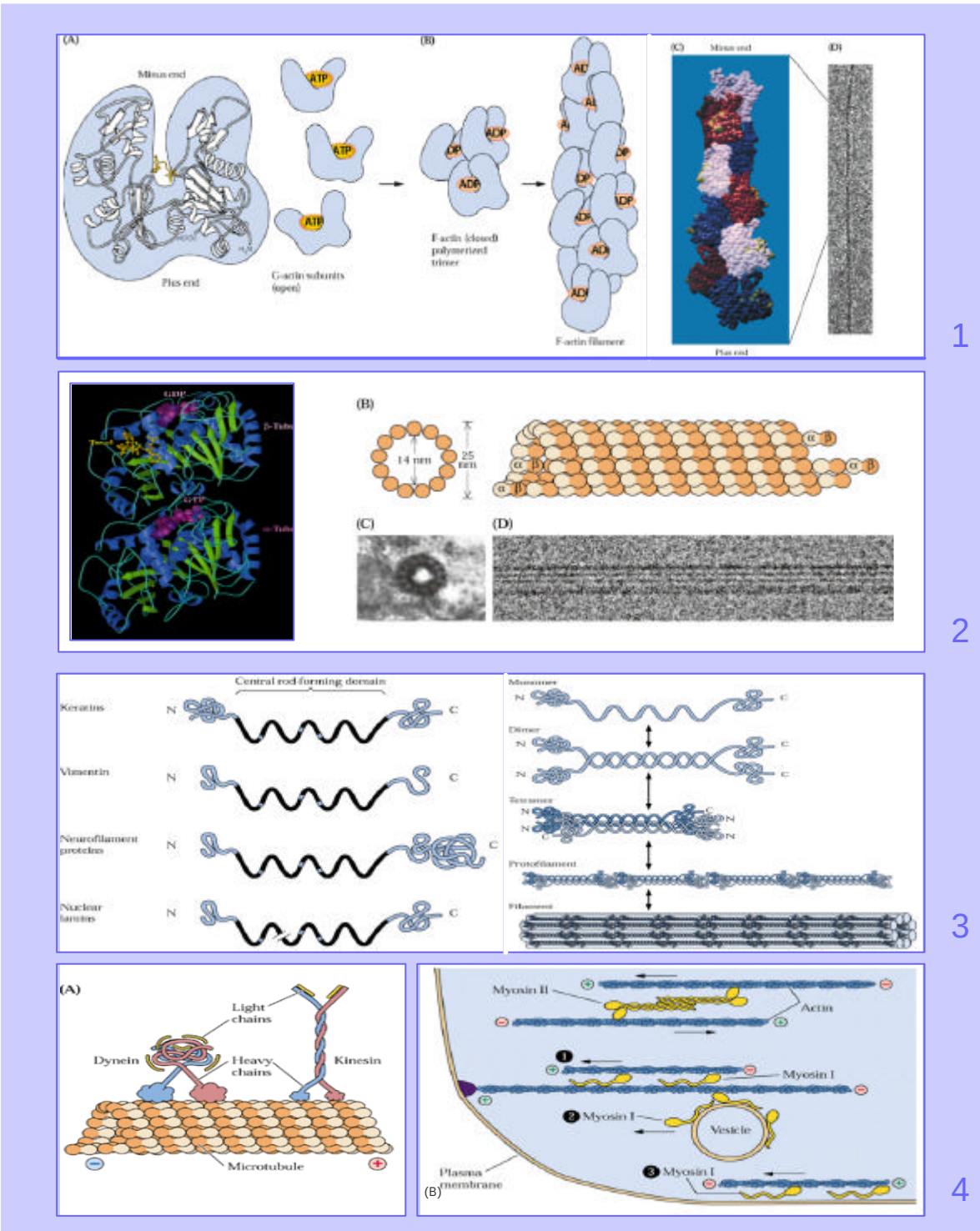


Figure 2: Basic components of the cytoskeleton

- Structure of actin and actin filaments, where panel A represents a single unit, B the formation of the filament, C a three-dimensional model and (D) a cryoelectron microscopical picture
- Structure of microtubules. Panel A is three-dimensional model, B a diagram of a cross section and side view and C and D corresponding electron micrographs
- Intermediate filaments. A represents the monomer and B the assembly of the filament
- Motorproteins. Panel A cytoplasmic dynein and kinesin, interacting with tubulins, B myosins in interaction with actin

1.1.2.2. Basic features of the mitotic spindle

1.1.2.2.1. General considerations

The mitotic spindle is a bipolar, dynamic structure (figure 3) formed at the onset of mitosis (Inoué, 1981; Baskin and Cande, 1990). It is mainly built of microtubules, radiating away from each pole. Microtubules are hollow fibers of a/ß tubulin heterodimers, polymerized in a linear fashion with two distinct ends: A fast-growing positive end where ß-tubulins are terminally exposed and a slow growing minus end, where a-tubulins are complexed with ?-tubulins. The minus ends are frequently associated at the sites where microtubuli nucleate, while the rapidly changing plus ends are often observed to occur freely in the cytoplasm. The microtubuli in the mitotic spindle are basically a scaffold for execution of force. For instance, polar microtubules exert a pushing force needed to drive the poles of the spindle apart, whereas a concerted pushing and pulling of the so-called kinetochore microtubules takes care of the alignment of the chromosomes at the metaphase plate and their subsequent pole ward migration in anaphase. When the nuclear envelope breaks down in early prometaphase, the kinetochore microtubules connect to the chromosomes. The interaction is mediated by the configuration of a large protein complex, named kinetochore, on the centromere region of the condensed chromatids. The process, that can be compared to a fisherman casting a fishing rod, randomly probing a pond, to catch a fish that every now and again passes close enough to the bait. Likewise, microtubule ends are probed and captured by the chromosomes. Subsequently, the chromosomes are aligned at the central plane of the mitotic spindle, forming the metaphase plate. Mammalian kinetochores have been extensively studied and are known to have a cup-shaped, trilaminar structure (Warburton, 2001). In plants, the ultra-structural appearance of the kinetochore resembles that of animals (Hong-Guo *et al.*, 2000). Moreover, recent data indicate that the plant kinetochores contain homologs of many of the proteins implicated in animal and fungal kinetochore function (Yu *et al.*, 2000). In yeast, there is no visible indication for the existence of kinetochores. However, it is manifest that they also do possess a kinetochore-like activity as chromosomes can bind the microtubules and a centromere-associated microtubule binding activity has been isolated (Lechner and Carbon, 1991; Sorger *et al.*, 1994; Saunders, 1999).

Microtubules are tremendously dynamic structures, marked by an abrupt alternation between phases of polymerization and depolymerization, often referred to as microtubule instability and mostly occurring at their plus ends, what makes it possible to assemble new structures (Mitchison and Kirschner, 1984; Gelfand and Bershadsky, 1991). This has already been demonstrated in animal systems but remains still to be determined for plant cells (Vantard *et al.*, 2000). During interphase, this turnover rate is moderate for animal cells, but for plant cells it has been demonstrated to be three times faster (Vantard *et al.*, 2000). At onset of M-phase, for both cell types this rate increases spectacularly and is clearly observed in the formed mitotic spindle. Concluding from research in the animal field, it was said that the origin of the spindle and chromosome movements, necessary for appropriate execution of mitosis, is residing in this dynamic, instable feature of the mitotic

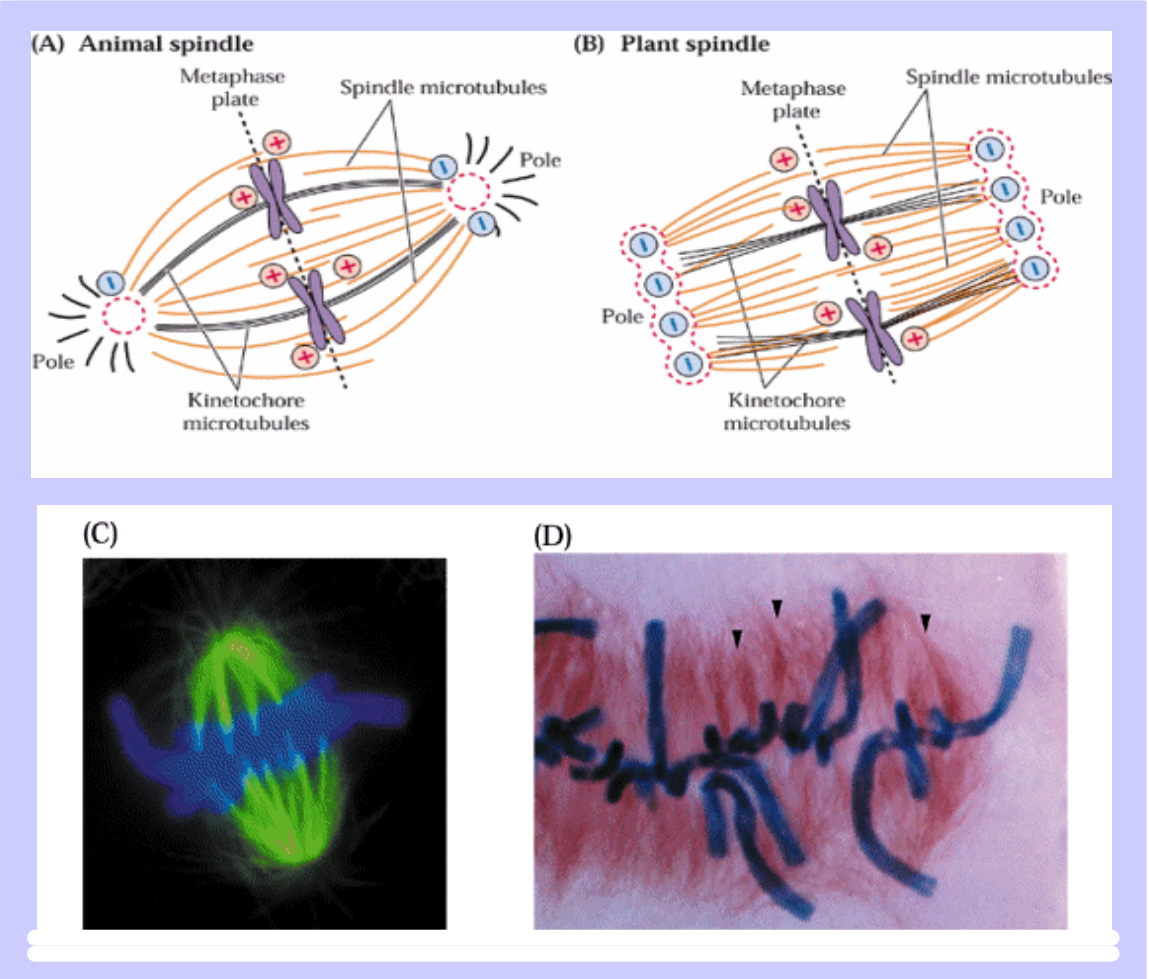


Figure 3: Cytokinesis is in animal versus plant cells

A and B represent a schematic comparison, while C is a a fluorescence micrograph of a metaphase spindle from a vertebrate tissue culture cell (chromosomes are blue and the microtubules are green). D is a light micrograph of a region of the metahase spindle of an endosperm cell from *Haemanthus katherinii*

spindle in concert with the phenomenon where individual microtubules fibers slide against one another. It has been estimated that these processes are satisfactory to produce the compulsory forces to assemble the spindle and to drive the pole ward movement of the chromosomes in anaphase (Koshland *et al.*, 1988; Inoué and Salmon, 1995; Hyman and Karsenti, 1996; Endow, 1999). For plant cells, it is thought that the dynamic instability of the microtubuli serves the same purposes (Vantard *et al.*, 2000).

1.1.2.2.2. The role of microtubuli organizing centers in animal and plant mitotic spindle morphogenesis

The major difference between the mitotic spindles of animals and plants is the structure of their poles (figure 3). In animals, the spindle pole is tightly focused, while in plants the pole usually tends to be very diffuse and broad, leading to a barrel-shaped mitotic spindle (Baskin and Cande, 1990). The animal spindle pole is built around a conspicuous structure called the

centrosome (Glover *et al*., 1993). It consists of a pair of centrioles in the core (a cylindrical array of triplets of microtubules), embedded in a mass of amorphous, pericentriolar material (Marshall and Brown, 1999). The centrosome is known to be the primary center of microtubule nucleation in animal cells and is associated with radial arrays of microtubuli, the astral microtubules (Stearns *et al*., 1991). At interphase, it is situated near the nucleus. The location of the centrioles at the core of the centrosome hints a role for these structures in nucleation. It was suggested that they might act as organizing centers by recruiting nucleating factors into a single focus within the centrosome (Marshal and Rosenbaum, 1999). Approximately a decade ago, an additional member of the tubulin super-family named ?-tubulin has been found to be specifically located in the centrosome (Oakley and Oakley, 1989; Stearns *et al,* 1991). Together with the genetic interactions between ?-tubulin mutations and those of ß-tubulin, it has been proposed that ?-tubulin might act as a nucleating factor (Oakley *et al*., 1990; Joshi *et al*., 1992). For yeast cells and fungi, a similar structure to the centrosome has been reported, called the spindle pole body. Although the structure is different to its mammalian counterpart, the basic mechanism of microtubule nucleation is known to be similar (Saunders, 1999). In contrast, plants do not possess centrosomes or any other discrete observable microtubule organizing center. Rather, the concept of multiple microtubuli nucleating sites, dispersed through the plant cell has been proposed (Mazia, 1984). During the late G2-phase, the nuclear envelope of a plant cell has been shown to be an important site of microtubule nucleation (Stoppin *et al*., 1996). After the breakdown of this nuclear envelope, the spindle is formed predominantly by kinetochore fibers (Palevitz, 1993; Smirnova and Bajer, 1998). In plants, genes encoding ?-tubulin have been cloned both in *Arabidopsis* and maize (Liu *et al*., 1993; Lopez *et al*., 1995). The presence of ?-tubulin has also been demonstrated in these species, found in association with all microtubule arrays and on kinetochores of isolated plant chromosomes (Liu *et al*., 1994; Binarova *et al*., 1998; Stoppin *et al*., 2000; Dibbayawa *et al*., 2001). Detailed analysis suggests a preferential localization of ?-tubulin in region with minus ends of microtubules (Liu *et* al, 1994). It seems that for plant cells, there is always a large amount of ?-tubulin present, although its localization changes depending on the cell cycle stage (Vantard *et al*., 2000). In interphase, they are localized within all cortical microtubule arrays. During G2, there is a preferential localization at the perinuclear region. As cells progress into mitosis, ?-tubulin is concentrated at caps near the poles of the prophase and late anaphase spindles, whereas it is localized in kinetochore fibers during metaphase. ?-tubulins are also found to be localized to other M-phase specific cytoskeletal structures such as the preprophase band and the phragmoplast (see further sections). The association of the nuclear form of ?-tubulin with chromatin has proved to be an important factor of plant-specific microtubule arrays and acentriolar plant cell spindles (Binarova *et al*., 2000). The fact that plant cells lack the presence of centrosomes and associated centrioles is a first indication that these complexes are not required for the cell cycle to be executed. Similar conclusions were made from experiments in mammalian oocytes, where the bipolar meiotic spindle can be formed in the absence of a centrosome (Szollosi *et al*., 1972; McKim and Hawley, 1995; Marshall and Rosenbaum, 1999; Khodjacov *et al*., 2000). Moreover, it has been

observed that centrosomes can detach from spindle poles without disrupting the tight focus of microtubule minus ends at the poles (Compton, 1998). Third, high resolution electron microscopic analysis of the spindle pole showed that many spindle microtubules are not embedded in the centrosome and consequently must be bound by other factors (Compton, 1998). Finally, several non-centrosomal spindle components have been identified that play a role in focusing microtubule ends in mitotic and meiotic systems (Mountain and Compton, 2000).

1.1.2.2.3. Importance of motor proteins for mitotic spindle assembly and operation

By experimental evidence, the molecular motor proteins of the kinesin and dynein super families were shown to be essential in the functional microanatomy of cell division, since they power various aspects of spindle assembly and functioning (Hildebrandt and Hoyt, 2000; Mountain and Compton, 2000; Sharp *et al.*, 2000). At present, cytoplasmic dynein and multiple members of important kinesin subfamilies implied in mitosis have been identified in both animals and yeast. For plants, several mitotic motors have been identified to date, mostly in *Arabidopsis thaliana* or tobacco (Vantard *et al.*, 2000). In one of the earliest events of the establishment of the mitotic spindle, the centrosomes of animal cells, that have been duplicated prior to mitosis, need to be pushed apart in order to establish spindle bipolarity. Bipolar kinesins have been known to be implicated in such a process by sliding and cross-linking of antiparrallel microtubuli (Kashina *et al.*, 1997; Sharp *et al.*, 1999). However, there are experimental observations that indicate the incompleteness of this model, since they indicate that additional motors are involved, such as certain kinesin subfamilies and cytoplasmic dynein (Sharp *et al.*, 1999; Mountain and Compton, 2000). Cytoplasmic dynein is one of the most important factors involved in the process of spindle pole organization (Mountain and Compton, 2000; Sharp *et al.*, 2000). Experiments with frog egg and mammalian mitotic extracts provide strong support for cytoplasmic dynein to cross-link microtubules, and uses its minus-end directed motor activity to drive the convergence of microtubule minus ends at the spindle poles. Additionally, it delivers also a key structural protein, NuMA, to the spindle poles. In interphase, this protein is a constituent of the nuclear matrix, but after breakdown of the nuclear envelope, it associates with microtubuli and concentrates at the spindle poles (Mountain and Compton, 2000). Some kinesin types have also been known to organize the minus ends of microtubuli (Walczak *et al.*, 1998; Endow, 1999; Mountain and Compton, 2000). Another important feature of motor proteins regarding mitotic progress is performed by chromosome-associated kinesins. Their generated motor activity has been implicated in chromosome attachment to the spindle and microtubule movement throughout progress of mitosis (Inoué and Salmon, 1995). Additionally, cytoplasmic dynein has been localized to the kinetochores as well as CENP-E, an undefined kinesin member (Holzbauer and Vallee, 1994). Finally, some kinesins are known to regulate microtubule dynamics (Walczak *et al.*, 1998; Endow, 1999). In conclusion, in spite of the known functions of some molecular motor proteins in the organization of mitosis, it is important to emphasize that the precise mechanisms by which they govern spindle assembly and chromosome segregation remain largely

unknown. Additionally, studies in mammalians revealed a high degree of redundancy among motor function during spindle assembly. Probably, this redundancy arose out of the need to maintain high fidelity of chromosome segregation for cell viability (Mountain and Compton, 2000).

1.1.2.3. The cytoskeleton and the cytokinesis process

Although cytokinesis is known to occur in all life forms, different strategies are pursued to achieve the formation of two daughter cells. An important aspect of the contrasting cytokinetic apparatuses is residing in the different cytoskeletal structures needed to guide this process. The next part will highlight those divergences hereby stressing on plant and animal systems (reviewed by Field *et al*., 1999; Smith, 1999; Straight and Field, 2000)

1.1.2.3.1. Positioning of the cell division plane

As a rule, the cleavage plane has to be positioned to bisect the axis of chromosomal segregation in order to ensure that each daughter cell will contain a nucleus. This fundamental requirement can be achieved either by specifying the cleavage site during mitosis based on the location of the mitotic spindle or by determination before onset of M-phase by a mechanism independent of spindle position.

In animal cells, the importance of microtubules in positioning the site of cell division has been demonstrated by chemical perturbation and micromanipulation in embryos of marine species like sand dollar and sea urchin (Rappaport, 1996). The mitotic apparatus dictates the position of the cleavage plane midway the two asters formed by the corresponding astral microtubules that originate from the spindle poles. There is evidence that interaction is necessary between these microtubuli and cortical sites (White and Strome, 1996). However, few molecular details of this communication have been worked out in animal cells. The proper separation of the asters seems to be the only requirement for accurate cleavage plane positioning and is probably the single function of these structures regarding cytokinesis (Hiramoto, 1956). Additional studies in grasshopper neuroblasts and newt epithelial cells indicated situations where signals originating from the spindle midzone are essential as an alternative to astral microtubules (Rappaport 1996). In the absence of any asymmetric cues (like reported for *Caenorhabditis elegans*), the mitotic spindle is known to be centered in animal cells, thus cytokinesis ultimately will result in a near exact cleavage of the mother cell into two equal parts.

For plants, the situation is somehow different since here the future cell division site already becomes determined at the end of G2 phase (figure 4). At this moment of the cell cycle, a transient array, the preprophase band (PPB), appears which originates from the interphase microtubule skeleton (microtubuli distributed throughout the cell cortex) and encircles the nucleus (reviewed by Mineyuki, 1999). It will disassemble prior to the appearance of the mitotic spindle at the end of prophase. Several kinds of molecules are reported to occur in the PPB, such as actin and ?-tubulin (Liu *et al*., 1993; Mineyuki, 1999). The PPB is thought to leave a landmark in the cortex, thereby specifying the future site for the new cell wall to be formed, since it has been observed that this always

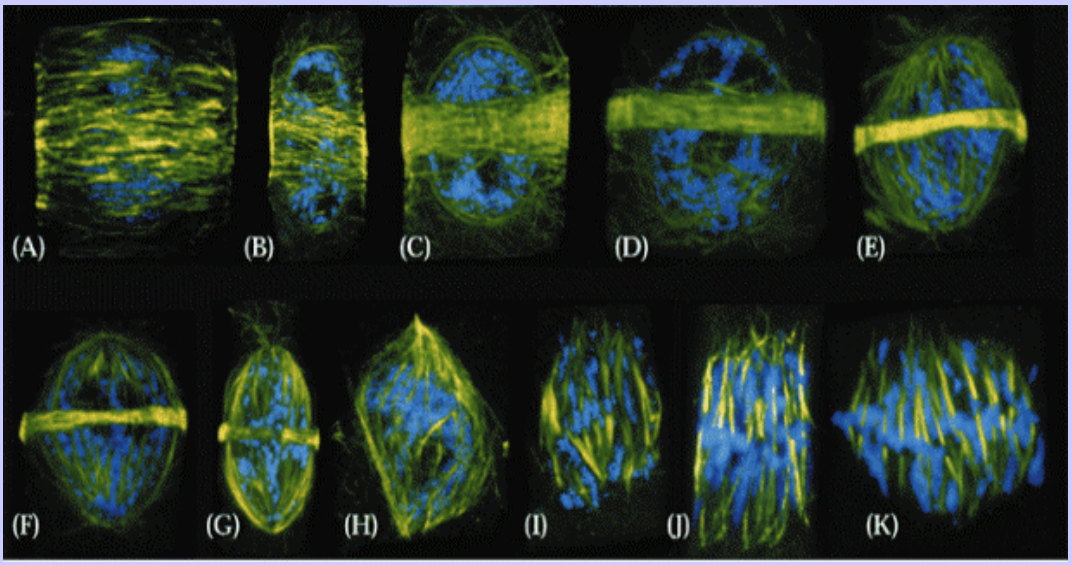


Figure 4: Transient formation of the preprophase band (PPB) during assembly of the plant mitotic spindle
(A-D) Development of PPB
(E-H) The prophase spindle forms from foci of micotubules at either end of the nucleus
(G,H) Disassembly of PPB in late prophase
(I-K) Formation of the mitotic spindle after breakdown of the nuclear envelope. Kinetochores capture bundles of spindle microtubules

occurs at the identical site where the PPB was located earlier in mitosis. However, the precise form of the deposited information is still unknown, but the occurrence of an actin-depleted zone in the PPB region during mitosis has been proposed to be involved (Cleary *et al*., 1992). Consistent with a crucial role for the PPB in orienting new cell walls, mutants have been isolated in *A. thaliana*, named *ton* and *fass*, that fail to form PPBs and as a consequence are disoriented in the formation of the cell wall (Torres-Ruiz and Jürgens, 1994; Traas *et al*., 1995; Nacry *et al*., 2000). However, it has to be mentioned that PPB formation probably is only one of many strategies utilized within the plant kingdom for establishment of the plane of cell division. Indeed, there are some plant species known not to form PPBs in their cells prior to mitosis (Pickett-Heaps *et al*., 1999). Moreover, PPBs are absent in the cytoskeletal organization necessary for meiosis (Staiger and Cande, 1992).

1.1.2.3.2. Spatial control of cytokinesis

Concerning cytokinesis, again a contrasting strategy has been reported for animal systems when compared to the events happening in plant cells.

In animal cells, cytokinesis occurs in an outside-in mode by a contraction of the plasma membrane between the daughter nuclei (Field *et al*., 1999; Straight and Field, 2000). In a first stage, a cleavage furrow becomes assembled by protein recruitment at the site of cell division (figure 5). Subsequently, it will become narrowed by the action of a contractile actomyosin-based ring that will be formed after breakdown of the

mitotic spindle. It is this ring that is responsible for the pulling of the existing plasma membrane inward the center of the plane of cell division. The exact mechanism resulting in the accumulation of the actin and myosin filaments that make up the contractile ring remains largely unknown (Field *et al*., 1999). Based on the availability of some imaging data, a role of directed transport in the plane of the cortex has been proposed (Cao and Wang, 1990; DeBiasio *et al*., 1996). In addition to actin and myosin, other evolutionary conserved proteins, the septins, are required for cytokinesis. However their role remains unclear (Field *et al*., 1999). It is thought that microtubuli are also involved in the cytokinetic apparatus by communicating with the cell cortex to stimulate the ingression of the cleavage furrow and by assembling the midzone and a structure containing proteins indispensable for completion of cytokinesis, named the midbody. The midzone is a term referring to the region between the separated anaphase chromosomes and is characterized by arrays of 10 to 30 antiparrallel bundled microtubules, formed of interzonal microtubules of the mitotic apparatus or *de novo* polymerized in cells that never contained a bipolar spindle (Canman *et al*., 2000). The microtubuli bundles in the midzone are similar organized as those in the plant phragmoplast (discussed in next section). Therefore, it has been suggested that they are required for the accumulation of vesicles and membrane disposition during furrowing (Field *et al*., 1999). Kinesins, such as ZEN-4, XKLP1 and CENP-E have been shown to associate with the microtubuli and probably assist these structures in their function (Raich *et al*., 1998; Straight and Field, 2000).

In contrast to animals, cytokinesis in most cells of flowering plants is achieved throughout the *de novo* construction of a cell wall and plasma membrane progressing from the center to the periphery of the cell (Smith, 1999; Nacry *et al*., 2000; Otegui and Staehelin, 2000; Sylvester, 2000; Verma, 2001; figure 6). The phragmoplast is the cytoskeletal apparatus that directs this event by targeting membrane vesicles to the center of the division plane where they fuse with one another to form a transient membrane compartment, the cell plate (Bowerman and Severson, 1999; Nacry *et al*., 2000). This cell plate will expand laterally by continuous vesicle fusion to its margins and finally will reach the parental cell wall. By a complex process of maturation, including the deposition of cell wall material into its lumen, it will finally turn into a new cell wall that cuts the mother cell into two parts (Nacry *et al*., 2000). The phragmoplast consists of two sets of opposing microtubuli, actin filaments and a multitude of mostly unknown proteins (Otegui and Staehelin, 2000). It appears to initiate from microtubuli in the center of the anaphase spindle and, upon progress of cytokinesis, grows centrifugally (Zhang *et al*., 1993; Granger and Cyr, 2000). Although arrays of actin filaments are prominent components of the phragmoplast, their function remains largely unknown. A role in phragmoplast assembly and turnover has been suggested, but some experiments do not exclude an eventual functioning in cell plate formation (Smith, 1999; Otegui and Staehelin, 2000). At present, three molecular motors of the kinesin family have been localized to phragmoplast: TKRP125 (Asada *et al*., 1997), KCBP (Bowser and Reddy, 1997; Kao *et al*., 2000), and a kinesin-related protein encoded by *KatA, -B, and –C* in *Arabidopsis* (Liu *et al*., 1996). One would expect them to be involved in the movement of vesicles to the emerging cell plate, but their co localization to the

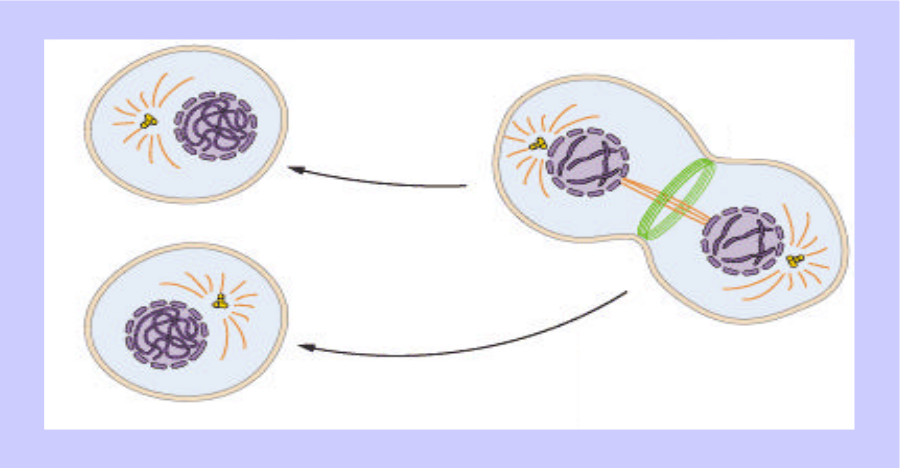


Figure 5: Cytokines is in animal cells, by the pinching of the cell by the contractile ring (consisting of actin filaments and myosin)

phragmoplast microtubuli contradicts this possibility. Moreover, the injection of KCBP antibodies that keep these proteins constitutively active into *Tradescentia* stamen hair cells suggested a role for this motor in mitotic spindle and phragmoplast organization (Vos *et al*., 2000). In an up-to-date study, using tobacco BY-2 cells that stably express a GFP-construct that labels microtubuli, observations where made that support the involvement of motors, such as TKRP125, in the process of phragmoplast formation out of the mitotic spindle in late anaphase (Granger and Cyr, 2000). To conclude, instead of directing vesicle transport, they are supposed to be involved in the organization of the phragmoplast itself and the motors that are truly responsible for formation of the cell plate are yet to be identified (Smith, 1999; Otegui and Staehelin, 2000; Sylvester, 2000).

The mutation of *knolle*, a syntaxin-encoding gene of *A. thaliana*, was found to result in cytokinesis defects during embryogenesis: Fragmented cell walls and wall stubs indicative of cytokinesis effects could be observed in these plants (Lukowitz *et al.*, 1996; Nacry *et al*., 2000). The type of syntaxin encoded by the *knolle* gene is involved in the fusion of secretory vesicles with appropriate target membranes in animal cells, indicating that membrane addition may be required specifically for cytokinesis. Further support for this thesis has come from ultra structural studies, which have shown that membrane vesicles accumulate at presumptive sites of cell wall synthesis in these *knolle* mutant embryos (Lauber *et al*., 1997). The mutant phenotype of *KNOLLE* is shared with other mutants in *Arabidopsis*, such as *KEULE* (Nacry *et al*., 2000). The protein localization of the *knolle* derived gene product is unaffected in *KEULE* mutants. Genetic interactions between the two proteins were analyzed in double mutant embryos, indicating that the two genes have partially redundant functions and interact specifically in vesicle fusion during cytokinesis in somatic cells (Waizenegger *et al*., 2000). Studies in *Xenopus* and more recently in *C. elegans* provided evidence that membrane addition also may be required for animal cytokinesis. (Blueminck and Laat, 1973; Jantsch-Plunger and Glotzer, 2000). In *Xenopus*, the addition of newly synthesized membrane during cytokinesis could be easily monitored (Blueminck and Laat, 1973).

In *C. elegans*, a syntaxin has been found to be indispensable for cytokinesis (Jantsch-Plunger and Glotzer, 2000). So, despite of their apparent very different cytokinetic strategies, plants and animals may use conserved genetic programs to incorporate new membranes during cytokinesis.

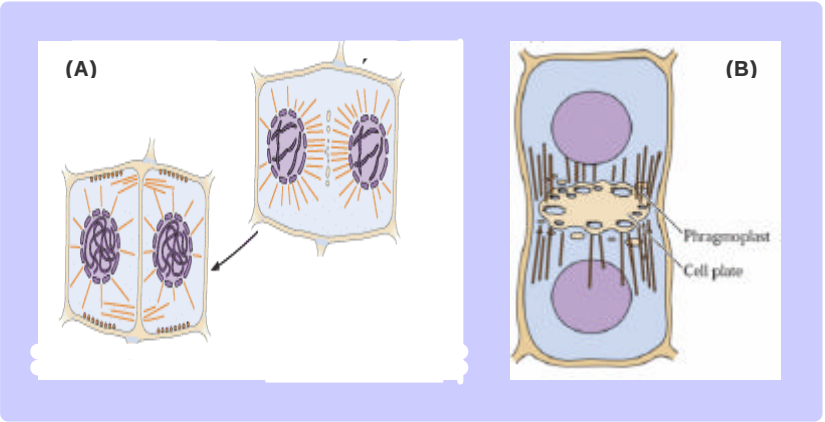


Figure 6: Cytokinesis in plant cells by formation of the phragmoplast (panel (A)-right and (B)). This structure will reach the parental cell wall to build a new cell wall between the daughter cells. It consists mainly of actin filaments and microtubules

1.2. Regulation mechanisms are an indispensable module of the cell cycle apparatus

The essential processes of the cell cycle, such as DNA replication, mitosis and cytokinesis are triggered by a central control system. Although the controller in principle could operate as a simple timer by allowing a fixed amount of time for each process to be executed, this proves not to be the case in reality. As a matter of fact, it becomes regulated at critical points of the cycle by feedback from the processes that are being performed. This is necessary to prevent the next process to become initiated before the former has been terminated (Hartwell and Weinert, 1989). Therefore, the controlling agent can be considered to follow an analogous procedure as a programmed washing machine, by behaving like an indicator that rotates clockwise (G1→S→G2→M) and hereby triggers the appropriate process only when it reaches specific checkpoints. The most important checkpoints are acknowledged to be the checkpoint at G1/S transition where the cell will commit to a new round of propagation (commonly referred to as the restriction point in yeast or START in higher eukaryotes), the so-called G2/M checkpoint before entry into mitosis and the checkpoint at the onset of anaphase to guide proper exit from mitosis. Figure 7 illustrates how cell cycle control is achieved in plants and animals. The different modules involved will be thoroughly discussed in the upcoming sections of this chapter.

1.2.1. The cell cycle control system is a protein kinase-based machine

1.2.1.1. Cyclin/Cdk complexes are the basic constituents of the cell cycle control system

1.2.1.1.1. The early days: Fundamental research in frogs and yeast provide the keys to unravel the basic principles of cell cycle regulation

A major breakthrough towards the understanding of the control system governing the proper execution of cell division was achieved by the analysis of M-phase inductive activities in amphibian eggs and early embryos (figure 8). By working with these model systems which have a simplified cell-cycle control system, one was able to isolate the crucial component to induce mitosis in G2-arrested embryos, named the maturation-promoting factor (MPF; Masui and Markert, 1971; Smith and Ecker, 1971). By applying biochemical strategies on purified MPF preparations, it became clear that its activity depends on the presence in equimolar amounts of two proteins. The first component, $p34^{cdc2}$, belongs to the family of the cyclin-dependent protein kinases (CDK for short) and upon activation, it induces the downstream processes by phosphorylating selected proteins on specific serine or threonine residues (Gauthier *et al.*, 1988). The second is a member of a family of specialized activating proteins called cyclins (Gauthier *et al.*, 1990). The first cyclins were discovered in marine invertebrates and were named after their characteristic cell-cycle dependent pattern of synthesis and destruction (Evans *et al.*, 1983). Entry into S-phase is only feasible upon assembly of these two components into a functional complex, where the activity of the CDK is reliant on the physical interaction with its cyclin partner (Norbury and Nurse, 1992; figure 8).

Contributions originating from genetical approaches were also of great importance to establish the functional significance of important members of the cell cycle control apparatus. Yeast genetics proved to be a very valuable tool in this field, since a systematic search for temperature sensitive mutations in genes encoding for components of the cell cycle machinery lead to the identification and cloning of the coding sequences for the cyclin and CDK subunits of MPF. In fission yeast (*Schizosaccharomyces pombe*), Cdc2 was identified to be the kinase subunit, whereas the *cdc13* gene encodes the cyclin constituent of the MPF complex (Nurse, 1975; Nurse and Thuriaux, 1980). In budding yeast (*Saccharomyces cerevisiae*), the CDK subunit is encoded by the gene *CDC28* (Nasmyth *et al.*, 1993; Lew *et al.*, 1997). Cdc2 and Cdc28 are the only CDKs that are occupied with cell cycle control in yeast. The Cdc2/CDC28 protein amount stays constant throughout the cell cycle and they are able to bind a diversity of cyclins accumulating at different phases, hereby determining their functioning at specific moments. More CDK proteins have been isolated from yeast species, such as PHO85 in *S. cerevisiae*, but they are known to have other functions than cell cycle control (Nigg, 1995; Measday *et al.*, 1997, Andrews and Measday, 1998). Cyclins are roughly divided into two classes: B-type or mitotic cyclins and G1-cyclins. At present, four different cyclins are known to associate with Cdc2 in fission yeast (Moser and Russell, 2000). Cig1, Cig2 and Cdc13 belong to the so-called group of

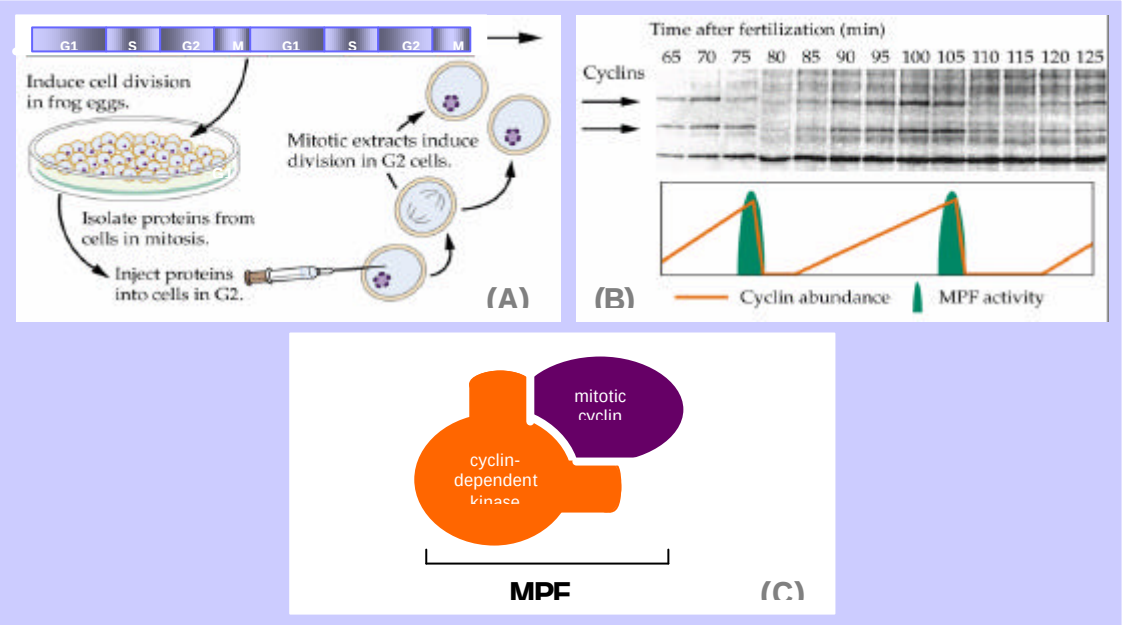


Figure 8: Research in *Xenopus* leads to the isolation of the MPF

A. Extracts taken from mitotic oocytes contain proteins that induce G2 cells to undergo mitosis. This and similar experiments were used to isolate a mitosis-promoting factor (MPF) capable of inducing G2 cells to divide

B. Biochemical analyses of fractions of cell extract taken at various stages of the cell cycle reveal the presence of cyclins. Cyclin abundance correlates with the activity of MPF

C. The two key subunits of MPF

B-type cyclins. Cig2 is known to be the major S-phase cyclin, accumulating late in G1-phase and disappearing upon exit from S-phase (Mondesert *et al.*, 1996). Cdc13 is required for Cdc2 activity at the onset of M-phase. Consequently, its protein levels are low in G1-phase but they will increase in G2-phase and stay at this level until the end of M-phase (Moreno *et al.*, 1989). Another cyclin, Puc1, is impaired in the regulation of G1 phase in response to their cell size (Martin-Castellanos *et al.*, 2000). In budding yeast, 9 cyclins are of importance for progression through the cell cycle and their expression profiles are fluctuating according to their function (Nigg, 1995; Andrews and Measday, 1998). CLN1,-2, and –3 are related to Puc1 of *S. pombe* and take care of the activation of CDC28 at START. They are thought to have overlapping roles, since these genes are genetically redundant (Nasmyth, 1993). *CLN1/2* expression is strongly cell cycle-activated, with peak expression in late G1 (Tyers *et al.*, 1993). CLN3 is only distantly related to the other two G1 cyclins. Its protein and transcript levels prove to be present throughout the cell cycle, but an elevation of expression occurs in early G1 phase, before the expression peaks of the *CLN1* and *–2* genes appear (Nasmyth, 1993; Tyers *et al.*, 1993). CLB1-6 are cyclins of the mitotic type (Nigg, 1995; Andrews and Measday, 1998). The cyclins of the CLB-group can be subdivided into two groups according to their proposed function: CLB5 and –6 are involved in DNA replication, whereas CLB1–4 are essential for mitosis. The observed expression patterns of these genes are similar as those observed for their functional counterparts in *S. pombe* (Nigg, 1995).

1.2.1.1.2. The basic mechanism for cell cycle control first discovered in unicellular yeast can be extrapolated to multicellular higher eukaryotes

Research in higher eukaryotes confirmed the conservation of cyclins and CDKs as the key factors of a universal cell cycle control mechanism. However, as will be demonstrated by the following sections, the situation for these multicellular organisms proves to be more complex since gene duplications and divergence apparently have generated multiple variants of the basic cell cycle genes. These variants exist side by side in one single cell and are specialized to function in slightly different ways.

Research in the mammalian field…

In mammalian organisms, the identification and isolation of cell cycle genes was achieved using conditional mutant animal cell lines as well as by complementation experiments with yeast mutant strains (Norbury and Nurse, 1992). At present, at least five mammalian CDKs are known to be involved in cell cycle control (CDK1-4 and –6; Murray and Marks, 2001). CDK1 was found to be the catalytic subunit of the vertebrate MPF and therefore is a true $p34^{cdc2}$ ortholog (Norbury and Nurse, 1992; King *et al*., 1994; Nigg, 1995). The other CDKs are known to function at the G1/S transition (Ekholm and Reed, 2000).

The mammalian cyclins constitute a super family, classified according to the deduced peptide sequence of the so-called “cyclin box”, the motif that is responsible for the interaction with the CDK partner. To date, the direct involvement in cell cycle regulation has been verified for four cyclin classes (cyclinA,-B,-C, and, -D; Nigg, 1995). CyclinA is involved in S-phase progression and DNA-replication, but synergistically with cyclinB is also essential to promote the entry of the cells into mitosis (Pagano *et al*., 1992; Knoblich *et al*., 1993; King *et al*., 1994). CyclinD is the principal cyclin that regulates G1 phase progression (Sherr, 1994). Finally, CyclinE is involved in the transition from G1 to the S-phase (Ohtsubo and Roberts, 1993; Knoblich *et al*., 1994). Recently, an indirect involvement of cyclinF in cell cycle control was proven by the fact that it seems to regulate the localization of cyclinB1 by physical interaction (Kong *et al*., 2000). Other classes are impaired with the regulation of transcription and therefore act independently of cell division. Furthermore, still many classes do exist where the exact function remains to be resolved. The availability of the human genome draft sequence allowed the identification of potential additional members based on homology data (Murray and Marks, 2001). CyclinA, -B, , and –F (also referred to as mitotic cyclins) levels are mainly under transcriptional control leading to a phase-dependent expression pattern, whereas the expression levels of the genes encoding D-type cyclins show little or no change during cell cycle progress (Sewing *et al*., 1993; Muller, 1995; Nigg, 1995). Peak mRNA and protein levels for cyclinA, -B, and –F are measured around G2/M transition. CyclinA accumulates slightly before the other mitotic cyclins and becomes destroyed earlier in mitosis. Finally, one additional mammalian cyclin, termed CyclinE, was isolated and found to be dominantly expressed at G1/S transition (Nigg, 1995).

… and how about plants?

The first clue suggesting that cyclin/CDK complexes also govern plant cell division appeared approximately 10 years ago with the discovery of a plant protein related to animal CDKs (John *et al*., 1989). This finding was almost simultaneously accompanied with the cloning of the first plant cDNA encoding a CDK (Feiler and Jacobs, 1990). Mainly by applying cloning strategies, many CDK-like proteins in both monocotyledonous and dicotyledonous plant species have been identified during the last years (Joubès *et al*., 2000; Mironov *et al*., 1999; Stals *et al*., 2000). Based on the comparison of their cDNA sequences with their animal and yeast counterparts, these CDK-related protein kinases from plants were allowed to be subdivided into 5 different classes (Mironov *et al*., 1999; Joubès *et al*., 2000). The A-type class forms the biggest collection and is most related to the mammalian CDK1 and –2 by the presence of the conserved PSTAIRE cyclin binding domain. Moreover, the protein kinases of this group are able to complement temperature sensitive mutants in yeast (Joubès *et al*., 2000; Stals *et al*., 2000). Additionally, a plant-specific class of CDK-related protein kinases can be distinguished (CDKB). The plant CDKs belonging to this group have a divergent cyclin binding motif (PPTALRE or PPTTLRE). Their expression levels are fluctuating during the cell cycle, which is unlike CDKs isolated from animals and yeast (Fobert *et al*., 1996; Magyar *et al*., 1997). In yeast *cdc2/cdc28* mutant complementation assays, they are not able to re-establish a functional phenotype. Based on expression analysis and sequence homologies, the CDKB group can be further subdivided into 2 subgroups (Mironov *et al*., 1999; Joubès *et al*., 2000; Stals *et al*., 2000). The members of the first two CDK classes are the most thoroughly characterized plant CDKs and to date are the only groups for which the relevance in cell cycle control has been experimentally verified (Mironov *et al*., 1999). The other three classes (CDKC-E) are less densely populated. The CDKD class includes the plant homologues of mammalian CDK7 (Joubès *et al*., 2000). For the CDK-like proteins belonging to the CDKC and –E class, sharing the PITAIRE and SPTAIRE hallmark respectively, there is no indication for a possible function in the plant cell cycle (Mironov *et al*., 1999). Experimental results for a member of the CDKC class in *Arabidopsis* even contradict a possible involvement of this protein in cell division control, since no expression associated with actively dividing cells can be detected by *in situ* hybridization experiments (de Pinho Barroco *et al*., submitted).

The first plant cyclins were discovered by Hata *et al.* in 1991 and constantly new cyclin cDNA clones become isolated in a wide variety of plants (Ito, 2000). The availability of the genomic sequence of *A. thaliana* additionally reveals new members (Vandepoele *et al*., submitted). As mentioned earlier for mammalian cyclins, also in plants there is wide sequence diversity for cyclins, leading to the categorization into different classes with a similar nomenclature as for mammalian cyclins. A unified classification for plant A-, B-, and D-types based on comparative sequence analysis was suggested by Renaudin *et al.* (1996), leading to a clustering into 8 branches (A1-3; B1 and –2; D1-3) and hereby revealing a more complex organization as for their mammalian counterparts. Additionally, it has to be stressed that this classification based on homology does not imply that the plant cyclins are functional homologs of the cyclins in the animal kingdom. Recently, a new class of D-type cyclins has been characterized in *Arabidopsis*, consequently named D4 (De Veylder *et al*.,

1999). CyclinE has not yet been identified in plants. Unpublished results from Vandepoele and colleagues indicate the presence of a putative H-type cyclin in the available sequence data of the *Arabidopsis* genome. Similar as for mammalian cyclins, a cell cycle-dependent expression pattern is measured for the plant cyclins of the A- and B-class contrasting to the cyclinD class members, which in general are expressed in a cell cycle-independent manner (Ito *et al*., 2000). Interestingly, there is a degree of correlation between this temporal expression pattern and a given cyclin class as defined by primary structure. Both plant B-type cyclins (B1 and B2) expression levels peak in the M-phase and therefore are thought to be the principal cyclins involved in mitotic progress (Fuerst *et al*., 1996; Reichheld *et al*., 1996; Shaul *et al*., 1996; Qin *et al*., 1996; Ito *et al*., 1997; Mironov *et al*., 1999; Ito *et al*., 2000). The cyclins of the A-class are induced and turned off in sequential waves prior to the B-type cyclins. Those of the A3-class are expressed first at G1/S transition, followed by similar peaks of expression from mid-S-phase till mid-mitosis for both the A1- and A2-class cyclins (Fuerst *et al*., 1996; Reichheld *et al*., 1996; Shaul *et al*., 1996; Ito *et al*., 1997; Setiady *et al*., 1997; Chaubet-Gigot, 2000). When compared to animal cyclins of the A-type, the expression profile of A3-type cyclins are similar, leading to the proposition that the cyclins of these groups might fulfil the dual functions in both replication and G2/M transition as described in animal systems (Shaul *et al*., 1996; Chaubet-Gigot, 2000). The expression pattern of cyclinA1 and –A2 is intermediate between those of the animal A and B cyclins. They probably play a role at both S/G2 and G2/M transitions (Chaubet-Gigot, 2000). One exception to this general observation has been reported for an alfalfa A2-class cyclin (CYCA2;1), which shows to be constitutively expressed during cell cycle progression (Meskiene *et al*., 1995). Consequently, it has been proposed that it plays a role in G1 phase transition. Nevertheless, recent research results contradict this thesis (Roudier *et al*., 2000): The associated protein kinase-activity occurred to be biphasic with a first peak in S-phase and a second major peak in M-phase. Moreover, immunolabelling experiments indicated a nuclear presence in interphase cells, whereas the protein completely disappeared in mitotic cells. For that reason, it has to be concluded that it most likely has the same function as a mammalian cyclinA protein. Up till now, no homolog of this cyclin has been reported to be isolated in other plant species. The plant D-type cyclins are suspected to play a similar role as their homologs in animals, acting in response to growth factors and nutrients, since cyclinD homologs in *Arabidopsis* and alfalfa are able to complement *cln* mutations in *S. cerevisiae* (Soni *et al*., 1995). Sorrell and colleagues (1999) characterized the expression pattern of some tobacco cycling genes in the synchronous cultures of BY-2 cells, where they showed that 2 mRNAs (corresponding to *cycD2*;1 and *cycD3*;1) appeared to be predominantly present during the G-to-M transition. This indicates an additional role for certain cyclinD gene products in entry into mitosis (Sorrell *et al*., 1999). However, these results should be regarded with caution, since they might originate from the consequence of the specialization of tobacco BY-2 cells to propagate at an extremely high rate, implying that these results are possible not to be extrapolated to other situations (Nagata *et al*., 1992; Nagata and Kumagai, 1999).

CDK/cyclin complex formation turns out to be more complicated for multicellular organisms

The fact that in animals and plants, more than one CDK seems to be directly occupied with progression through the different phases of the cell cycle makes the situation more multifaceted than in yeast, where only one CDK is present and can associate with all the available cyclins at a given checkpoint in the cell cycle. This potential presence of many different complexes makes their analysis more difficult.

For animal systems, already a considerable stack of data about CDK/cyclin complexes operating in specific phases of the cell cycle is available. It has been generally accepted that the activity of CDK4 and –6 is driven by the association with the three D-type cyclins to obtain a proper progression of the G1-phase (Nigg, 1995; Eckholm and Reed, 2000). CyclinE, which is known to accumulate very close to the G1/S-phase transition, is specifically concerned with the activation of CDK2, as is cyclinA later on in S-phase (Nigg, 1995; Eckholm and Reed, 2000). The mammalian CDK-component of MPF, CDK1, has experimentally been shown to have the B-type cyclins as an activating partner (Nigg, 1995). The partner of CDK3 has not yet been identified to date.

Papers describing functional data regarding CDK/cyclin complexes in plant cell cycle research are still very scarce. By performing a 2-hybrid screen, using an *Arabidopsis* CDKA-type CDK (CDC2aAt) as a bait, it was possible to identify a number of interacting proteins such as CYCD1;1 and CYCD4;1 (De Veylder *et al*., 1999). However, one has to bear in mind that it still remains to be demonstrated that these kinds of complexes actually become formed and activated under *in vivo* circumstances. An additional report indicates that -in *Arabidopsis*- CYCD2 and CYCD3 interact with the CDC2a protein *in vivo* (Healy *et al*., 2001). The interaction of these two CYCD kinases with PSTAIRE containing CDKs was already demonstrated before in tobacco plants (Nakagami *et al.*, 1999; Cockroft *et al*., 2000). The interaction of an A-type cyclin with a CDCA-class CDK was demonstrated in alfalfa as well as tomato (Roudier *et al*., 2000; Joubès *et al*., 2001). Very limited data are on hand about the formation of CDK/cyclin complexes around G2/M transition (Mironov *et al*., 1999). Some experimental information was obtained in an approach where a combination of multiple affinity matrices and chromatographic steps was used (Stals *et al*., 2000). In this way, active kinase complexes could be isolated from *Arabidopsis* suspension cells. CYCB1;1, CYCB2;1 and CYCB2;2 were found to preferentially co-elute with CDKA;1 whereas CYCB1;1 and CYCB2;2 became purified together with CDKA;2. As a summary, this collection of results in different experimental systems indicates that, at the G2/M progression of the plant cell cycle, complexes are formed with both CDKA and CDKB-type CDKs. On the other hand, CDKA type proteins seem to be the exclusive partner of the cyclins operating at G1/S.

1.2.1.2 Post-translational modifications are essential for CDK/cyclin activity

As outlined earlier in the text, the most important aspect of cell cycle regulation is the association of a CDK with its cyclin partner. Hence, the periodic availability of cyclins as a

result of transcriptional activity determines at which phase of the cell cycle a given complex can be formed and is considered to be the first level of regulation of the resulting CDK/cyclin complex. Although the interaction of both partners is indispensable to induce the kinase activity of the CDK subunit, it has been proven not to be sufficient to phosphorylate their substrates. It turned out that posttranslational modifications are required for the ultimate full activity necessary to induce downstream events (Norbury and Nurse, 1992). Additionally, CDK activity can be antagonized by the binding of inhibitory proteins (Lees, 1995). Consequently, posttranslational regulation mechanisms of the CDK/cyclin complex can be referred to as a secondary level of CDK kinase activity regulation.

1.2.1.2.1. Phosphorylation and dephosphorylation mechanisms regulate CDK/cyclin complex activation

In the case of $p34^{cdc2}$, phosphorylation of threonine 14 and tyrosine 15, two neighboring residues occurring within the ATP binding site of cdc2, causes the cdc2/cdc13 complexes to stay inactive upon assembly (Norbury and Nurse, 1992; Nigg 1995). This situation lasts during the last part of interphase until the G2/M transition point. The responsible kinase proteins for phosphorylation of tyrosine 15 were first identified in fission yeast and are the gene products of the *wee1* and *mik1* genes (Russell and Nurse, 1987; Booher *et al*., 1993). Slightly later, they became also identified in budding yeast cells. The region of $p34^{cdc2}$ that spans Tyr15 has been shown to be identical in its amino acid sequence for all eukaryotic species (Lee *et al*., 1987). Additionally, both in animals and plants, evidence has been presented that the phosphorylation of this site in the native CDK also functions as a control mechanism in multicellular eukaryotes (Norbury and Nurse, 1992; Zhang *et al*., 1996). Additionally, Tyrosine15 phosphorylation of CDKs has been implicated in water stressed wheat plants and salt stress conditioned *Arabidopsis* plants (Schuppler *et al*., 1998; Stals *et al*., 2000). Consequently, a number of Wee1/Mik1 type kinases were identified in animals, such as humans, *Drosophila*, *Xenopus* and mouse (Campbell *et al*., 1995; Honda *et al*., 1995; McGowan and Russell, 1995; Mueller *et al*., 1995; Watanabe *et al*., 1995). Recently, the first Wee1 homologue was isolated from maize, which is capable to inhibit CDK activity *in vitro* (Sun *et al*., 1999). Although the research on Wee1 kinases has been focused on its role in the regulation of MPF, it has become clear that this protein also could function at the entry into S-phase. It has been shown in animals that Wee1 is capable not only to phosphorylate the mitotic CDK/cyclin complexes CDK1/cyclinB, but also the CDK2/cyclinE and CDK2/cyclinA complexes at G1/S transition (Watanabe *et al*., 1995; Booher *et al*., 1997). Moreover, Tyr15 phosphorylation of CDK2 reaches a maximum during Sphase, primarily when complexed with cyclinA, whereas a CDK2 mutant that cannot be phosphorylated by Wee1 has a lethal phenotype in human cells (Gu *et al*., 1992; Jin *et al*., 1996). The maize Wee1 is up-regulated in endosperm development, hereby suggesting to influence CDK activity during the endoreduplication process which is known to involve a simplified cell cycle with only S- and gap phases (see further sections in this chapter; Sun *et al*., 1999).

In order to enter the eukaryotic cell into mitosis, the activity of the phosphorylating enzymes will decline. Simultaneously, a dual

specific phosphatase will become activated, removing both phosphate groups on $p34^{cdc2}$ and hereby causing the abrupt activation of the kinase (Norbury and Nurse, 1992; Nigg, 1995). By a genetic screen of temperature sensitive mutants, the protein encoded by the *cdc25* gene of *S. pombe* was identified to be responsible for this activity. It is assumed that the rate-limiting molecular process during the G2 timer is conferred by the dephosphorylation of Tyrosine15 by the CDC25 phosphatase (Sveiczer *et al*., 1999). In animals and budding yeast, functional homologues of this gene have already been cloned (Norbury and Nurse, 1992). To date, three different CDC25-related proteins have been identified in mammalian cells that complement fission yeast cells lacking the *cdc25* gene (Sadhu *et al*., 1990; Nagata *et al*., 1991). While CDC25B and –C are required for the regulation of G2/M transition, it appeared that CDC25A exerts its dephosphorylating activity to enter cells into S-phase (Molinari *et al*., 2000). Zhang and colleagues (1996) provided evidence for the relevance of a plant *cdc25* homologue in activation of the MPF in cytokinin-depleted tobacco cells, which are consequently arrested in G2-phase. When treated with purified yeast Cdc25 phosphatase, they will become dephosphorylated on Tyr15 and subsequently enter into mitosis. Nevertheless, to date no plant homologue of the yeast *cdc25* has been cloned.

Phosphorylation of threonine161 is absolutely required for CDC2 activity and is believed to regulate G1 exit in mammalian cells (Nigg, 1995; Wu *et al*., 2001). This residue is located within a domain (the T-loop) that is present in many kinases and is implicated in controlling the access of potential substrates to the catalytic site (Morgan and De Bondt, 1994). Accordingly, many efforts have been done to purify the enzyme that is able to phosphorylate this residue in CDC2 and other CDKs (termed CAK for CDK-Activating Kinase). The catalytic subunit of CAK was found to be itself a member of the CDK family. Originally, when first isolated in mammals and *Xenopus*, it was named MO15 (Fesquet *et al*., 1993; Solomon *et al*., 1993), but became later on referred to as CDK7 (Nigg, 1996). The eukaryotic CAK is believed to operate as the CDK7/cyclinH/MAT1 complex (Nigg, 1996). The MAT1 factor is responsible for the assembly and subsequent targeting of the CDK7/cyclinH complex by its RING finger motif (Wu *et al*., 2001). In yeast, CAK activity is exerted *in vitro* by Crk1/Mop1 in association with the cyclinH homologue Mcs2 (*S. pombe*) and in *S. cerevisiae* by CAK1/Civ1, whose activity does not require association with a cyclin (Buck *et al*., 1995; Damagnez *et al*., 1995; Mendenhall and Hodge, 1998). In budding yeast another protein kinase named KIN28, which is a very close relative of CDK7, has been isolated (Nigg, 1995 and 1996). However, it does not seem to possess any CAK activity and is only able to phosphorylate the C-terminal repeat domain of RNA polymeraseII, which is an activity that CDK7 and Crk1/Mop1 also have acquired (Buck *et al*., 1995; Damagnez *et al*., 1995; Nigg, 1995 and 1996). The mechanisms seem to be divergent for certain plant species. An *Arabidopsis* cDNA (CAK1At) was isolated as a repressor of CAK mutations in budding yeast and fission yeast (Umeda *et al*., 1998). It was demonstrated that the encoded protein possesses CAK activity toward human CDK2/cyclinA complexes. This protein is only distantly related to animal CAKs and is not dependent upon the association with a cyclin. In rice, the CDK7 homologue R2 (Hata, 1991) was found to be able to complement CAK deficiency in budding yeast, but not in fission

yeast. Additionally, in *in vitro* phosphorylation assays, the human CDK2 and the rice counterpart CDC2Os1 became kinated by this enzyme (Yamaguchi *et al*., 1998). A more recent report from the same research group presents evidence for the interaction of R2 with H-type cyclins that have been isolated from rice and poplar (Yamaguchi *et al*., 2000). The R2 protein is classified as member of the CDKC family of CDKs in plants (Joubès *et al*., 2000).

1.2.1.2.2. CKS proteins interact with CDK/cyclin complexes to serve as a docking factor

The CKS protein family constitutes of small proteins that perform essential conserved functions in the cell cycle. The first to be isolated was the *suc1* gene product in fission yeast, which is a suppressor of defective *cdc2* alleles (Hayles *et al*., 1986). The counterpart of this protein was soon isolated in budding yeast by grace of its ability to interact with CDC28 and was designated as Cks1 (Cdc28 kinase subunit 1; Hadwiger *et al*., 1989). The fact that two human homologues could be isolated from human cells by a PCR-based approach revealing that the CKS proteins are structurally conserved among eukaryotes. Each of them is able to functionally replace Cks1 in budding yeast and they associate specifically with CDK1, -2 ad –3 (Richardson *et al*., 1990). The precise role of these proteins remains obscure. However, based on their crystallographic structure and the fact that upon binding of the CKS proteins the CDK complex is stimulated to become phosphorylated by Wee1 and CDC25, it is suggested that they might act as a docking factor for positive and negative regulators impinging on the CDK complex (Pines, 1996; Patra *et al.,* 1999). In this respect, there are studies that indicate the role of CKS in the phosphorylation of the APC complex, responsible for the ultimate degradation of the CDK/cyclinB complex at anaphase (see further in the text; Sudakin *et al*., 1997). Such studies additionally indicated that CKS proteins and cyclins interact simultaneously and independently with CDKs (Pines, 1996). By using a 2-hybrid approach with CDKA;1 as a bait, an *Arabidopsis Suc1/Cks1* structural and functional homologue was identified (*CKS1At*; De Veylder *et al.*, 1997). Its functionality was demonstrated by the fact that the expression of *CKS1At* is able to rescue the temperature sensitive *cdc2*-mutant of fission yeast and that the introduction of this gene complements a *Suc1*-deleted strain. Moreover, CKS1At binds the CDK proteins CDKA;1 and CDKB1;1 under *in vitro* and *in vivo* circumstances (De Veylder *et al*., 1997). A second CKS homologue was identified in *Arabidopsis* by a sequence homology search (Stals *et al.*, 2000). Overexpression of *CKS1At* in *Arabidopsis* has been shown to inhibit the progression of the cell cycle, hereby equally extending both gap phases, which is specifically due to the continuous binding of the CKS protein to the CDK subunit (De Veylder *et al*., 2001a). As a result, the plant growth rate is inhibited by a strong reduction of the meristem size of the transgenic plants.

1.2.1.2.3. The CDK/cyclin complex becomes inhibited upon association with a Cyclin Kinase Inhibitor

The first CDK inhibitors, named CKI for short, were identified in yeasts, where they function to mediate cell cycle arrest in response to antimitogenic factors and ensure that particular cell cycle events do not initiate before others are

completed in G1 phase (Nigg, 1995). In *S. cerevisiae*, these functions are performed by FAR1 and SIC1 respectively: FAR1 is known to cause arrest in G1 phase via inhibition of the CDC28-CLN1 and –CLN2 complexes (Peter and Herkowitz, 1994), whereas SIC1 inhibits CDC28-CLB5 and -CLB6 resulting in the prevention of DNA synthesis until their degradation at G1/S transition (Schwob *et al*., 1994). In *S. pombe*, the *rum1* gene product is responsible for the correct ordering of events in the cell cycle by acting as an inhibitor of both the G1 and M phase forms of CDC2 (Moreno and Nurse, 1994). At present, 7 CKIs have been identified in mammals, divided into 2 families based on their structural and functional similarities: the Cip/Kip and INK4 families (Nakayama and Nakayama, 1999). They are structurally unrelated to the CKIs found in yeast. The Cip/Kip family consists of 3 proteins, named $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ and the Ink4 family represents the proteins $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. The members of the Cip/Kip family of CKIs have a broad specificity for CDK/cyclin complexes that they are known to inhibit, such as all known G1/S and G1-specific CDKs. The CKIs of the INK4 family only exert inhibitory effects on CDK4 and CDK6. The first plant CDK inhibitor genes were cloned from *Arabidopsis* (ICK 1 and-2; Wang *et al*., 1997 and 1998) and are known to inhibit CDK activity in *in vitro* assays. The C-terminal domain of the CKI1 protein shares an important consensus sequence with the mammalian CKI $p27^{Kip1}$ but the rest of the deduced sequence shows little similarity with any mammalian CKI. Together with the fact that this plant CKI is not able to inhibit the mammalian CDK1/cyclinB complex *in vitro,* one can conclude that plant CKI protein have diverged from their mammalian counterparts (Wang *et al*., 1997). Overexpression of the *ick1* gene resulted in reduced CDK activity and reduced the number of cells in these transgenic, dwarfed plants (Wang *et al.*, 2000). Evidence has been provided that this plant CKI is able to interact with CDC2aAt and cyclinD3 upon induction by the phytohormone abscissic acid (Wang *et al*., 1998). By performing a 2-hybrid screen with the *Arabidopsis* CDKA;1, 1 new putative CKIs was isolated (De Veylder *et al*., 2001b). In parallel, 4 related genes were identified by screening of the *Arabidopsis* genomic database and the corresponding cDNAs were isolated by a RT-PCR strategy (De Veylder *et al*., 2001b). All proteins showed the same identity to the mammalian $p27^{Kip1}$ in the C-terminal domain (as mentioned before for ICK1 and –2) and are therefore referred to as Kip-related proteins (KRPs, numbered 1 to 7). The KRPs have no significant homology to any other protein present in the databases and display only a low homology among each other. With the exception of KRP5, all KRPs exhibit the same CDK-binding specificity toward CDKA;1 in yeast (De Veylder *et al*., 2001b). When KRP2 is overexpressed, it dramatically inhibited cell cycle progression in the cells of leaf primordia without affecting the temporal pattern of cell division and differentiation. As a result, mature leaves of these transgenic plants are serrated and consist of larger cells (De Veylder *et al*., 2001b). Surprisingly, transgenic leaves were serrated, illustrating a role for cell division in leaf morphology.

1.2.2. Fine-tuning of the basic cell cycle control machinery

The fact that the activity of the CDK/cyclin complexes becomes posttranslationally regulated provides the cell cycle machinery with a multitude of possibilities to orchestrate the correct follow-up of the different phases and their associated processes during eukaryotic cell division. This is compulsory, since in living beings biochemical reactions (e.g. DNA replication) do not always proceed to completion and the integrity of complex structures (e.g. mitotic spindle, adhesion of sister chromatids) needs to be checked to ensure their correct functioning. Furthermore, a cell can experience adverse conditions and hereby damage its DNA. Finally, cell growth needs to be regulated with the intention of producing daughter cells of a constant size. When encountering these kinds of conditions, the cell must be able to stop the division process at certain dedicated checkpoints in order to finish reactions or to repair. In the following sections, the two most important checkpoints (G1/S and G2/M) will be highlighted by discussing the different control mechanisms impinging on the CDK/cyclin complexes and hereby arresting cell cycle progression when necessary.

At some critical moments, specific mechanisms in the cell will induce an overall destruction of the available cyclin levels at that given moment. By this process, a third level of CDK regulation is achieved by the cell cycle control mechanism, since the destruction of the cyclin partner of certain CDK/Cyclin complexes will contribute to the passage of cells through checkpoints and phase boundaries.

1.2.2.1. G1/S checkpoint

Cell proliferation is primarily regulated during the G1 phase of the cell cycle (Norbury and Nurse, 1992). At this moment, the cells monitor their environment and judge whether the conditions are favorable to replicate their DNA in a next round of cell division. Alternatively, the cell can decide to leave the cell cycle and undergo a differentiation process to a specific function. This decisive moment during G1 phase is called START in lower eukaryotes, such as yeast, or the restriction point in animal cells. For the majority of the eukaryotic organisms, the duration of this gap phase can be adjusted in order to gain more cell mass when a critical cell size has not been reached. Additionally, the G1/S transition provides the cell with the chance to check for DNA damage and counter with appropriate repair mechanisms.

1.2.2.1.1. Progress through G1/S: The decision to divide once again

Eukaryotic cells have evolved complex regulatory mechanisms in order to obtain an adequate interpretation of the cues governing their decision to divide or to differentiate. Ultimately, the gathered information will become translated to the basic cell cycle machinery, which is reviewed in this section. In unicellular eukaryotes, the molecules involved are different from those in multicellular organisms. Therefore, the discussion will be restricted to animal and plant cells that share a conserved mechanism for G1 progression and G1/S transition (Meijer and Murray, 2000; Durfee *et al.,* 2000).

CDK/cyclin complexes of importance for G1 progression and G1/S transition

Progression through the restriction point is mediated by the action of the cyclinD proteins in association with CDK4 and –6 in animals or CDKA-type proteins in plant cells (Meijer and Murray, 2000; Stals *et al*., 2000; Stals and Inzé, 2001). In mammalians, quiescent cells will enter the cell cycle at this moment upon addition of serum type growth factors (Matsushime *et al*., 1991; Sherr, 1994; Jones and Kazlauskas, 2001). The transcription CyclinD1 is absolutely dependent of the addition of these molecules. Upon removal of these growth factors, the transcript levels will decline. As outlined earlier in the text, the abundance of CyclinD1 transcripts won't change significantly in an already cycling cell (Matsushime *et al*., 1991). This suggests that their activity is post-transcriptionally regulated. In the current working models, mammalian D-type cyclins are believed to act as sensors with an expression profile that depends more on the extra cellular, impinging stimuli than on the position of the cell cycle (Sherr, 1994). This hypothesis is supported by the observation of cells where D-type cyclins are overexpressed: The length of the G1 phase is strongly reduced, whereas the need of dividing cells for mitogens is partially lost (Kato and Sherr, 1993). For plant cells, the situation is fairly similar (Meijer and Murray, 2000). Plant CycD-type cyclins are induced in response to exogenous signals which are known to affect plant cell growth. Among these products are the plant hormones auxin and cytokinin (Meijer and Murray, 2000; Stals and Inzé, 2001). Addition of cytokinins to plant cell cultures regulates the G1/S progression by induction of *CycD3;1* transcription and the continuous expression of this gene enables *Arabidopsis* transgenics to bypass the need for this hormone in tissue culture (Riou-Khamlichi *et al*., 1999). As at G2/M transition, the influence of auxins is situated at the level of CDKA;1 transcription and activation (Zhang *et al*., 1996; Stals and Inzé, 2001). However, these activating capacities of auxin occur only in concert with satisfactory levels of cytokinins. The application of brassinosteroid-type plant hormones is also sufficient to induce *CycD3;1* transcription (Hu *et al*., 2000). Nonetheless, this promotive effect would involve a distinct induction pathway dependent on protein synthesis instead of protein phosphorylation (den Boer and Murray, 2000; Hu *et al*., 2000). In addition of plant hormones, several reports indicate the importance of sucrose for *CycD* expression. Different studies demonstrate the induction of *CycD2, CycD3,* and *CycD4* genes upon addition of sucrose (Soni *et al*., 1995; De Veylder *et al*., 1999; Meijer and Murray, 2000). The induction of *CycD3* and *–D4* by cytokinins and/or sucrose is independent of *de novo* protein synthesis, which indicates a sensing role of these cyclins for the nutritional status of the cell as demonstrated in animals (Meijer and Murray, 2000). However, the signal transduction pathways leading to their response are suggested to be separated. Finally, gibberellins and abscissic acid have been reported to affect G1/S progression by proteins that affect the activity of the assembled CDKA/CycD complexes at this moment. In water submerged rice plants, gibberellins have been shown to induce *CDKA*-type genes and R2-CAK related mRNAs (Lorbiecke and Sauter, 1999). Abscissic acid inhibits cell division in *A. thaliana* by decreasing the amounts of *CDKA;1* mRNAs and the induction of expression its specific inhibitor gene *ICK1* (Hemerly *et al*., 1993; Wang *et al*., 1998; Stals and Inzé, 2001).

In animal cell systems, a transient wave of E-type cyclins will occur in late G1-phase, forming kinase complexes with CDK2. Their action will drive the cells irreversibly across the G1/S boundary (Sherr, 1996). To date, no plant homolog has been reported. A possible explanation could be the fact that in plants only one type of CDK has been reported to be involved at G1-transition, compared to three different animal CDKs. Moreover, the presence of a more diversified group of D-type cyclins in plant species suggests the possible occurrence of sequential waves of plant CycD proteins which can be responsible for the function of the CycE proteins in animal cells.

The pRb/E2F pathway is essential to initiate a new round of cell proliferation

The relative importance of the retinoblastoma tumor suppressor protein (pRb) for cell cycle control was first demonstrated by the identification of homozygous loss-of-function mutations at the *RB* locus in mammalians. This type of genetic defect is the rate-limiting step for development of an intraocular tumor affecting young children (Weinberg, 1995; Harbour and Dean, 2000). It was shown that pRb normally functions as an antagonist of cell proliferation near the restriction point (Goodrich *et al*., 1991). For that reason, this protein can be considered as a gatekeeper that will block G1/S progression until the appropriate signals for propagation are received by the basic cell cycle machinery. In mammalians, the family of Rb proteins has two other members, named p107 and p130. These proteins appear to have some overlapping functions with pRb (Harbour and Dean, 2000). pRb homologues have also been found in other multicellular organisms, including plants (Huntley *et al*., 1998; Gutierrez, 1998; Durfee *et al*., 2000; Bartek and Lucas, 2001). The absence of yeast members suggests that this type of proteins is not involved in G1/S transition in yeast. The central region of pRb contains two large conserved domains, which are crucial for interaction with most of the associated protein thus far. Consequently, they are thought to be providing the link between these interactions and pRb function (Harbour and Dean, 2000). It has been experimentally demonstrated that peptides with the motif LxCxE are competent for interaction with one of these conserved domains of the pRb (Lee *et al*., 1998; Bartek and Lucas, 2001). Analysis of the structure of all D- and E-type cyclins identified to date revealed this motif and makes them potential interaction partners. Their interaction has already been demonstrated *in vitro* both in animals and plants (Weinberg, 1995; Nakagami *et al*., 1999). The Rb protein can be phosphorylated on multiple sites in the region flanking the two conserved interaction domains. The majority of these phosphorylations can be attributed to the activity of CDKs. Such modifications have been shown to negatively regulate its growth-suppressing activity (reviewed by Weinberg, 1995; Bartek and Lucas, 2001). The phosphorylation degree of pRb is cell cycle-dependent: During most of G1 phase, the pRb is present in a hypophosphorylated form in animal cells. Near the restriction point, it will become phosphorylated by the action of the first CDK4/CycD complex and subsequently this phosphorylation becomes accelerated by the action of the CDK2/CycE complexes, driving the cells into a new round of cell division. In reality, the achievements of the CDK4(6)/CycD complex will be responsible for the subsequent activation of CDK2/CycE since the *cycE* gene becomes expressed upon the initial inactivation of pRb.

Additionally, since CyclinE is sequestered by pRb in its hypophosphorylated form, the action of the CDK4/CycD1 complex will indirectly lead to functional CyclinE levels. The CDK2/CycA complexes, appearing slightly later, have also been reported to contribute into the kination of pRb. pRb continues to exist in its hyperphosphorylated form until M-phase. At this moment, it will become dephophorylated by the action of a type 1 phosphatase. Based on the fact that in plants there is also evidence for the interaction of CycD with pRb, the same mode of action is suggested to be involved in the G1/S transition of plant cells (Meijer and Murray, 2000; Durfee *et al*., 2000).

The mechanism of how pRb executes the blocking of G1/S transition until the appropriate moment approaches (figure 9) was discovered by the fact that it physically interacts with members of the E2F family of transcription factors (E2F1-5; reviewed by Black and Azizka-Clifford, 1999; Harbour and Dean, 2000; Bartek and Lucas, 2001). E2F binding sites are found in a number of promoters of genes that are known to control DNA synthesis and cell cycle regulation genes such as *cycE* and *cdc25A*. E2F mediates the transcription of these genes, acting as a heterodimer with DP-type transcription factors (La Thangue, 1994). The vision that E2F indeed is involved in G1/S progression was enforced by the fact that ectopic expression of the E2F-1 protein is responsible for a premature entry into S-phase in cultured cells and under *in vivo* circumstances (Johnson *et al*., 1993; Shan *et al*., 1996). Interaction of the transactivation domain of the E2F protein with pRb inhibits its activity (Shan *et al*., 1996). The sequestration of the transcription factor alone is not the only way in which pRb manages to delay G1/S progression: It has been shown that E2F directs pRb to promoters that must be kept transcriptionally inactive until the proper stimulus is received. This happens by both masking of the E2F transactivation domain and the modification of the local chromatin structure by the activity of a histone acetylase (HDAC1) that also interacts with the Rb protein (Harbour and Dean, 2000). E2F homologues have been isolated from plant tissues (Durfee *et al*., 2000) and more recently, the first plant members of the DP family of transcription factors have been isolated in *Arabidopsis* (Magyar *et al*., 2001). Furthermore, the interaction between the plant homologues of E2F and DP has been confirmed in *Arabidopsis* plants (De Veylder, submitted). This further enforces the vision that the plant strategy to achieve a G1/S transition is similar to this occurring in animal systems.

The mammalian Myc protein is involved in a process working parallel of Rb and E2F

Recently, it became clear that the Rb pathway on its own couldn't account for the execution of G1/S control in mammalian systems. Evidence is accumulating that the Myc proto-oncogene should be implicated as a central element of a parallel pathway that cooperates with the pRb-E2F axis (Bartek and Lucas, 2001). The *c-myc* gene encodes a transcription factor and its expression is induced by mitogens (Nasi *et al*., 2001). Upon expression of this gene, quiescent cells are enabled to enter the cell cycle again (Bouchard *et al*., 1998). The role of Myc in proliferation promotion has been generally accepted. However, the identity of the target genes that are responsible for the mediation of these mitogenic effects and the precise mechanistic links with the basic cell cycle machinery are only lately coming to light. Recent

reports show that among the downstream target genes of Myc that are relevant for G1/S control, one can find the those which encode D- and E-type cyclins and the dual-specific Cdc25A phosphatase (Santoni-Rugiu *et al*., 2000; Bartek and Lucas, 2001; Nasi *et al*., 2001). It has been demonstrated that Myc is involved in the regulation of the CDK2/CycE complex and E2F-dependent transcription. Myc is suggested to be responsible for the direct transcription of the *cyclinE* and *cdc25A* genes. Indirectly, it acts through the sequestration of the CDK inhibitor $p27^{kip1}$, which blocks CDK4(6)/CycD complexes, and by the phosphorylation and subsequent degradation of this CKI at G1/S transition (Bartek and Lucas, 2001 and references herein). To summarize, these new discoveries on the pRb/E2F and Myc cascades indicate that the

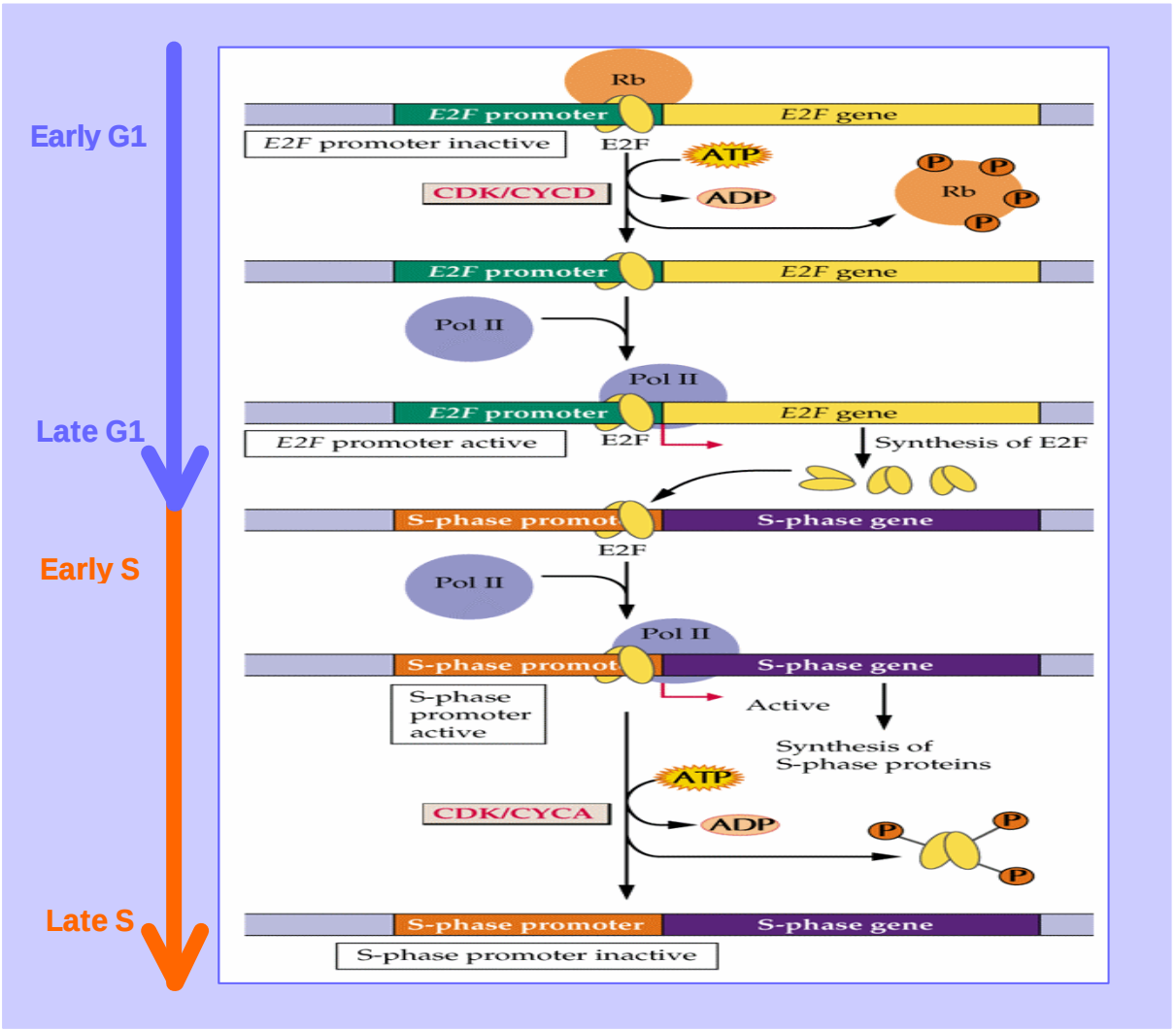


Figure 9: E2F transcription factor activation at G1/S enforces the commitment to S-phase (animal cells). In G1, E2F activity is suppressed by binding of the retinoblastoma protein (Rb). As a consequence of the passage through the restriction point, the CDK4/CycD complex phosphorylates Rb, resulting in dissociation of E2F, hereby relieving the inhibition of this transcription factor. By a positive feedback loop, large amounts of E2F are formed and E2F also activates the transcription of many S-phase-specific proteins. Ultimately, E2F activity will be inhibited by phosphorylation of the CDK2-cyclinA complex. PolII stands for RNA

CDK2/CycE complex can be considered as a convergence point of G1/S control. The activity of this key G1/S-promoting enzyme is both rate limiting and essential for entry into S-phase.

1.2.2.1.2. Pathways that check for DNA damage and the possible link with apoptosis

In response to DNA damage, cell cycle checkpoints integrate cell cycle control with DNA repair. In multicellular organisms, DNA damage has a variety of effects on cell division control. Next to the transient arrest before mitosis (as described in section 1.2.2.2.1.), permanent arrest in the G1 phase of the cell cycle occurs as well as apoptosis, a cell death program that is unique to metazoans. Although there is evidence that DNA repair mechanisms similar to animal species are present in plants (Britt, 1999), it is not known whether such a G1 checkpoint control point exists in the plant kingdom.

Activation of the mammalian transcription factor p53 is sufficient to induce cell cycle arrest and apoptotic cell death (Walworth, 2000; Ryan *et al*., 2001). The CKI $p21^{Cip1}$ is a direct p53 target and deletion of this gene significantly reduces the cell cycle arrest response to p53 (El-Deiry, 1998). Moreover, p53 has been demonstrated to participate directly in DNA repair by regulating the expression of a ribonucleotide reductase gene, named *p53R2* (Nakano *et al.*, 2000; Tanaka *et al*., 2000). Although p53 has been shown to activate the expression of many genes that could contribute to the apoptotic response, no single predominant effector has been identified. It has been shown that p53 induces the expression of proteins that target both the mitochondrial and the death-receptor-induced apoptotic pathways (Ryan *et al*., 2001). Very recently, evidence has been reported that p53 also is involved in the checkpoints that control mitotic entry (Taylor and Stark, 2001). The mechanism by which p53 blocks cells with damaged or incompletely replicated DNA involves the inhibition of Cdc2 through the action of 3 of its transcriptional targets, $p21^{cip1}$, Gadd45 and 14-3-3. Additionally, p53 represses the transcription of the *cyclinB1* and *cdc2* genes. It is believed that the pathway induced by p53 works independently of other pathways involved in G2/M checkpoint control as discussed in 1.2.2.2.1..

The analysis of the *Arabidopsis* genomic sequence did not reveal any ORF with homology to p53. Its potential absence in plants suggests the possibility that here different links between the cell cycle, DNA damage and programmed cell death exist.

In mammalians, not all aspects of the G1 checkpoint responses to genotoxic stress can be attributed to the p53 pathway since at least a transient inhibition of Cdk2 takes place in cells that lack p53 or p21 (Rotmann and Shiloh, 1999; Carr, 2000). Moreover, when exposed to DNA damage, cells are able to reduce the rate of ongoing DNA synthesis by the so-called intra-S-phase checkpoint. This pathway, regardless of p53 action, results in a persistent inhibitory phosphorylation of Cdk2 on Thr14/Tyr15. This leads to the inhibition of the CDK2/CycE and CDK2/CycA complexes and G1/S arrest. This becomes possible through the rapid decrease of Cdc25A activity (Molinari *et al*., 2000; Bartek and Lucas, 2001).

1.2.2.2. The G2/M checkpoint

For all eukaryotic dividing cells, entry into mitosis is tightly controlled by the

p34^{cdc2}/cyclinB complex (or MPF). Initially, the assembled complex stays inactive by inhibitory phosphorylations, but ultimately it becomes activated through the dephosphorylating action of a cdc25 homologue. This will only occur when several prerequisites are fulfilled by the cell: In order to maintain the integrity of the genome, the duplication of the DNA-content of the mother cell has to be finished and were damage has occurred, it needs to be repaired. Additionally, mitotic control has clearly been shown to link cell growth to the cell cycle in *S. pombe*, allowing progression to M-phase only when the cells have achieved a certain threshold size. The major focus of the discussion about G2/M transition will be on fission yeast research where most work has been done, but comparisons will be made with work in higher eukaryotes such as *Drosophila*, *Xenopus* or mammalian cells. For plants, this aspect of the cell cycle control machinery remains mainly unexplored. Nevertheless, the results of some studies in plants do point toward the influence of certain growth hormones on the activation of the MPF.

1.2.2.2.1. The replication checkpoint and strategies to avoid definitive DNA damage

Chk1 and Cdc25 are the principal players in DNA damage control at G2/M in eukaryotes

The identification and subsequent characterization of loss-of-function mutants that fail to delay mitotic entry following induced DNA damage lead to the isolation of the components responsible for the perception and transduction of the appropriate checkpoint signal for DNA damage (reviewed by Humphrey, 2000; Murakami and Nurse, 2000; Walworth, 2001; figure 10). They are commonly termed the *rad* checkpoint genes (corresponding to Rad1, Rad3, Rad9, Rad17 and Hus1 protein) and have been cloned in both fission yeast and higher eukaryotes such as humans. The Rad26 protein is considered as an additional member of this family, but a human counterpart has not yet been identified. Roles for these proteins have been postulated, based on their similarity to proteins with defined biochemical properties. It has been suggested that Rad1, Rad9 and Hus1 might act together as a PCNA-like structure (Proliferating-Cell Nuclear Antigen, a component of the DNA replication machinery; Caspari *et al*., 2000). This suggests that it could provide processivity for the DNA-repair and replication enzymes. Rad17 resembles a subunit of replication factor C (Green *et al*., 1999). The fact that some of the Rad proteins are related to proteins of the replication machinery could be an indication that they are components of complexes interacting with the DNA and hereby act as sensors for the DNA status. Rad3 belongs to the ATM family of proteins, which have been demonstrated to show protein kinase activity. Rad26 is known to form a complex with these proteins and therefore it has been thought to act as a substrate for it (Edwards *et al*., 1999).

The ultimate target of checkpoint signaling pathways should be the Cdc2 component of the MPF. Although it has been suspected for a long time, it was only proven recently that the inhibitory phosphorylation of Tyr15 is required in order to allow repair of damaged DNA at G2/M phase transition (Russell, 1998). Simultaneous inactivation of Wee1 and Mik1, overproduction of cdc25 or the mutation of Tyr15 to a Phe is sufficient to abrogate the checkpoint (Rhind *et al*., 1997). However, this finding does not prove the direct regulation of Wee1, Mik1 or Cdc25 by the DNA damage

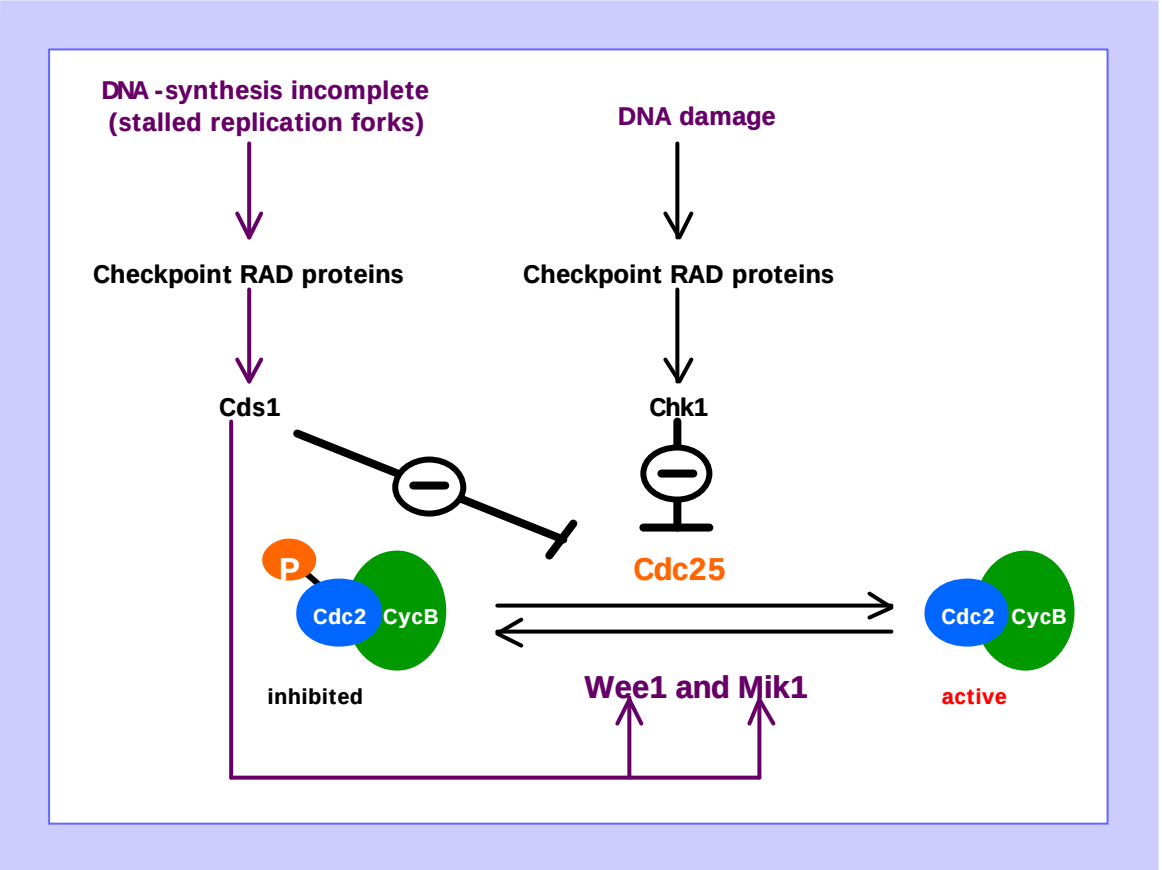


Figure 10: The replication and DNA damage checkpoints are closely related

checkpoint. A protein termed Chk1 was at first isolated as a high copy suppressor of a cold-sensitive allele of *cdc2* (Walworth *et al.*, 1993). It encodes a serine/threonine kinase and its loss of function results in moderate sensitivity to DNA damaging agents correlating with a loss of G2-arrest. As a result of the activity of the some Rad proteins, it becomes phosphorylated and will physically interact with other Rad proteins, Rad24 and Rad25, which are 14-3-3 proteins that were also identified to be implied in checkpoint control (Ford *et al.*, 1994; Chen *et al.*, 1999). A possible target of the *S. pombe* or human Chk1 was demonstrated to be Cdc25, since *in vitro* studies successfully indicated the phosphorylation of human Cdc25C by the Chk1 protein kinase of both eukaryotes (Sanchez *et al.*, 1997). Interestingly, the site of phosphorylation on the Cdc25C protein (Ser216) is the same site that mediates the interaction of these proteins with 14-3-3 proteins (Peng *et al.*, 1997). Moreover, the dephosphorylation of this site is coincidental with mitotic entry observed in mammalian cells, leading to the suggestion that this will accompany the activation of the Cdc25 protein and consequently Cdc2. In response to DNA damage, Cdc25 is phosphorylated by Chk1 and promotes the interaction with 14-3-3 proteins. Similar conclusions could be drawn from experiments in *Xenopus* (Walworth, 2001). However, the model didn't seem to fit under all circumstances because not all experimental results are consistent. When *Xenopus* extracts were immunodepleted of Chk1, the phosphorylation on the Ser216 residue became only slightly reduced (Kumagai *et al.*, 1998a). In

S. pombe, the phosphorylation pattern at this site was unaffected by checkpoint activation which suggests that 14-3-3 binding site phosphorylation is not an inducible event (Zeng *et al.*, 1998). Moreover, the amount of Cdc25 that is bound with 14-3-3 proteins upon DNA damage is not different from that under normal circumstances, a fact that was also demonstrated in fission yeast (Kumagai *et al.*, 1998b; Chen *et al.*, 1999). Nonetheless, although the association of the 14-3-3 binding site is not a checkpoint-regulated event, it appears to be indispensable to prevent Cdc25 from overriding the function of the checkpoint. This was demonstrated by the fact that overexpression of *cdc25* alleles where Ser215 has been mutated is able to compromise the checkpoint more severely than occurs by the overexpression of wild-type alleles (Walworth , 2001).

What might than be the specific role of the 14-3-3 protein? The observation that Cdc25 proteins are localized in the cytoplasm during interphase and become relocalized in the nucleus shortly before mitosis offered some novel insights (Lopez-Girona, 1999). Since both the 14-3-3 and Cdc25 proteins appear to occlude a nuclear localization signal (NLS), it was speculated that their interaction might inhibit the nuclear import of Cdc25 and hereby will block its nuclear binding with the $p34^{cdc2}$/CyclinB complex. Furthermore, exposure to DNA-damaging agents leads to the prevention of the targeting of Cdc25 to the nucleus. *Xenopus* studies suggest that the role of 14-3-3 proteins might be the masking of the Cdc25 NLS upon sequestering of this protein in the cytoplasm (Kumagai and Dunphy, 1999). When tested in fission yeast, this hypothesis initially was supported, since the elimination of the NLS in Cdc25 leads to the exclusion of this protein and subsequent mitotic delay. However, the forced nuclear inclusion achieved by attachment of an exogenous NLS failed to override the damage checkpoint, proving that this phenomenon is independent of the DNA damage checkpoint (Lopez-Girona *et al.*, 2001). Therefore, the authors of this report propose that direct inhibition of Cdc25 phosphatase activity by the Chk1 protein is sufficient to achieve a G2 arrest mediated by Cdc25 upon DNA damage. Direct phosphorylation of Cdc25 by the Chk1 kinase has already been demonstrated *in vitro* (Blasina *et al.*, 1999; Furnari *et al.*, 1999). Moreover, both reports suggest the presence of another protein kinase, Cds1, that is able to regulate the activity of Cdc25 by phosphorylation (as discussed further in the text).

To conclude, it has to be pointed out that Chk1 presumably has supplementary targets different from Cdc25. It has been shown already quite some time ago that fission yeast cells, which lack Cdc25 proteins, are checkpoint competent (al-Khodairy and Carr, 1992). The most obvious candidates are the Wee1 and Mik1 proteins by means of their inhibitory phosphorylating activity on the Tyr15 residue of Cdc2. Reports have been published, where a role has been indicated for one or the other (Baber-Furnari *et al.*, 2000; Rhaleigh and O'Connell, 2000; Rhind and Russell, 2001; Lee *et al.*, 2001). Lee and coworkers (2001) were furthermore able to demonstrate the activation of Wee1 by the positive influence of the 14-3-3 proteins. Studies in mammals point out that at least one other target exists: The Polo1 kinase becomes inactivated in response to DNA damage and is thought to regulate Cdc25 activity (Smits *et al.*, 2000).

The DNA replication checkpoint partially overlaps with the one that controls DNA damage

Although one of the most important goals of cell division is to distribute complete replicas of the genome to daughter cells, there is no physical requirement of these chromosomes to be replicated prior to nuclear division. For this reason, a checkpoint controlling the replication status of the DNA is obligatory before onset of mitosis.

Studies in *S. pombe* clearly demonstrated that the replication checkpoint shares many features with the DNA damage control point (Russell, 1998; Walworth, 2001; Murakami and Nurse, 2000, see figure 10): The same Rad proteins seem to be required to detect and transduce the signal for incomplete DNA replication and the checkpoint also requires the phosphorylation of the CDK-subunit of the MPF on Tyr15. Yet, the Chk1 protein on its self seems not to be essential for the replication pathway (Walworth *et al.*, 1993). A protein kinase, named Cds1, is responsible for the establishment of the replication checkpoint in a Chk1 negative yeast strain and has been shown to become highly activated by a mechanism that requires Rad proteins. The replication checkpoint becomes only overruled in a *chk1 cds1* double mutant (Boddy *et al.*, 1998). Consequently, it can be concluded from these experiments that Cds1 and Chk1 jointly enforce the replication checkpoint. However, reports indicate that the Chk1 protein only becomes involved in situations where the replication checkpoint is threatened, such as in a *cds1* mutant (Lindsay *et al.*, 1998). As outlined earlier in the text, Cds1 has been suggested to perform inhibitory phosphorylations on a human Cdc25 homologue (Blasina *et al.*, 1999), hereby demonstrating further the resemblance of the DNA damage and replication checkpoints at the G2/M transition. In fission yeast, the Cds1 kinase has additionally been demonstrated to bind and phosphorylate Wee1 in cell lysates (Boddy *et al.*, 1998). Moreover, the accumulation of Mik1 protein levels requires the action of both Rad3 and Cds1 proteins. The multiple targets of the Cds1 protein are an elegant explanation for the earlier observation in *wee1* and *cdc25* double mutants where the replication checkpoint remains intact (Enoch *et al.*, 1993). As a summary, the replication checkpoint can be presented as a pathway with similar components as the DNA damage checkpoint with the Cds1 protein as the phosphorylating factor of the regulating agents of the $p34^{cdc2}$/CyclinB complex, whereas the DNA damage checkpoint seems to make use of the Chk1 protein to fulfill this function. Under circumstances where one of these kinases fails to function, the other can function as a backup to guarantee the integrity and completion of the duplicated genome.

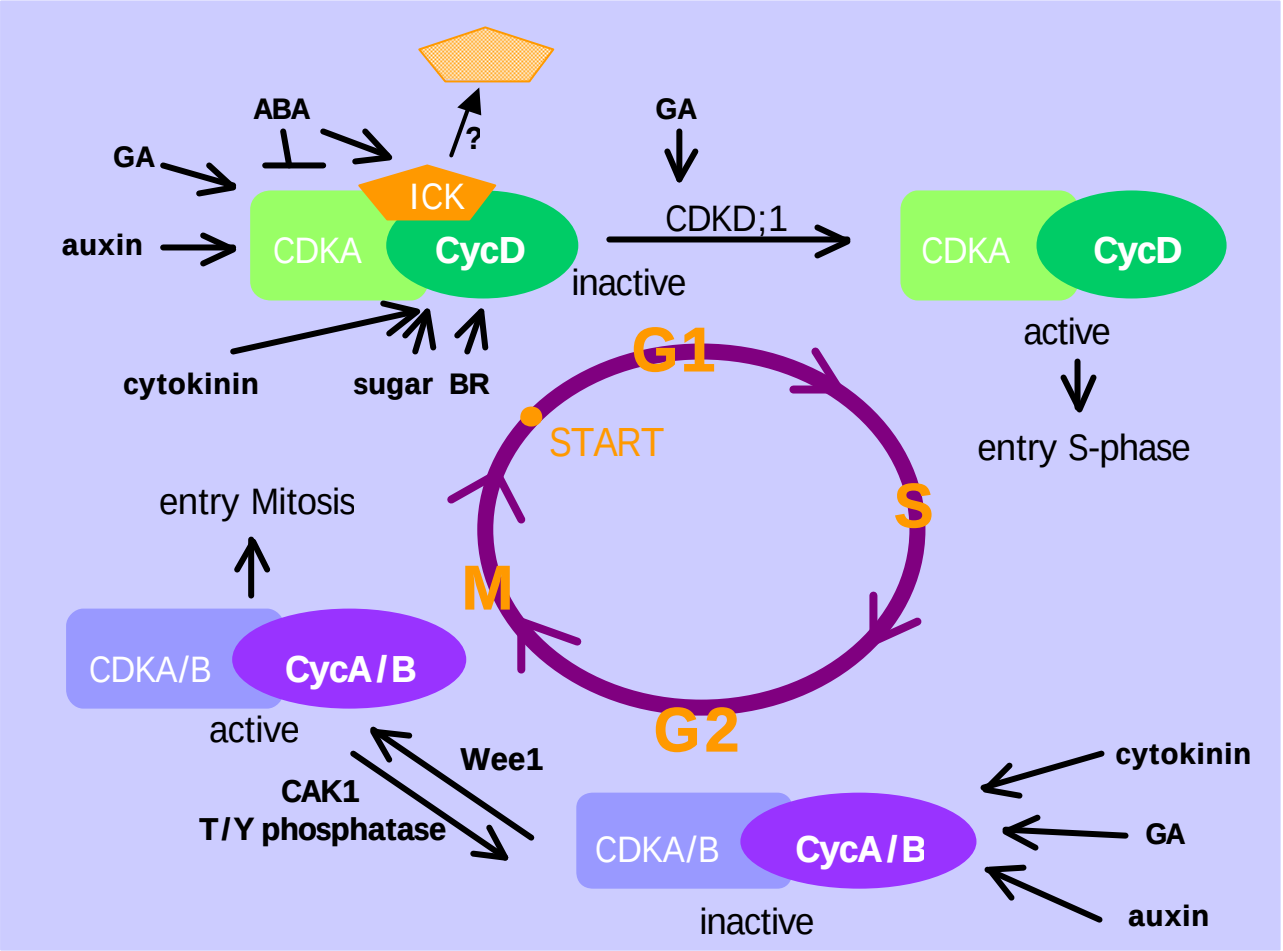


Figure 11: Influence of hormones on plant cell cycle progress. GA: gibberellic acid, BR: brassinosteroids, ABA: abscissic acid

1.2.2.2.2. In plant cells, mitotic entry is under hormonal control

As outlined before, the connection between the plant hormones auxin and cytokinin and cell division has been made already some decades ago by the application of *in vitro* culture protocols of tissue explants (Skoog *et al*., 1957). Classical studies on cell suspension cultures show that plant cells, in contrast to animals, not only are able to arrest in G1 phase, but also in G2 when they are depleted of these hormones (see also figure 11). Nevertheless, the G1 arrest proves to be the most stringent one as reported for animal cells. It has been shown that auxin affects cell division at the cellular level, but it needs to act in concert with cytokinins to stimulate cell division in most cultured cells and *in planta*, where their action is needed at both G2/M and G1/S transitions (Zhang *et al*., 1996; Stals and Inzé, 2001). At the G2/M transition, addition of these hormones leads to an increase of *CDKA;1* expression and activity. Addition of auxin alone is sufficient for the induction of CDK expression, but for the activation of the CDK protein the presence of satisfactory cytokinin levels is compulsory. This activation is achieved through the dephosphorylation of the inhibitory phosphate residue on the Tyr15 site of *CDKA;1*.

In rice, a monocotyledonous plant which is cultivated in submerging conditions, it has been proven that cell division and subsequent elongation is accelerated by the action of gibberellins in both gap phases (Lorbiecke and Sauter, 1999; Fabian *et al*., 2000). Prior to the increased availability of B-type cyclins at late G2, gibberellin addition leads to the induction of the *CycA1;1* and *CDKB1;1* genes. This suggests a specific role of this hormone in the G2/M transition (Fabian *et al*., 2000; Stals and Inzé. 2001).

1.2.2.3. Mitotic checkpoints

When cells undergo mitosis, their replicated genetic material becomes evenly distributed between the two daughter cells. This is achieved through the features of the mitotic apparatus, consisting of a bipolar spindle where the condensed sister chromatids are attached by their kinetochores and will be pulled to the opposite poles as they undergo anaphase. This process needs to be tightly controlled, since the missegregation of sister chromatids will lead to aneuploidy. Therefore, checkpoints have been established to monitor the completion of certain steps in mitotic progress. The so-called spindle checkpoint is responsible for the inhibition of sister chromatid separation until all of the kinetochores are attached to the microtubules that make up the mitotic spindle. This occurs at metaphase-to-anaphase (figure 12). Subsequent exit form mitosis is controlled by a pathway that inhibits the action of the mitotic exit network until the completion of chromosome separation. The pathways and proteins that are involved in these checkpoints have been most extensively studied in budding yeast. Recently, experiments in higher eukaryotes have provided additional important insights. In plant cells the organization of mitotic checkpoints remains a black box.

1.2.2.3.1. The spindle checkpoint

A spindle checkpoint exists to ensure that chromosome assembly, orientation and segregation occurs with optimal fidelity. When cells are treated with a microtubule-depolymerizing drug such as nocodazole, they become arrested prior to anaphase. By a screening in *S. cerevisiae* for mutants where this checkpoint becomes overruled under such conditions, 7 genes were identified, named *BUB1, BUB2, BUB3* and *MAD1, MAD2, MAD3 and MPS1* (Burke, 2000; Amon, 1999). The *BUB* and *MAD* genes are non-essential, but *MPS1* proves to be essential and has a function in both spindle pole assembly and spindle checkpoint signaling (Weiss and Winey, 1996). Homologues of these genes were identified in several organisms, including mammalians (Amon, 1999). When analyzed by microscopical means, they are observed at the kinetochore during the first stages of mitosis. Once the anaphase has started, rapid degradation occurs (Burke, 2000). Kinetochores seem to be dynamically involved in the activity of the checkpoint, since the protein Ndc10 is an integral component of this protein complex. Mutants in which this protein is not present (*ndc10* mutants) are not blocked at the metaphase-to-anaphase transition upon addition of nocodazole. In the current working hypothesis of the spindle checkpoint, the presence of unattached chromosomes is responsible for the induction of cell cycle arrest. The kinetochore could be envisioned as the sensor recognizing this situation and as a consequence initiating a signal to inhibit onset of anaphase (Amon, 1999). This signal is mediated by a mechanism where

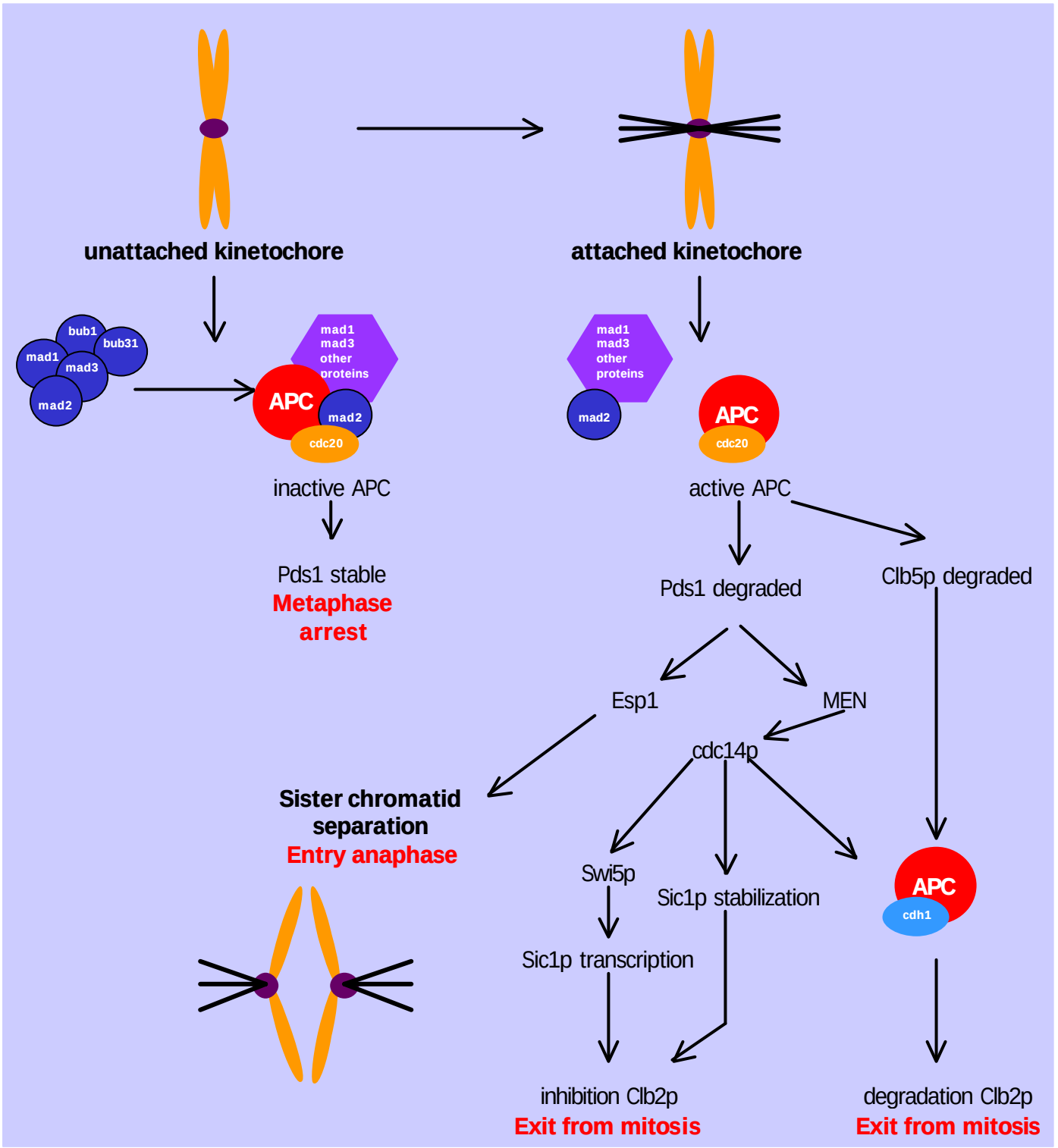


Figure 12: The spindle checkpoint and regulation of exit from mitosis in *S. cerevisiae*

the BUB and MAD proteins are involved. The MAD proteins will inhibit the activity of Cdc20. Cdc20 is an accessory protein of an ubiquitin-dependent protein degradation mechanism,

called the Anaphase Promoting Complex (APC for short). When the activity of the APC becomes inhibited, one of their substrates, the Pds1 protein, will not become degraded and therefore will stay able to sequester the anaphase initiator protein Esp1 in an inactive complex (Ciosk *et al.*, 1998). When all chromosomes are attached to the mitotic spindle, cell cycle progression becomes allowed by the checkpoint and the Pds will be destroyed after the association of the Cdc20 with the APC. Accordingly, the protein becomes activated and will be able to direct the proteolytic cleavage of the Scc1/Mcd1 complex, hereby causing their release from the chromosomes and permitting sister chromatid separation (Burke, 2000). The action of the APC interacting with cdc20 will also lead to the destruction of the Clb5 cyclin (Cerutti and Simanis, 2000; Shirayama *et al.,* 2000). As a result, a next associating partner of the APC will become activated which is involved in the exit from mitosis in late anaphase (as discussed in next section).

1.2.2.3.2. The guidance of daughter cells toward exit from mitosis

Mutants in the *S. cerevisiae* genes *Cdc5, Cdc14, Cdc15, Dbf2, Dbf20, Mob1, Lte1* and *Tem1* become arrested in late anaphase. Their cells all contain separated chromosomes and an elevated level of CDC29/CLB kinase, which is similar to conditions where non-degradable forms of mitotic cyclins are expressed (Cerutti and Simanis, 2000). Moreover, cytokinesis does not occur and the cells will never enter the next Sphase. These genes are collectively referred to as the Mitotic Exit Network (MEN) and are believed to co-operate to regulate the destruction of the mitotic cyclins, the exit from mitosis and cytokinesis (Jaspersen *et al.*, 1998). There are indications that the MEN genes are evolutionary conserved. In fission yeast, homologues of *Cdc15, Cdc5, Dbf2* and *Tem1* have already been identified (Cerutti and Simanis, 2000). The *Cdc5* gene product is a homologue of the *Drosophila* polo kinase. These proteins are present in all eukaryotes and seem to play multiple roles in both mitosis and cytokinesis (Glover *et al.*, 1999). Furthermore, proteins with structural homology to Cdc14 have been identified in higher eukaryotes, such as human cells. In some cases, they have been shown to functionally complement a budding yeast *cdc14* mutant (Li *et al.*, 1997).

When exit from mitosis is allowed, the Cdc14 protein, which is a phosphatase, becomes activated through the action of the other MEN proteins (Cerutti and Simanis, 2000). The process of exit from mitosis is tightly linked to the spindle checkpoint: As long as this checkpoint arrests the cell at metaphase-to-anaphase transition, Pds1 will inhibit the action of the MEN (Tinker-Kulberg and Morgan, 1999; Cohen-Fix and Koshland, 1999; figure 12). Upon entry into anaphase, the active form of the Esp1 will be responsible for the activation of the MEN next to its function in sister chromatid cleavage (Tinker-Kulberg and Morgan, 1999). The phosphatase activity of Cdc14 has three consequences (Cerutti and Simanis, 2000; Wang *et al.*, 2000). First, it will dephosphorylate the transcription factor Swi5 that will be allowed to the nucleus where one of its targets, the *Sic1* gene, becomes transcribed. The resulting protein is a CKI, which is responsible for the inactivation of the CDC28/CLB2 complex (Wang *et al.*, 2000). Second, the action of the Cdc14 protein antagonizes the phosphorylation of the CKI Sic1, hereby preventing its proteolytic destruction

leading to increased levels of the protein. Finally, the APC accessory factor Cdh1 becomes dephosphorylated, which will lead to the activation of the APC and subsequent degradation of Clb2. Together, these events will lead to the destruction of the CDC28/CLB2 complex and allow exit from mitosis and execution of cytokinesis.

1.2.2.4. Ubiquitin-dependent proteolysis is responsible for the ultimate breakdown of cyclin/CDK complexes

Crucial for cell cycle regulation at certain moments of the cell cycle is the timed destruction of some of its key proteins. The proteolytic machinery responsible for the determination and destruction of such proteins is conserved among all eukaryotes and is mediated by ubiquitination (reviewed by Peters, 1998; Zachariae and Nasmyth, 1999). Targets for proteolytic destruction include mitotic and G1 cyclins, CKIs, p53, proteins involved in sister chromatid separation and spindle components (Hersko and Chiechanover, 1998). Basically, two different ubiquitination complexes are active during cell cycle progress: the SKP1-cullin-F-box-protein complex (SCF for short) at G1/S transition and the anaphase-promoting complex/cyclosome (APC/C) during mitosis (figure 13).

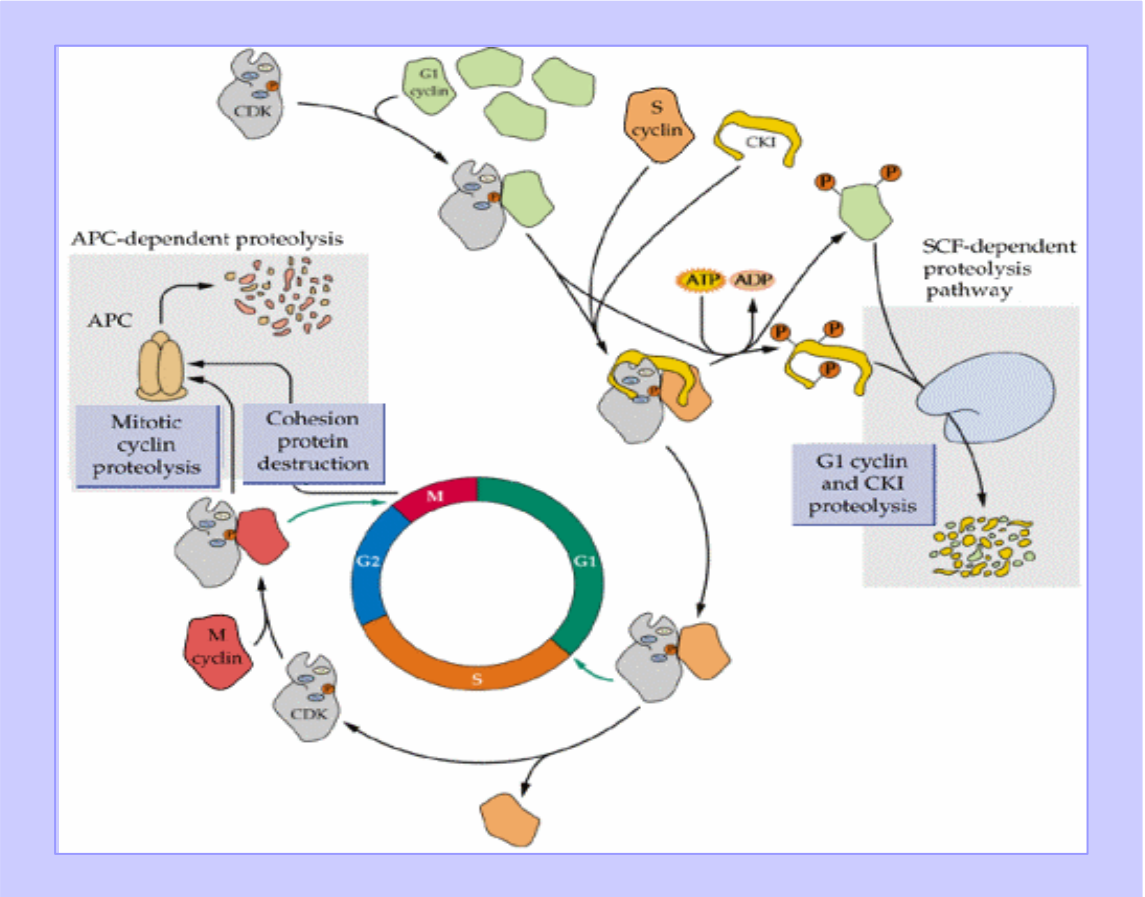


Figure 13: Mechanisms whereby ubiquitin-dependent proteolysis regulates cell cycle progression

1.2.2.4.1. Basic principles of ubiquitin-dependent protein degradation

The degradation of many short-lived eukaryotic proteins is carried out by the ubiquitin system (figure 14), where proteins are targeted for destruction by the covalent ligation of a small conserved polypeptide of 76 amino acids, named ubiquitin (reviewed by Hershko and Chiechanover, 1998). Proteins with these ubiquitin tags are subsequently recognized by the 26S proteasome complex, which will digest the protein into polypeptides. This ubiquitin-mediated degradation of regulatory proteins controls many processes, including cell cycle progression, signal transduction cascades, transcriptional regulation, receptor-down regulation and endocytosis. Ubiquitin-ligation requires the chronological action of three enzymes. In a first step, a specific activating enzyme, E1, will activate the Gly-residue of ubiquitin in an ATP-dependent fashion. Next, the activated ubiquitin becomes tansferred to an active site Cys-residue of an ubiquitin-carrier protein, E2 (or alternatively called Ubiquitin-conjugating enzymes or Ubc for short). Finally, the ubiquitin will be linked by its C-terminus to the substrate protein's Lys residues. This step requires the action of a so-called E3 enzyme. Usually, there is only one E1 present, but there are many species of E2 available in a given cell as well as multiple families of E3 enzymes or E3 multiprotein complexes. Specific E3s seem to be mainly responsible for the selectivity of the ubiquitin-target protein ligation. The APC/Cyclosome and SCF are examples of complexes with E3 activity.

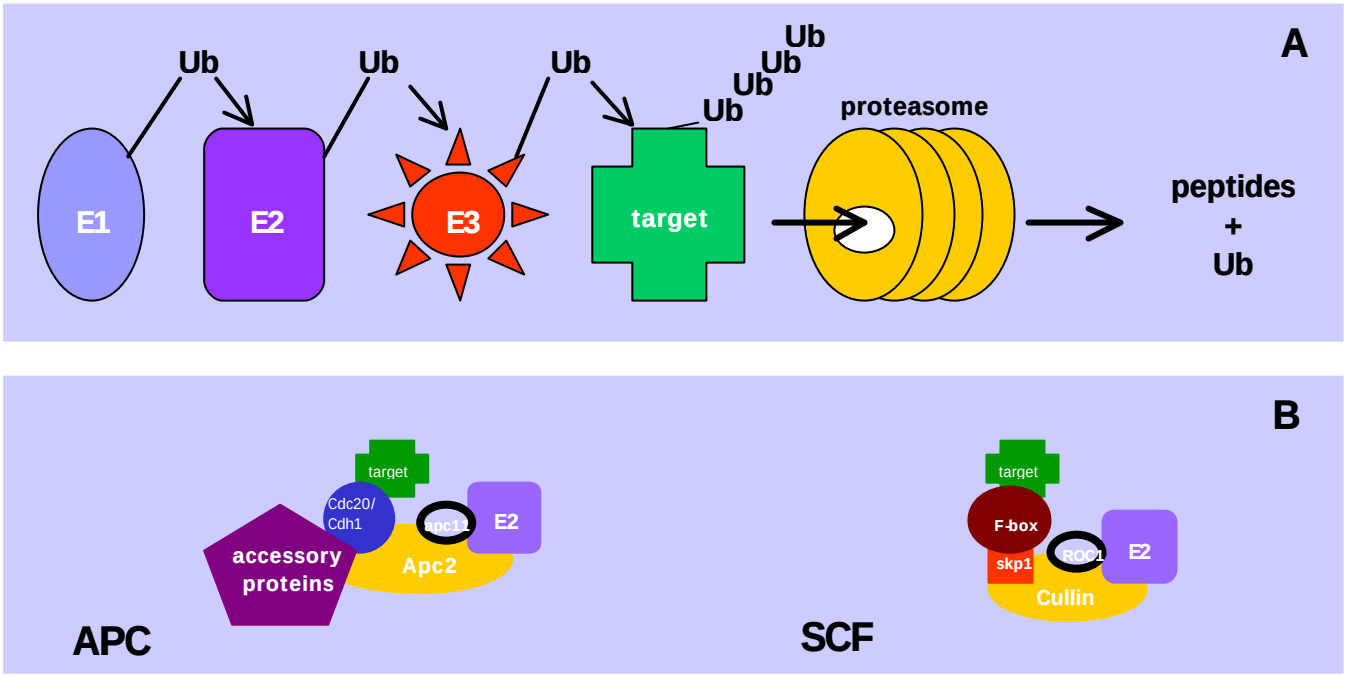


Figure 14: ubiquitin-dependent proteolysis
A. basic ubiquitin-dependent proteolysis machinery
B. Structure of the APC and SCF

1.2.2.4.2. The Anaphase Promoting Complex

The APC is the specific proteolytic machinery (figure 14b) responsible for the degradation of anaphase inhibitory proteins, such as Pds1 in budding yeast (see section 1.2.2.3.1), leading to the triggering of sister chromatid separation. Additionally, it is responsible for destruction of the mitotic cyclins. As a consequence, CDK activity is significantly reduced and will allow cytokinesis (Zachariae and Nasmyth, 1999). This is demonstrated by the expression of no degradable cyclin variants: CDK1 activity, preventing cytokinesis and spindle disassembly (Holloway *et al*., 1993). The APC works in close collaboration with the E2 enzymes Ubc10 and Ubc4 (Zachariae and Nasmyth, 1999).

A more close inspection of the N-terminus of mitotic cyclins in animals revealed a degenerate motif of nine amino acids, named the destruction box. When mutated, cyclinB proves to be resistant to degradation (Glotzer *et al*., 1991). CyclinA becomes degraded in metaphase, whereas CyclinB will be destroyed slightly later in the meta-to-anaphase transition (Zachariae and Nasmyth, 1999). Plant A- and B-type cyclins also feature the destruction box hallmark (Genschik *et al*., 1998). Genschik and his colleagues fused the N-terminal domain of plant mitotic cyclins to the *CAT* reporter gene and expressed them in tobacco BY-2 cells. When assaying for *CAT* expression, they were able to registrate a cell cycle-specific oscillation of the fusion proteins, suggesting that mitotic cyclins in plants are subjected to translational control. Moreover, when a proteasome inhibitor was added to the culture medium, the BY-2 cells become arrested in metaphase and the fusion proteins remain stable. Analogous results are obtained by Criqui and coworkers (2000). However, a recent report about plant A-type cyclins indicates the presence of constant levels of these cyclins during cell cycle progression (Chaubet-Gigot, 2000). Corresponding results were presented by Mews *et al.* (1997), who were able to detect A-type cyclins by immunolocalization in late mitosis both in BY-2 cells or sectioned maize root tips. One has to conclude that although proteolytic degradation is involved in plant cell cycle control, other mechanisms of degradation will also be anticipating.

The exact identity of the APC multisubunit E3 ubiquitin ligase was only discovered recently (reviewed by Zachariae and Nasmyth, 1999; Peters, 1998). By immunopurification, the composition of the APC/C was investigated in yeast, *Xenopus* and human. Human and *Xenopus* particles contain more than 10 subunits while those of yeast contain more than 12 subunits. When comparing the subunits from the yeast APC with those from higher eukaryotes, one observed that almost all subunits do have a counterpart in vertebrates. This suggests a high degree of conservation. The subunits tend to stay associated throughout the cell cycle. The major subunit is considered to be formed by the Apc1 protein (Peters *et al.*, 1996). Association with regulatory subunits, such as the WD activator proteins Cdc20 and Cdh1, is cell cycle regulated. In plants, some homologs of APC-related proteins have been described, such as the WD-repeat protein Fizzy in maize (Luo *et al*., 1997). However, a possible destructive function or an actual role of this protein in cell cycle control remains speculative.

1.2.2.4.3. The SCF proteolytic complex

In order to enter a new round of cell division, G1 cyclins and CKIs need to be degraded at G1/S transition (reviewed by DeSalle and Pagano, 2001; Yew, 2001; figure 14b). This requires the action of the E3 ligase complex SCF and the E2 enzyme Cdc34 (alternatively named Ubc3).

The rapid turnover of yeast and mammal G1 cyclin is dependent on the presence of so-called PEST sequences, regions that are rich in these four amino acids (Rechsteiner and Rogers, 1996). Examination of the cyclinD protein sequences in plants, revealed the same motif in all cyclins of this type, except for the tobacco CycD2;1 (Sorrell *et al*., 1999; Meijer and Murray, 2000). However, in no case the role of these PEST sequences in the half-life of these proteins has been experimentally verified in plants.

The composition of the SCF has been thoroughly studied in budding yeast and mammals and contains three major subunits (reviewed in DeSalle and Pagano, 2001). The F-box protein confers substrate specificity by recruiting a particular target to the core ubiquitination machinery. The hallmark of these kinds of proteins is the existence of a conserved domain of approximately 40 amino acids that mediates binding to the Skp1 subunit of the SCF, the F-box. In yeast, the Fbox protein in the complex can be Cdc4, Grr1 or Met30. Consequently, this leads to the degradation of a certain type of protein: the CKI Sic1 becomes destroyed through the association with Cdc4, while Cln1 becomes targeted for proteolysis when Brr1 is part of the SCF. Mammalian homologues of F-box proteins involved in cell cycle control are Skp2 and ßTrcp. Skp2 is responsible for SCF specificity for p27. The Skp1 subunit is regarded to be an indispensable scaffold protein binding the F-box protein and the cullin subunit of the SCF. The presence of this protein stabilizes the complex. Finally, the proteins of the cullin family constitute the third part of the SCF complex. Proteins of this type are known to interact with associating factors of the SCF, such as the E2 enzymes. The budding yeast homologue of the mammalian cullins is called Cdc53. In plants, homologues of the SCF proteolytic machinery have been identified, such as Skp1 (Porat *et al*., 1998). The plant gene products, Axr1 and Tir1, are implicated in auxin signaling (del Pozo *et al*., 1998; Ruegger *et al*., 1998). The *axr* gene encodes a subunit of the Rub1 activating enzyme (del Pozo *et al*., 1998). Rub1 is an ubiquitin-like protein and has been shown to become attached to a cullin-like protein of *Arabidopsis in vitro*. The Tir1 protein belongs to the family of F-box proteins (Ruegger *et al*., 1998). For that reason, it is tempting to speculate that auxins promote cell division through the degradation of CKIs at the G1/S transition (Gray and Estelle, 2000).

Research Objectives

Research objectives

Research during the last decades already elucidated many molecular aspects of the cell cycle control apparatus, mostly in yeast and animals. Research on genes involved in the plant cell cycle made enormous progress the last decade and revealed many similarities with the higher eukaryotes mentioned above. Remarkably is the fact that for the gene classes of importance for the basic cell cycle machinery plant-specific members were isolated, which clearly suggests the existence of regulatory pathways that are exclusive for plants. Most probably this is due to their non-motile lifestyle as well with the physical limitations caused by the presence of a rigid, surrounding cell wall. The primary objective of the work presented in this thesis is to extend the present knowledge of plant-specific processes by means of the large-scale isolation of novel plant genes involved in cell cycle-specific processes. The strategy of choice to achieve this purpose is a whole-genome expression analysis approach based on the AFLP technique. The analysis of the comprehensive collection of cell cycle modulated genes, resulting from this screening, will be done by means of the quantification of their expression patterns and the characterization of the corresponding sequence by gene annotation. As such, the data achieved will allow us to have a general overview of the transcriptome associated with cell division and to get to know more about the specific pathways involved in the process of plant cell propagation. Moreover, they will provide us with essential information, necessary to select interesting candidates for further molecular-genetic characterization. In addition, the results will allow us to create a global picture of both the similarities and differences of cell cycle progress between plant and other higher eukaryotes.

Chapter Two

Genome-wide expression analysis and gene discovery in plants using cDNA-AFLP-based transcript profiling

Abstract

A cDNA-AFLP-based transcript profiling method has been optimized and applied in a genome-wide screen of cell cycle modulated genes in synchronized tobacco BY2 cells. In subsequent global and in-depth analyses, abundant as well as scarce messengers could be visualized. In addition to known genes, several transcript tags corresponding to putative novel sequences have been identified, demonstrating the sensitivity and the power of the technology for discovering new genes in organisms for which no sequence information is available. Furthermore, the cDNA-AFLP technique has been demonstrated to be highly specific and competent to discriminate between very homologous gene copies, what is an important prerequisite for detailed gene expression studies. Apart from being an efficient method for gene discovery, we have also verified that cDNA-AFLP is an appropriate tool for determining quantitative gene expression profiles. Based on the profiles, several discrete transcript patterns were recognized and subtle temporal differences in transcript accumulation during cell cycle progression could reliably be distinguished. Based on the obtained results, it can be concluded that cDNA-AFLP is a valid tool for genome-wide expression analysis at both the qualitative and quantitative level.

The contents of this chapter are submitted to **Nature Biotechnology** by **Breyne, Dreesen, Cannoot, Rombaut, Vandepoele, Rombauts, and Zabeau** under the title **"Cell cycle modulated gene expression in plants monitored by cDNA-AFLP-based transcript profiling"**
The data were also presented as a poster at the 6th meeting of the ISPMB in Quebec (Canada), June 2000

2.1. Introduction

2.1.1. An overview of the major technologies to screen for differential gene expression

A scientist on its way to unravel a certain biological process will be most successful when trying to obtain experimental data about the genes involved. Indeed, the temporal and developmental expression patterns of such genes are important clues to their role in a given process. Hence, the search for genes that are differentially expressed under defined circumstances is one of the most straightforward strategies in the study of a biological system.

One of the first attempts to screen for differential expression, the differential hybridization method, was based on the comparison of the hybridization patterns of cDNA libraries derived from different tissues or treatments (Maniatis, 1978). When performing experiments with this technique, one will soon realize that only few of the isolated clones are interesting for further characterization, illustrating clearly the foremost encountered difficulty when screening for differential expressed genes. The transcriptome of a common eukaryotic cell is estimated to contain thousands of different mRNA species that can be present in one or up to multiple copies. Moreover, approximately half of the transcript mass is made up by only a few hundred transcripts, commonly referred to as housekeeping genes, while at least 99% of the transcripts constitutes the other half and is considered to be rather rare (Furl and Paul, 2000). As a result, the search for a gene responsible for a specialized function can be seen as "fishing" in a pool. The fact that the coda libraries used for the differential hybridization technique also contain abundant transcripts, explains the low efficiency in finding biological significant genes. Although this major drawback can be improved by normalization and subtraction procedures (subtractive hybridization, Hedrick *et al*., 1984), one is not able to isolate interesting genes, which are expressed at a low level. This is due to the fact that the hybridization signal cannot be amplified and as a consequence will be missed.

Gel-based RNA profiling techniques as a strategy to detect differential expression

The difficulties encountered by the previously described traditional methods could be circumvented by the establishment of techniques that are based on PCR technology. A first approach, named differential display, uses a 3' anchored oligo(dT) primer and short 5' arbitrary primers to amplify portions of cDNAs which become subsequently fractionated on a high-resolution gel (Liang and Pardee, 1992). It has been are estimated that 80 primer combinations should be sufficient to cover the complete eukaryotic transcriptome (Liang *et al*.,1993). At the same time, a similar technique, AP-PCR, was introduced by Welsh and coworkers (1992), based on the same principle as differential display, but using exclusively arbitrary primers for PCR amplification. Because of its fast performance, sensitivity and easiness, such techniques soon became an established value in

the toolbox of a molecular biologist. However, a number of disadvantages were experienced. The ability of the technique to detect rare transcripts was questioned, since some experiments indicated that only mRNAs with a mass of more than 1.2% of the total sample could be visualized by differential display (Bertolli *et al*., 1995). This would be due to the competition for dNTPs of different PCR products to be assembled or the limited amount of primers present in the reaction mixture, leading to a more prevalent amplification of abundant transcripts than rare ones, which stay under the detection limit. A second drawback is the high number of false positives generated as a result of the annealing of the primers at relatively low temperatures, hereby reducing its specificity (Zhao *et al*., 1995). Another problem arises when cloning an isolated fragment. Here fore, bands of interest need to be isolated from the gel and the eluted DNA has to be sequenced to determine the identity of the corresponding gene. Even when very accurately excised from the gel, several cDNA species will be present in the cloning mixture resulting in a mixed population of clones. Additional techniques, such as Northern or RT-PCR analysis, are needed to identify the truly differential cDNA.

A part of these problems can be overcome when performing the AFLP technique on cDNA populations as described by Bachem *et al.* (1996). Here, the PCR templates consist of restriction fragments to which adapters are ligated. These adapters serve as annealing sites for the primers making it possible to perform PCR reactions under more stringent conditions. The addition of selective bases to the 5' ends of the primers offers the possibility to reduce the numbers of cDNA fragments that will be displayed. As a consequence, this technique has been experienced to be highly sensitive and specific. Moreover, the gene expression patterns produced by this technique have been demonstrated to be equivalent to results obtained by Northern analysis since the intensity of the bands is a reliable measure of the relative differences in gene expression levels. cDNA-AFLP has been successfully applied to detect differential gene expression, mainly in plant systems (Durrant *et al*., 2000; Qin *et al*., 2000; van der Biezen *et al*., 2000). However, the problem of heterogeneity in the reamplified bands still needs to be addressed in this technique.

As a conclusion, the PCR-based techniques described above have proven their valuability for the systematic analysis of genes in particular biological processes. However, in most published studies, only a small fraction of the transcriptome was analyzed and quantitative expression data could not be obtained

A look at gene expression from the perspective of the whole genome

In recent years, a paradigm shift occurred in molecular biology by the development of methods to generate large amounts of expression data from many genes. This makes it possible to look at differences in global gene expression and to overview the response of all genes in an organism to a certain stimulus. In the early nineties, the automatization of the sequencing protocol allowed scientists for the first time to build up a large data collection of so-called expressed sequence tags (ESTs) in model organisms. Practically, this occurs by the single pass sequencing of randomly selected cDNAs (Adams *et al*., 1991; 1995). Although promising, it soon became clear that this

approach was by far too laborious and expensive to be implemented in routine gene expression analysis. Moreover, low abundant messengers are difficult to detect. A second sequence-based technology to monitor differences in gene expression is SAGE (Serial Analysis of Gene Expression; Velculescu *et al*., 2000). This technique relies on the digestion of cDNA libraries with a selection of restriction enzymes. This generates sets containing fragments of 12 bp lengths, which are ligated together and sequenced. Absolute measures of the prevalence of certain transcripts can be made by counting the number of tags derived from each cDNA. To date, the information of about 47 human SAGE libraries is already available to the scientific community through the SAGEmap expression resource (Lash *et al*., 2000). By these means, it has become effortless to merge newly obtained data with those already present in this database. By recent technical improvements of the SAGE method, one is now able to perform analyses with very small quantities of tissue (Breyne and Zabeau, 2001). The main limitation of this technique resides in the fact that too many tags need to be sequenced when the detection of scarcely expressed genes is aimed. Moreover, homology searches with the short tag sequences are limited and dependent on the availability of large databases of well-characterized ESTs to match the tag precisely with a cDNA. More recently, a novel sequence-based technology was introduced, called Massively Parallel Signature Sequencing (MPSS; Brenner *et al*., 2000). Here, millions of DNA-signatured microbeads, each carrying a different cDNA attached by *in vitro* cloning, are repeatedly cycled between restriction type II cleavage, ligation steps and hybridization reactions to add decoder probes for reading the signatures. Subsequently, the amount of microbeads carrying identical cDNAs is counted using a flow cell. In this way, approximately 250,000 microbeads can be processed at once. Therefore, MPSS can be considered to be a major improvement over SAGE. Moreover, even rare mRNAs can be assessed without prior knowledge of their sequence, since longer signatures of 16-20 nucleotides are generated. However, as a major drawback it should be noted that the technical execution of this method is very complex. Consequently, it will take some time before this technology will become available for the broad scientific community.

The development of the microarray technology is considered to be a breakthrough for genome-wide expression analysis. These arrays consist of series of different cDNAs or gene specific oligonucleotides attached to a solid support like glass. As a consequence they provide a format for the simultaneous measurement of the expression level of thousands of genes in one single hybridization assay. For certain model organisms, such as yeast, some bacterial species and *Caenorhabditis elegans*, the public availability of the complete annotated genome sequence enabled the manufacturing of microarrays with entire gene collections (Wodicka *et al.*, 1997; Laub *et al*., 2000; Selinger *et al*., 2000; Jiang *et al*., 2001). Microarrays are also available for other well-studied eukaryotic species, including all major species such as mouse, human and *Arabidopsis,* and consist of that part of the genome for which a cDNA clone or an EST sequence is available (Ruan *et al*., 1998; Breyne and Zabeau, 2001). Nowadays, the microarray technology is routinely used for the identification of genes involved in many biological processes, such as the study of systemic acquired

resistance, key pathways involved in the circadian clock in *A. thaliana*, the analysis of developmental and sex-regulated gene expression in *C. elegans* or the global transcriptional response of *S. cerevisiae* to ionic radiation (Harmer *et al*., 2000; Schenk *et al*., 2000; Jiang *et al*., 2001; De Sanctis *et al*., 2001). An important aspect of this technology is the possibility to combine the results of different experiments in one single database, allowing a straightforward comparison of the expression profiles of different tissue samples or material that has been subjected to different treatments. As such, Hughes and coworkers (2000) were able to build an extensive compendium of expression profiles in yeast. However, microarrays have their limitations. Besides the fact that only those genes of a given organism can be surveyed for which a clone or sequence has been isolated, the over-representation of abundant messengers in cDNA libraries avoids the detection of rare messengers using this approach. Therefore, genome-wide expression profiling by microarray analysis is so far limited to a small group of model organisms. Another drawback is the fact that distinguishment between transcripts belonging to a gene family is rather difficult since the technique depends on hybridization. Finally, the rather poor sensitivity of the hybridization creates a restriction for the usefulness of microarrays in whole-genome differential expression screenings.

Very recently, two fragments-based methods for whole-genome expression analysis were introduced by which cDNAs can be displayed combined with a high sample throughput. A first technology is called GeneCalling™, which assays transcript abundance by processing poly-A+ RNA-derived cDNA through a restriction digestion utilizing optimized pairs of 6-base-pair recognition restriction enzymes. Resultant gene fragments are ligated and end-labeled with 5'-fluorescamine and 3'-biotin adapters and subjected to PCR amplification. Subsequently, the fragments are resolved by gel electrophoresis (Shimkets *et al*., 1999). Differentially expressed fragments are identified and confirmed against a virtual digest of the appropriate databases. By combining the sequence knowledge derived from the two restriction enzymes with the length of a given fragment, one is able to "call" putative gene assignments for each fragment. This information is sufficient for database cross-referencing to determine the identity or novelty (in case no "Gene Call" can be made in the database) of a particular gene. This technique proves to be very sensitive, but on the other hand is very laborious and time-consuming. A second method is named TOGA, an acronym for Total Gene expression Analysis (Sutcliffe *et al*., 2000). This approach utilizes sequences near the 3' ends of mRNA molecules to give each mRNA species present in the organism a single identity. This is achieved by exact matching by an equimolar mixture of 48 anchor primers to initiate reverse transcription. One member of this mixture initiates synthesis at a fixed position of each mRNA species, hereby defining a 3' endpoint. By restriction digestion with two enzymes, uniform fragment lengths for each cDNA species are obtained. After PCR amplification, the fragments are visualized and the status of each mRNA can be compared among samples in an automated fashion. Its identity can be electronically matched with sequences of known mRNAs in databases. This method is sensitive and its single-product-per-mRNA feature allows a near complete coverage of all mRNAs present in a given organism.

However, as like GeneCalling™, this method requires a high input of labor and time.

2.1.2. Land marking the plant cell cycle by means of a whole-genome expression profiling strategy

Cell division is a highly conserved cyclic event. Both the timing and execution of the successive processes of DNA replication, mitosis and cytokinesis are rigorously controlled (Amon, 1998; Leatherwood, 1998). Earlier studies revealed that the regulation of transcript levels is an important aspect of the activity of several cell cycle genes. RNA messengers of key regulatory genes such as cyclins and cyclin dependent kinases have been shown to accumulate at discrete moments during the cell cycle (Dynlacht, 1997; Mironov *et al*., 1999). More recently, genome-wide expression analysis in yeast, bacteria and human fibroblasts using microarrays revealed that about 10% of all transcripts differentially accumulate during the mitotic cell cycle (Spellman *et al*., 1998; Laub *et al*., 2000; Cho *et al*., 2001). Most genes necessary for the execution of a particular process tend to be strictly expressed during or immediately prior to the cell cycle phase in which this process occurs. Moreover, groups of co-regulated genes tend to follow the same expression pattern.

Assessing transcriptional modulation of gene activity during cell division requires highly synchronized cells in which discrete fluctuations in mRNA levels efficiently can be monitored. In plants, a number of cell lines with varying levels of growth rate and synchronicity in cell cycle progression have been established (Kapros *et al*., 1992; Nagata *et al*., 1992; Fuerst *et al*., 1996; Peres *et al*., 1999). Among these, the *Nicotiana tabacum* BY2 cell line (Nagata *et al*., 1992) is by far the most superior one and therefore is a widely used system for cell cycle research.

However, the choice of the BY2 cell line as model system has implications for the design of genome-wide expression analysis experiments. Microarrays are excluded as the method of choice since only few molecular resources are available for tobacco. Technologies not relying on the availability of large collections of sequenced genes or cDNA clones provide thus an appropriate alternative. As already outlined above, the gel-based technology, cDNA-AFLP, has already proven to be useful for identifying differentially expressed genes (Durrant *et al*., 2000; Qin *et al*., 2000; van der Biezen *et al*., 2000). The results presented further in this chapter, discuss in more detail how the cDNA-AFLP method can be adapted for a qualitative as well as quantitative genome-wide expression analysis of cell cycle modulated genes in BY2 cells. Moreover, the robustness of this technology will be thoroughly discussed and illustrated by means of some preliminary clustering results.

2.2. Experimental Procedures

2.2.1. The maintenance and synchronization of BY2 cells

Tobacco BY2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) suspension cultured cells were maintained as described by Nagata *et al*., 1992. For synchronization, a 7 days old stationary culture was diluted ten times in fresh medium supplemented with aphidicolin (Sigma; 5 mg/liter), a DNA-polymerase inhibiting drug. After 24 hours, cells were released from the block by several washings with fresh medium and continued cell cycle progression starting from early S-phase.

2.2.2. Sampling of material and monitoring of synchrony

Sampling was done every hour, starting at the moment of release from the aphidicolin block (time 0) until 11 hours later. At each time point, 75 ml of cell culture was sampled. A sub sample was used to check cell cycle progression and synchrony levels. After DAPI staining of the DNA (Sigma, 1 mg/ml), the mitotic index was determined by counting the number of cells undergoing mitosis through fluorescence microscopy. A mitotic peak of about 40% was obtained at 8 hours after washing. For flow cytometry, cells were first incubated in a buffered enzyme solution (2% cellulase and 0.1% pectolyase in 0.66 M sorbitol) for 20 minutes at 37 ºC. After washing and resuspension in Galbraith buffer (Galbraith *et al*., 1983), the suspension was filtered through a 30µm nylon mesh to purify nuclei, which were stained with DAPI. The fluorescence intensity was measured using a BRYTE HS flow cytometer (Biorad). Exit from S-phase was observed at 4 hours after aphidicolin release. The level of synchrony was observed to be sufficiently high throughout the time course.

2.2.3. RNA extraction and cDNA synthesis

Total RNA was prepared from each sample using the LiCl precipitation protocol (Sambrook, 1988). Subsequently, polyA+ -RNA was extracted from 500 µg of total RNA using oligotex™ columns (Qiagen) according to the manufacturer's instructions.

First strand cDNA synthesis was done starting from 1 µg of polyA+ -RNA by reverse transcription using a biotinylated oligo-dT_{25} primer (Genset) and Superscript II (Gibco-BRL). Second strand synthesis was performed by strand displacement with *E. coli* ligase (Gibco-BRL), DNA polymerase I (USB) and RNase-H (USB). Further cleaning of the cDNA occurred by elution through DNA Spun columns (Amersham Pharmacia Biotech).

2.2.4. AFLP analysis

500 ng of double stranded cDNA was used for AFLP analysis as described by Vos *et al.* (1995) and Bachem *et al*. (1996) with modifications. The restriction enzymes used were BstYI and MseI (New England Biolabs) and the

digestion was done in 2 separate steps. After the first restriction digest with one of the enzymes, the 3' end fragments were collected on Dynabeads (Dynal) by means of their biotinylated tail, while the other fragments were washed away. After digestion with the second enzyme, the released restriction fragments were collected and used as templates in the subsequent AFLP steps. The adapters and primers used are:

BstYI-adapter:	5' - CTCGTAGACTGCGTAGT - 3'
	5' - GATCACTACGCAGTCTAC - 3'
MseI-adapter:	5' - GACGATGAGTCCTGAG - 3'
	5' - TACTCAGGACTCAT - 3'
BstYI-primers:	5' - GACTGCGTAGTGATC(T/C)N_{1-2} - 3'
MseI-primers:	5' - GATGAGTCCTGAGTAA N_{1-2} - 3'

For preamplifications, a MseI-primer without selective nucleotides was combined with a BstYI-primer containing either a T or a C as 3' most nucleotide. PCR conditions were applied as described (Vos *et al*., 1995). The obtained amplification mixtures were diluted 600-fold and 5 µl was used for selective amplifications using a P^{33}-labeled BstYI-primer and the Amplitaq-Gold polymerase (Roche) following the described procedure (Vos *et al*., 1995). Amplification products were separated on 5% polyacrylamide gels using the Sequigel system (Biorad). Sodium acetate (22mg/l) was added to the buffer in the bottom tank to avoid run-off of the smallest fragments. Dried gels were exposed to Kodak Biomax films and in an additional approach scanned in a Phosphor-Imager apparatus (Molecular Dynamics).

Alternatively, selective amplifications were performed using IRD-labeled primers and amplification products were separated on an IR^2 DNA analyzer (Li-Cor), allowing direct imaging of the banding patterns.

2.2.5. Characterization of AFLP fragments

Bands corresponding to differentially expressed transcripts were cut out from the gel, the DNA was eluted in TE buffer and reamplified using the same conditions as for selective amplification.

Sequence information was obtained by direct sequencing of the reamplified PCR product using the selective BstYI-primer or after cloning the fragments in the pGEM-T Easy (Promega) and sequencing of the individual clones. The obtained sequences were compared against nucleotide and protein sequences present in the publicly available databases by BLAST sequence alignments (Altschul *et al*., 1997).

2.2.6. Quantitative measurements of the expression profiles and data analysis

Scanned Molecular Dynamics gel images or IR^2 derived image files were quantitatively analyzed using the AFLP-Quantar-Pro™ image analysis software of Keygene. This software has been designed and optimized for accurate lane definition, fragment detection and quantification of band intensities. All visible AFLP fragments were scored and individual band intensities were measured per lane. The obtained data were used to determine the quantitative expression profile of each transcript. Raw data were first corrected for differences in total lane intensities which may occur due to loading errors or differences in the efficiency of PCR amplification with a given primer combination for one or more time points. The correction factors were calculated based on constant bands

throughout the time course. For each primer combination, a minimum of ten invariable bands was selected and the intensity values were summed per lane. Each of the summed values was divided by the maximal summed value to give the correction factors. Finally, all raw values generated by QuantarPro™ were divided by these correction factors.

Subsequently, each individual gene expression profile was variance-normalized by standard statistical approaches as previously used for microarray-derived data (Tavazoie *et al*., 1999). For each transcript, the mean expression value across the time course was subtracted from every individual data point after which the obtained value was divided by the standard deviation. A coefficient of variation (CV) was calculated by dividing the standard deviation by the mean. This CV was used to establish a cut-off value and all expression profiles with a CV less than 0.25 were considered to be constitutive throughout the time course. The Cluster and TreeView software developed by Eisen (Eisen *et al*., 1998) was used for hierarchical, average linkage clustering.

TABLE1 *In silico* analysis of available full length cDNAs from *Arabidopsis thaliana*

Restriction endonuclease used	# of sites/cDNA	Position of site to polyA	% of tags 1st digest	% of tags reverse digest	Total % of tags	Mean length of tags
Sau3A (GATC)	5.31	451.68	61.2	22.56	83.76	122.97
TaqI (TCGA)	3.98	522.85	63.78	18.04	81.82	137.19
DdeI (CTNAG)	3.9	536.93	62.4	18.9	81.3	132.59
MspI (CCGG)	2.54	618.39	61.28	9.68	70.96	159.92
EcoRII (CCWGG)	0.96	756.26	41.9	5.46	47.36	180.17
StyI (CCWWGG)	0.89	774.6	41.42	5.2	46.62	177.09
AflIII (ACRYGT)	0.6	811.2	27.76	6.6	34.36	165.56
BstYI (RGATCY)	1.48	697.36	51.6	8.54	60.14	165.54

2.3. Results

2.3.1. cDNA-AFLP-based expression profiling

The original cDNA-AFLP protocol, as described by Bachem and coworkers (1996), was modified into a quantitative transcript profiling technology (Fig. 1). This enables a genome-wide screen for differential expressed genes in tobacco BY2-cells (Breyne and Zabeau, 2001). Essentially, the cDNA-AFLP method comprises the generation of unique restriction fragments originating from cDNA followed by a series of selective PCR amplifications. This results in the amplification of distinct subsets of transcript tags that are subsequently separated and visualized on high-resolution gels.

As for any study of the transcriptome, a genome-wide screen for differentially expressed genes using cDNA-AFLP requires that as many transcripts as possible are analyzed in a unique

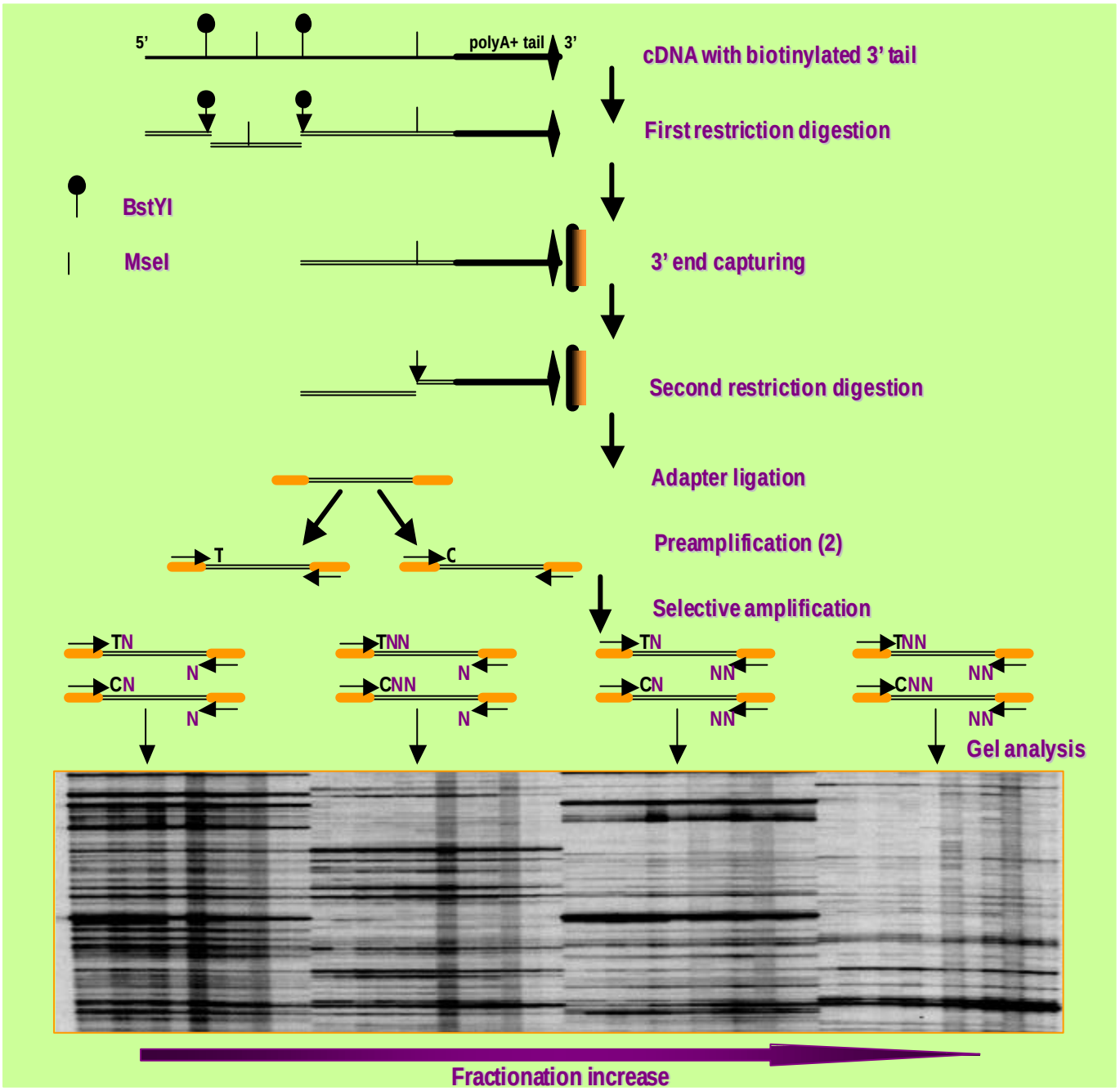


Figure 1: Overview of the cDNA -AFLP protocol and enhanced detection sensitivity by increased fractionation of template mixture

In the restriction procedure shown here, digestion by BstYI is followed by MseI. The AFLP templates were preamplified with BstYI+C or +T and Mse+0 primers, followed by selective amplifications using primers carrying 1 or 2 additional selective nucleotides, as described in experimental procedures. By gradual increasing of the number of selective nucleotides, less bands become amplified, while low abundant transcript become visible. This is demonstrated by the gel analysis shown: at the left, only two selective nucleotides are used (+A/+T), in the middle, 3 selective nucleotides are used (resp. +AC/+T and +A/+TC), while at the right, 4 selective nucleotides are used (+AC/+TC)

way. In our study, transcript tags were generated using the enzymes BstYI and MseI. *In silico* analysis of available full-length cDNAs from *Arabidopsis thaliana* has shown that 70% of all transcripts are cut by both BstYI and MseI (table 1). 59.6% of the transcripts is represented as a

BstYI-MseI 5'-3' fragment, while 11.4% of tags corresponds to a MseI-BstYI 5'-3' fragment. Almost 80% of the BstYI sites occur in the protein coding region of the genes and the average distance from the 3' most BstYI site to the poly-adenylation site is estimated to be 500 bp. Hence, the majority of the tags will be derived from the more informative coding sequences, facilitating the characterization of the tags. Five percent of the generated restriction fragments is either too small or too large to be analyzed on polyacrylamide gels, so that in principle around 65% of all tagged transcripts can be displayed. Repeating this analysis on a small number of available full-length cDNAs of tobacco resulted in comparable figures.

However, the actual number of fragments that can uniquely be scored on polyacrylamide gels also depends on the complexity of the amplification mixture. The average number of BstYI sites per cDNA is 1.5. By selecting the 3' most restriction fragment of the cDNAs, only one AFLP tag is obtained per transcript, reducing the number of restriction fragments 3-fold. Further reduction of the mixture complexity of amplified fragments is achieved through an increased fractionation of the template mixture in the selective amplifications (Fig. 1). Raising the number of additional nucleotides to the primers not only results in less fragments, but also enhances the detection sensitivity and enables to amplify tags originating from less abundant transcripts. The fractionation of the template mixture is performed in two subsequent steps. The complexity is reduced 2-fold in the preamplification step by the use of either a BstYI+T or a BstYI+C primer in combination with a MseI primer that does not carry any selective nucleotides. In a second round of amplification, between 2 and 4 additional selective nucleotides are added in order to screen for abundant and rare transcripts respectively.

2.3.2. Screening for cell cycle modulated genes

The cDNA-AFLP-based trancript profiling method has been applied to perform a quantitative and qualitative analysis of cell cycle modulated genes in plants. 12 time points were sampled after aphidicolin treatment of tobacco BY2 cells covering the cell cycle from early S-phase until M/G1 transition. By flow cytometry and microscopical determination of the mitotic index it was observed that the majority of the synchronized cells leaves S-phase after 4 hours, while the peak of mitosis is observed at 8 hours after release from blocking. The transition from G1 into S phase was not included in this study because of the rapid loss of synchrony after mitosis, therefore impeding a reliable analysis of differential gene expression.

Based on the knowledge obtained by the *in silico* analysis and pilot experiments, the following experimental set-up for the screen for cell cycle genes was designed: each of the samples was initially digested with BstYI, followed by MseI (referred to as Bst/Mse templates) or vice versa (Mse/Bst templates). After selective preamplification, screening for abundant messengers was performed with 1 or 2 additional selective nucleotides for respectively the Mse/Bst and the Bst/Mse templates. For the identification of scarce messengers, a total of respectively 2 and 3 additional selective nucleotides was used. When further increasing the number of selective nucleotides, the sensitivity of detecting new fragments was not significantly enhanced indicating that the majority

of cell cycle modulated transcripts already has been visualized (data not shown).

All possible primer combinations were used to screen for cell cycle modulated transcripts. The fingerprints were visually scored for AFLP fragments that accumulated in a differential fashion during the time course, in comparison to the majority of the bands, which remained constant (Fig. 2). Only bands that were present in at least two successive time points were considered for further analysis. In addition, all generated fingerprints were subjected to a computer-based analysis to identify true differential expression and to determine the corresponding quantitative expression profile (see below).

2.3.3. Quantitative analysis of cell cycle modulated gene expression

Phospho-images of the AFLP gels were analyzed using the QuantarPro™ software package in order to obtain quantitative expression data. All detectable AFLP fragments were scored and the intensity of every band in all lanes was determined. Subsequently, the obtained raw data were corrected for differences in total lane intensities and variance-normalized as described in the experimental procedures section. By these means, we were able to check the degree of differential expression of the transcript tags, which were identified to be cell cycle modulated after visual scoring. This was done by the calculation of their coefficient of variation (CV; see experimental procedures). Consequently, a small fraction was considered to be constitutively expressed since they have a CV of less than 0.25. Furthermore, the application of the QuantarPro™ quantification procedure enabled us to score approximately 400 additional differential bands that were missed in the visual scoring.

The cDNA-AFLP-based expression profiles of the transcript tags corresponding to

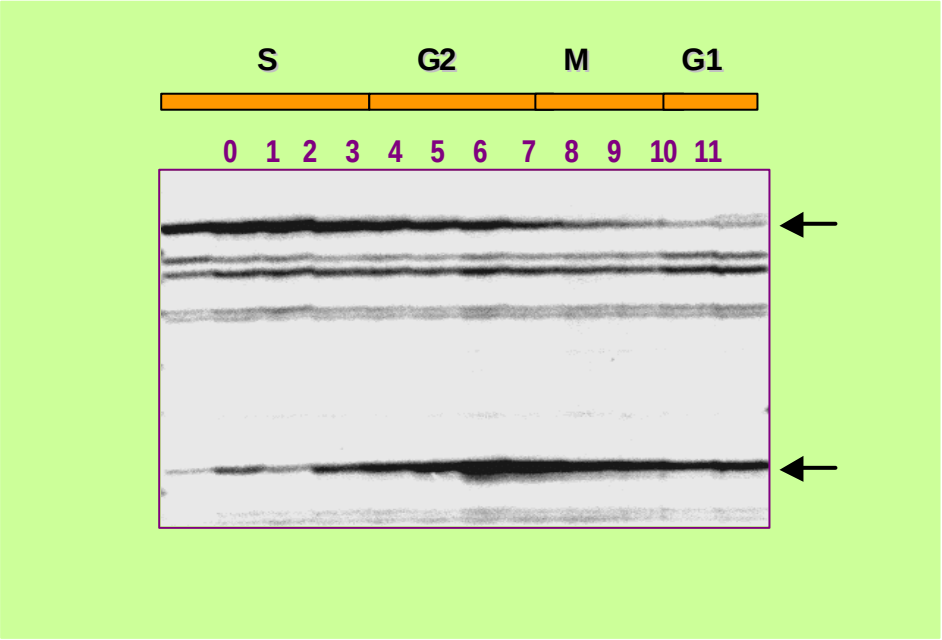


Figure 2: Screening for cell cycle modulated transcript tags
Samples obtained from 12 subsequent time points covering almost one complete cell cycle were analyzed. The profiles shown were obtained by using a BstYI+TT and a Mse+CC primer. Only a small part of the gel with fragments between 130 and 170 bp is shown. Arrows indicate differentially accumulating tags, the other bands were derived from genes that are considered as constitutively expressed

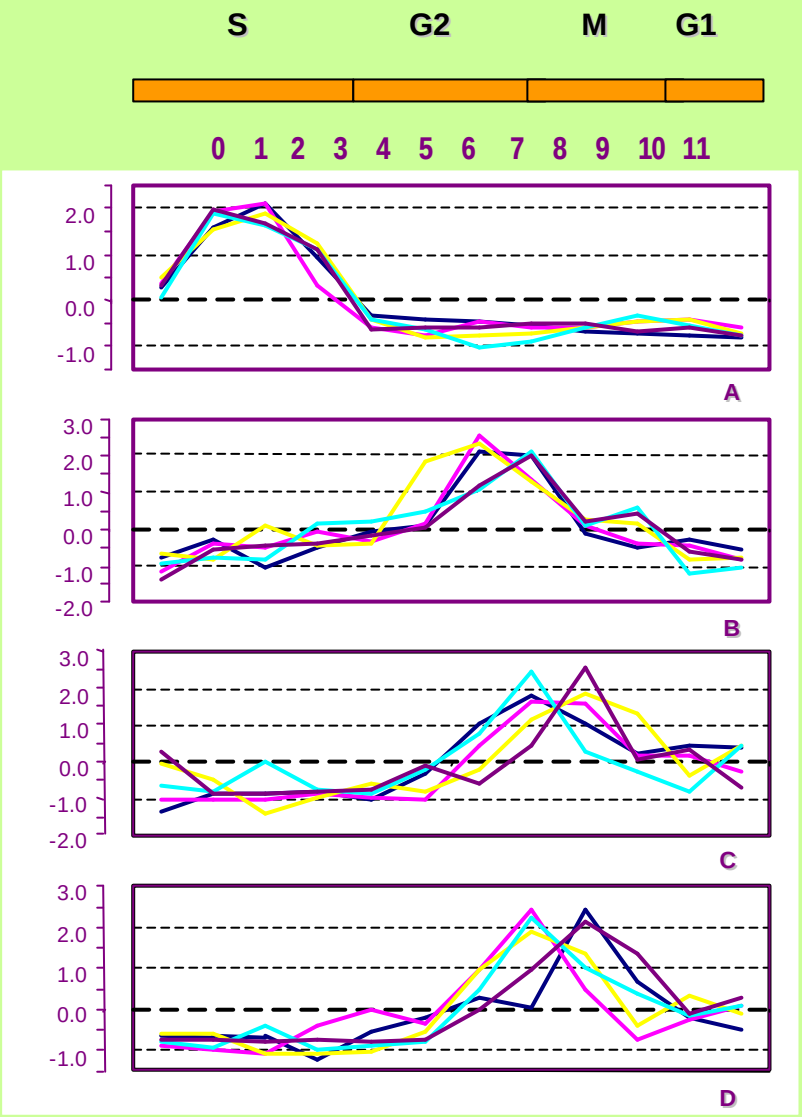


Figure 3: Quantitative expression profiles of cell cycle markers

The relative, variance normalized expression data (relative expression) of transcript tags derived from well-characterized genes known to exhibit a cell cyle modulated expression pattern were plotted against the different time points.

A. histone encoding genes
B. genes encoding B-tpye CDKs
C. cyclin genes
D. genes for tubulins

well known cell cycle modulated genes (Fig. 3), nicely fit with published data based on Northern analysis or RT-PCR, hereby indicating the reliability of the obtained data. Average linkage hierarchical clustering of the expression profiles grouped the genes in several large and small clusters, specific to the different cell cycle phases. A detailed analysis of the clusters and expression profiles will be presented in chapter four.

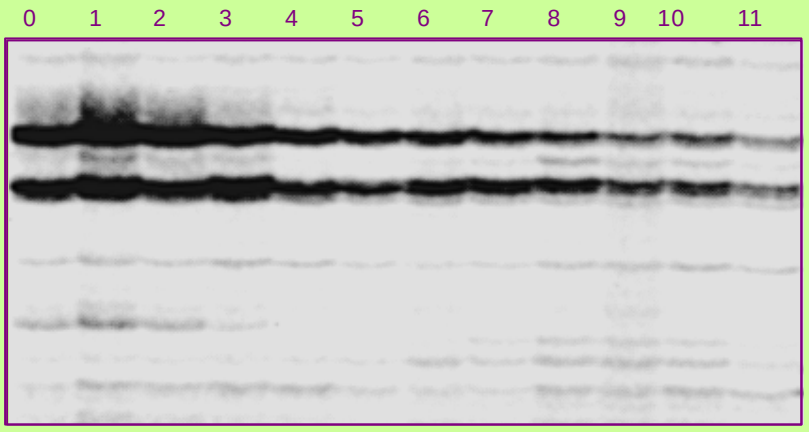
2.3.4. Characterization of cell cycle modulated transcripts

Bands corresponding to cell cycle modulated transcripts were isolated from gel, reamplified and sequenced. Direct sequencing of the PCR products resulted in good quality sequence for about 65% of the fragments. For the remaining bands, a contamination of co-migrating fragments or the fact that their length is too short lead to a bad quality of the obtained sequence. These bands were cloned after which the PCR products of 6 individual clones were sequenced. The individual colonies of the majority of these fragments contained different inserts. In such cases, the identity of the transcript tags could only unambiguously become resolved after performing complementary experiments. An alternative strategy to the cloning of AFLP bands is to isolate them from PCR amplifications performed on a pool of the 12 time point samples. The selective primers that are used carry one additional nucleotide. Under these circumstances, the complexity of the amplification mixture is reduced 4-fold, leading to the isolation of less contaminating bands from the gel and an increase to 80% of success rate when performing direct sequencing.

The sequences obtained from the tags were compared to those present in the Genbank database using the Blast software. Detailed results are presented in chapter three. Similarity with genes of known function was found for around 33% of the sequenced tags, while another 15% matches with a gene of unknown function (EST sequences or putative proteins). The remaining 52% may represent previously uncharacterized genes or the lack of homologues is a consequence of the fact that AFLP tags are too short and originate from the 3' end region of the transcripts. In addition, tags derived from the 3' untranslated region of a gene might be too diverged to find homologous sequences. However, as described above, *in silico* analysis revealed that the majority of the tags at least partially resides within the protein encoding regions. Further experimental evidence came from the sequence analysis of 66 cDNA clones that were isolated from a BY2 cDNA library using AFLP tags as probe. This analysis showed that only 23% of the tags is derived from the 3' untranslated region. The other tags totally or partially overlap with the coding sequences of the gene. Homology searches performed with the obtained cDNA sequences showed that of the 21 cDNAs corresponding to a tag without any significant homology, only 9 match with a sequence in the database. Although the number of cDNAs analyzed is small, these results indicate that 10 to 20% of all the cell cycle modulated transcript tags possibly are derived from novel genes.

2.3.5. Validation and evaluation of the data

To evaluate the fidelity of the cDNA-AFLP method for genome-wide expression analysis and to check the quality and reliability of the obtained data set, a partial complementary screen was performed. To this purpose, the analysis of the Bst/Mse templates with 3 additional selective nucleotides, which rendered the majority of cell cycle modulated transcripts, was done with both BstYI+2/MseI+1 and BstYI+1/MseI+2 primers. In such a duplicate screen, the generated data can directly be compared as they are derived from the same templates. Based on the length of the tags obtained with the BstYI+2/MseI+1 primer



A

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Bstt32-220  GCAGTGCTGTTGCTGCCCTACAAGAGGCTGCTGAGGCTTACCTTGTTGGCCT
Bstt32-225  GCAGTGCTGTGGCCGCTCTACAAGAGGCTGCTGAGGCTTACCTTGTTGGACT

Bstt32-220  CTTTGAAGATACAAATCTCTGTGCCATTCACGCTAAGAGGGTTACTATCATG
Bstt32-225  CTTTGAAGACACCAATCTCTGTGCTATTCACGCGAAGAGGGTCACCATAATG

Bstt32-220  CCTAAGGACATGCAGCTTGCTAGGAGGATTCGCGGTGAAAGGGCTTAGGATG
Bstt32-225  CCAAAGGACATTCAGCTGGCCAGGAGGATTCGTGGTGAAAGGGCTTAGGAAT

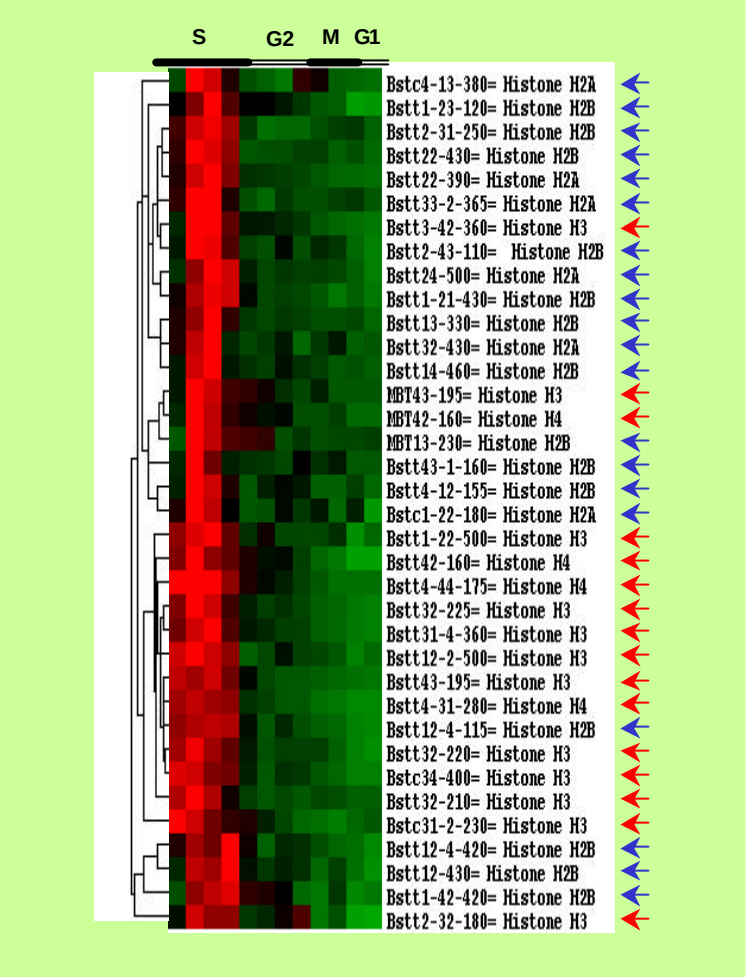
Bstt32-220  AAG.CTTGTTATGCTTATGGTTAA
Bstt32-225  ATGCCTCGCTGTGCTTGTTGTTATGGTTAA
```

B

Figure 4: Discrimination between paralogous genes
Two transcript tags obtained with the same primer combination, displaying nearly the same expression profile and with only a small size difference (panel A), were isolated, sequenced and identified as histone H3 genes. Panel B shows that the sequences are highly homologous and that the tags only differ 5 nucleotides in length

combinations and the sequence obtained from the corresponding fragments, the expected bands in the replicate BstYI+1/MseI+2 primer combination could be predicted. Subsequent sequence analysis of these bands confirmed the identity of the tags. The quantitative expression profiles of replicate bands were most often highly comparable, as judged by the evaluation of the relative correlation between the complementary expression patterns: The average Pearson correlation coefficient of a random subset of approximately 60 identical fragments has been determined to be 85.5%.

Furthermore, the synchronization experiment has been done twice independently, whereas several of the primer combinations were performed in duplicate on both series of samples. The banding patterns and expression profiles throughout the time course were always highly similar and sequence characterization of a number of cell cycle modulated transcript tags confirmed their indistinguishable identity. Analyzing a number of primer combinations on a Li-Cor IR2 DNA analyzer validated the quantitative data. QuantarPro analysis of the generated cDNA-AFLP images confirmed the quantitative expression profiles of the different transcript tags (data not shown). These results are a strong indication that cDNA-AFLP-based transcript profiling is robust and produces reliable



Figuur 5: Hierarchical clustering of histone gene tags based on their expression profiles

The variance normalized expression profiles of histone gene tags that were identified in our screen, were subjected to average linkage hierarchical clustering. Histone H3 or H4 tags and H2A or H2B tags are respectively marked by a red or a blue arrow

data. In addition, the quantitative analysis can be performed in a semi-automatic way, hereby facilitating and accelerating the throughput.

As described above, the generated expression profiles of known cell cycle modulated genes fit with previously published data and the correlation between the expression patterns of independently generated tags from the same transcript is high. Additional proof of the quality of the generated data is demonstrated by the analysis of a subset of tags corresponding to histone genes. The different histone subtypes are encoded by multigene families and their differential expression during the cell cycle has been well documented (Chaubet and Gigot, 1998; Meshi *et al.*, 2000). Several tags corresponding to genes encoding histone H2A, H2B, H3 or H4 have been identified in our screen for cell cycle modulated transcripts. Figure 4 illustrates one of the main characteristics of the cDNA-AFLP technology, namely the discrimination between paralogues. With the particular primer combination shown, two tags were generated with only a small difference in size and with an equal expression profile. Sequence analysis demonstrated that both tags

are highly homologous and derived from paralogous genes.

Quantitative analysis of the expression patterns indicated that the different histone gene transcripts behave very similar throughout the cell cycle and hierarchical clustering (Fig. 5) showed that they indeed tend to group together. Interestingly, the histone H3 and H4 tags are clearly separated from the H2A and H2B tags with the H2 genes being expressed slightly later than the H3 and H4 genes. These results do not only show that cDNA-AFLP is a very specific method to distinguish between closely related genes, but also that the generated quantitative data allow to discriminate between slightly different expression profiles.

2.4. Discussion

The use of the highly synchronizable tobacco BY2 cell line combined with cDNA-AFLP-based transcript profiling allowed for the first time the performance of a genome-wide screen for cell cycle modulated genes in plants. The identification of several hundreds of transcript tags corresponding to novel as well as to diverse classes of known genes, together with the generation of reliable quantitative expression data, proves that cDNA-AFLP is a valid tool for genome-wide expression analysis. The study performed and the data obtained exemplify most of the advantages and characteristics of cDNA-AFLP over other methods for transcript profiling. Without prior sequence information or available molecular resources, we were able to assess the majority of transcriptional changes occurring during cell cycle progression in BY2. *In silico* analysis of full-length cDNA clones showed that around 70% of all transcripts can be screened using the enzyme combination that was chosen. As tobacco is an amphidiploid species, the actual number of genes that has been screened for cell cycle modulated expression is likely even higher.

In agreement with recent studies (Durant *et al*., 2000; Qin *et al*., 2000; van der Biezen *et al*., 2000), our analysis has proven that cDNA-AFLP is an efficient technique to identify previously unknown transcripts. Although several of the tags don't show any homology to a known sequence since they are too short, the analysis of a limited number of isolated cDNA clones indicates that without a doubt many novel genes were identified. Among the transcript tags corresponding to known sequences, several genes that are scarcely expressed have been identified. This sensitivity of detecting low abundant messengers results from the increased fractionation of the mixture of transcript tags, which can be experimentally varied and demonstrates another asset of cDNA-AFLP.

An additional advantage of cDNA-AFLP is the high discriminatory strength of the technology. The specificity of the PCR amplifications and the resolving power of high-resolution gels, enable to distinguish between highly homologous transcripts. During the characterization of the transcript tags, several examples of tags derived from paralogous genes

were found. Especially for plants, this is an important aspect, as many gene duplications have occurred and large gene families exist in most species. Moreover, several species, including tobacco, have amphidiploid genomes originating from different species.

In addition to the aspect of gene identification, we demonstrated that reliable gene expression patterns can be obtained by a quantitative analysis of the generated cDNA-AFLP profiles. The differences in band intensity between the samples reflect fluctuations in transcript levels and allow determining the relative expression profile of the corresponding gene. Replicate experiments and statistical evaluation of the data validated the results, showing that the technological approach produces consistent quantitative data. The obtained gene expression profiles can be handled in the same way as is done for microarray data and allow for example the identification of groups of co regulated genes by cluster analysis. As exemplified by the detailed analysis of the transcript profiles of the different subtypes of histone genes, subtle temporal differences in expression patterns can be distinguished. The observation that the histone H3 and H4 transcripts accumulate a little earlier in the S-phase than H2A and H2B is in agreement with published results indicating that these histone types interact first in nucleosome assembly (Wolffe, 1995).

For studies that involve non-model species, cDNA-AFLP-based transcript profiling is a valid alternative to the widely used microarray technology. Although it requires a minimum of 160 primer combinations to screen 70% of all transcripts and to reach a sufficient level of sensitivity, the analysis can be accelerated and semi-automated using DNA fragment analyzers that can process several hundreds of samples every day, which makes a genome-wide analysis a feasible task. But also for organisms for which microarrays are available, cDNA-AFLP still can be a complementary tool in many studies. As it is much cheaper, large numbers of samples can be pre-screened using cDNA-AFLP with a limited set of primer combinations to determine which samples are the most informative ones for a detailed microarray analysis. Moreover, although the analysis performed in this study needed 1 µg of poly-A+ RNA as starting material, recent improvements to the technology enable to work with less than 100 ng of total RNA (unpublished results). These small amounts of starting material allow the analysis of little samples, what often has shown to be the bottleneck in many experiments.

A detailed, integrated analysis of the functional properties of the different cell cycle modulated genes identified and their quantitative expression profiles will be of assistance in describing the different processes that occur during the process of cell division in plants and might result in the identification of additional key regulatory genes that control cell cycle progression.

Acknowledgements

We wish to thank Bernard Cannoot and Debbie Rombaut for excellent technical assistance in the transcript profiling analysis. Sequencing of the retrieved AFLP tags was done by the concerted efforts of Rebecca Declercq, Caroline Buysschaert, Jan Gielen and Raimundo Villarroel. The *in silico* data were generated by Stephane Rombauts and Hans Constandt. Klaas Vandepoele assisted us in the characterization of the sequenced gene fragments. Discussions with Marnik Vuylsteke were very helpful to establish a suitable method for normalization of the expression data.

Chapter Three

The cDNA-AFLP-based transcript profiling technique enables the assembly of a comprehensive catalog of cell cycle modulated genes in tobacco BY-2 suspension cells

Abstract

By means of genome-wide expression analysis using cDNA-AFLP-based transcript profiling, a comprehensive collection of cell cycle modulated genes was established in tobacco BY-2 cells. By this approach, around 1500 unique gene fragments could be isolated, which differentially accumulate at certain phases of the cell cycle. This inventory includes known cell cycle genes as well as several new putative regulatory genes. Moreover, some plant-specific genes were for the first time observed to be cell cycle regulated while as yet unknown plant homologues of cell cycle related genes in other eukaryotic species were identified. The majority of the tagged genes encodes novel proteins or proteins of unknown function. In frame of the fundamental developmental differences between plants and other eukaryotes, our data set provides a solid basis for further unraveling of conserved pathways in addition to the characterization of plant-specific mechanisms of importance in plant cell cycle control.

A part of the results of this chapter will be submitted to **Nature Genetics** by **Breyne*, Dreesen*, De Veylder, Van Breusegem, Callewaert, Vandepoele, Rombauts, Raes, Engler, Inzé, and Zabeau** under the title **"Functional analysis of the transcriptome during cell division in plants"**

* these authors contributed equally to this work

3.1. Introduction

As a consequence of the fact that cell division is one of the fundamentals for the endurance of life, many characteristics of this procedure are analogous for both prokaryotes and eukaryotes (Amon, 1998; Leatherwood, 1998). This degree of evolutionary conservation is even more pronounced when the cell cycle mechanisms of different eukaryotic kingdoms are compared. The most striking resemblance is observed for the core cell cycle control machinery that governs the progression of a dividing cell through the successive phases of the cell cycle and which is represented by a widespread class of heterodimeric serine/threonine protein kinases (Nigg, 1995; Mironov *et al*., 1999). Such protein complexes consist of a catalytic subunit (termed cyclin dependent kinase or CDK) and an activating subunit (cyclin). Their activity is regulated at multiple levels, which makes them most suitable to integrate signals from different pathways that check the qualitative execution of cell division. Other essential processes, such as DNA replication and certain aspects of mitosis and cytokinesis, also tend to be similarly organized for all eukaryotes.

The last decade, it has become more and more clear that the molecular aspects of cell cycle control in plant species share many features with the machinery present in yeast and animals (Mironov *et al*., 1999; Joubès *et al*., 2000; Stals *et al*., 2000; Renaudin *et al*., 1996). However, despite the many resemblances, several plant-specific gene class members have been isolated up till now, suggesting the existence of regulatory pathways which are exclusive for plant species. This is not really surprising, since plant cells have adapted to a non-motile life-style during their evolution, associated with physical limitations as a result of the presence of a rigid, surrounding cell wall.

At present, the knowledge of the molecular aspects of the cell cycle in plants is still very limited when compared to other multicellular organisms such as yeast and animals. The majority of the already characterized genes have been isolated by traditional molecular techniques. The study presented here aims to be a first step towards the extension of the already existing collection of characterized cell cycle regulated plant genes by means of a whole-genome expression analysis approach. Such a strategy is guaranteed to be successful, since it has been demonstrated by earlier studies that the occurrence of many cell cycle genes at specific moments during the cell cycle is regulated by means of differences in transcript levels (Dynlacht, 1997). Furthermore, results of micro-array studies of the cell cycle in yeast cells and human fibroblasts revealed the differential accumulation of approximately 10% of all transcripts tested (Spellman *et al*., 1998; Cho *et al*., 2001). As outlined in the previous chapter, we optimized the cDNA-AFLP technology to a *bona fide* method to perform whole-genome expression studies in plant species for which only little sequence information is available, such as tobacco. This was necessary in order to work with tobacco BY-2 suspension cells, which is the

most accurate model system in which good synchronization levels can be obtained. This chapter presents the results of the complete screening for cell cycle modulated gene expression during different phases of the division cycle of tobacco BY-2 cell suspensions. In this way, gene fragments corresponding to approximately 1500 genes were visualized which possess a cell cycle regulated expression profile, making up the first comprehensive collection of plant cell cycle modulated genes. A significant part of these transcripts seems to be derived from novel or yet uncharacterized genes. Among the annotated transcripts, many new members of known gene classes involved in the cell cycle could be identified, of which some might be the first isolated plant member. In addition, for many other fragments with homology to well-characterized gene classes, a connection to the cell cycle machinery could be established. In this way, a basis is provided to unravel the basic mechanisms underlying the plant cell cycle.

3.2. Experimental procedures

3.2.1. Maintenance of BY-2 cell cultures. Synchronization experiment and harvesting of samples

Cell suspensions of tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) were cultivated as recommended by Nagata and coworkers (1992). Synchronization was achieved using aphidicolin as a blocking drug. Here fore, a stationary culture of 7 days old was diluted 10 times in fresh medium containing aphidicolin (Sigma) at a total concentration of 5 mg/liter. After 24 hours- when the complete cell population is blocked at G1/S-transition- the cells were released by performing several washes with fresh medium and subsequently continued progress through the cell division cycle in a synchronized fashion.

Sampling of cells occurred each hour, starting at the release from the aphidicolin block (time point 0) until 11 hours later. Each time, 75 ml of cells was harvested, after which the medium was removed by filtration. Cells were rapidly frozen in liquid nitrogen and stored at –80^{0}C until further processing.

Some sub sampling was done in order to evaluate cell cycle progression and the degree of synchronization. By DAPI-staining of the DNA content (Sigma; 1mg/ml), the Mitotic Index (MI) could be determined through fluorescence microscopy by counting the number of cells that exhibit mitotic figures. Additionally, flow cytrometric analysis was performed on a representative sample of cell nuclei. Here fore, the cells were initially incubated in a buffered enzyme solution (2% cellulase and 0.1% pectolyase in 0.66M sorbitol) for 20 minutes at 37^{0}C. After washing and resuspension in Galbraith buffer (Galbraith *et al*., 1983), the suspension was filtered through a 30µm nylon mesh to purify the nuclei that were stained by the DAPI solution. Using a BRYTE HS flow

cytometer (Biorad), the fluorescence intensity of individual nuclei was determined.

3.2.2. Sample preparation for transcript profiling

The cDNA-AFLP-based transcript profiling method requires cDNA as an initial template. Here fore, total RNA was prepared from each sample by a protocol based on LiCl precipitation (Sambrook, 1988). In the next step, polyA+-RNA was extracted from 500µg of total RNA. This was achieved by the use of oligotex™ columns (Qiagen) according to the manufacturer's instructions.

First strand DNA synthesis was performed starting from 1 µg of polyA+-RNA by reverse transcription with a biotinylated oligo-dT_{25} primer (Genset) and the Superscript II reverse transcript transcriptase (Gibco-BRL). By strand displacement with the enzymes *E. coli* ligase, DNA polymerase (USB) and RnaseH (USB), the second strand was synthesized. Additional cleanup of the resulting cDNA from all the reagents used in the preparing synthesis steps was performed by elution through DNA spun columns (Amersham Pharmacia Biotech).

3.2.3. cDNA-AFLP-based transcript profiling

For AFLP analysis, 500 ng of double stranded cDNA was used as starting template as reported by Vos *et al.* (1995) and Bachem and coworkers (1996). The protocols of these authors were applied with some modifications. The restriction enzymes used were BstYI and MseI (New England Biolabs) in a digestion that was performed in 2 separate steps. After the first restriction digest with one of the enzymes, the fragments resulting from the 3' end of the cDNA were collected on Dynabeads (Dynal) by means of their biotinylated polyA+-tail, while the other fragments were washed away. After digestion with the second enzyme, the released restriction fragments were collected and used as templates in the later AFLP steps. The adapters and primers used are:

BstYI-adapter:	5' - CTCGTAGACTGCGTAGT - 3'
	5' - GATCACTACGCAGTCTAC - 3'
MseI-adapter:	5' - GACGATGAGTCCTGAG - 3'
	5' - TACTCAGGACTCAT - 3'
BstYI-primers:	5' - GACTGCGTAGTGATC(T/C)N_{1-2} - 3'
MseI-primers:	5' - GATGAGTCCTGAGTAA N_{1-2} - 3'

For preamplifications, a MseI-primer without selective nucleotides was combined with a BstYI-primer containing either a T or a C as 3'-most nucleotide. PCR conditions were applied as described (Vos *et al*., 1995). The obtained amplification mixtures were diluted 600-fold and 5 µl was used for selective amplifications using a P^{33}-labeled BstYI-primer and the Amplitaq-Gold polymerase (Roche) following the described procedure (Vos *et al*., 1995). Amplification products were separated on 5% polyacrylamide gels using the Sequigel system (Biorad). Sodium acetate (22mg/l) was added to the buffer in the bottom tank to prohibit a run-off of the smallest fragments. Vacuum dried gels were exposed to Kodak Biomax films and in an additional approach scanned in a Phosphor-Imager™ machine (Molecular Dynamics).

3.2.4. Quantitative analysis by means of the Quantar-Pro™ software

Scanned Molecular Dynamics gel images were quantitatively analyzed using the

AFLP-Quantar-Pro™ image analysis software (Keygene). This software has been designed for accurate lane definition, fragment detection and quantification of band intensities. All visible AFLP fragments were scored and individual band intensities were measured per lane. The obtained data were used to determine the quantitative expression profile of each transcript. Raw data were first corrected for differences in total lane intensities which may occur due to loading errors or differences in the efficiency of PCR amplification with a given primer combinations in one or more time points. The correction factors were calculated based on constant band profiles throughout the time course. For each primer combination, a minimum of 10 invariable bands was selected and the intensity values were summed per lane. Each of the summed values was divided by the maximal summed value resulting in the correction factors. Finally, all raw values generated by QuantarPro™ were divided by these correction factors.

Subsequently, each individual gene expression profile was variance-normalized by standard statistical approaches as previously used for the normalization of microarray-derived data (Tavazoie et al., 1999). For each transcript, the mean expression value across the time course was subtracted from every individual data point after which the obtained value was divided by the standard deviation. A coefficient of variation (CV) was calculated by dividing the standard deviation by this mean value. Ultimately, a threshold CV was appointed to establish a cut-off value in order to define the significance of differential expression.

3.2.5. Recuperation and reamplification of AFLP fragments and sequencing strategies

Bands corresponding to differentially expressed transcripts were cut out from the dried gel. Hereafter, the DNA was eluted by gentle resuspension in 150µl of TE buffer. A small aliquot (1-5 µl) of this suspension was used for reamplification by PCR under the same conditions as for selective amplification.

Sequence information was obtained by direct sequencing of the reamplified PCR-product. Alternatively, fragments were cloned in the pGEM-T Easy vector (Promega) after which plasmid DNA of a random, small group of individual clones was sequenced.

3.2.6. Homology searching and gene annotation

By removing the adapter sequences on both edges of the raw DNA sequence, the tags were ready to be subjected to homology searching for further characterization. Therefore, the obtained sequences were compared against all nucleotide and protein sequences present in the publicly available databases (DNA: EMBL; Genbank; EPD Eukaryo and Vectors – Protein: SWISS-PROT; PIR; GenPept; TREMBL and SPTREMBL). For algorithms, the BLAST sequence alignments by Altschul *et al.* (1997) were applied.

The complementary design of the cDNA-AFLP-based transcript profiling experiments has as a consequence that a given cell cycle modulated gene can become tagged twice. In order to remove all redundant sequences, all our sequences were subjected to

a so-called selfblast homology search. For this purpose, a database consisting of the available sequences of our isolated gene fragments was established after which a blastn-search with the Altschul *et al.* (1997) parameters was performed for each individual tag.

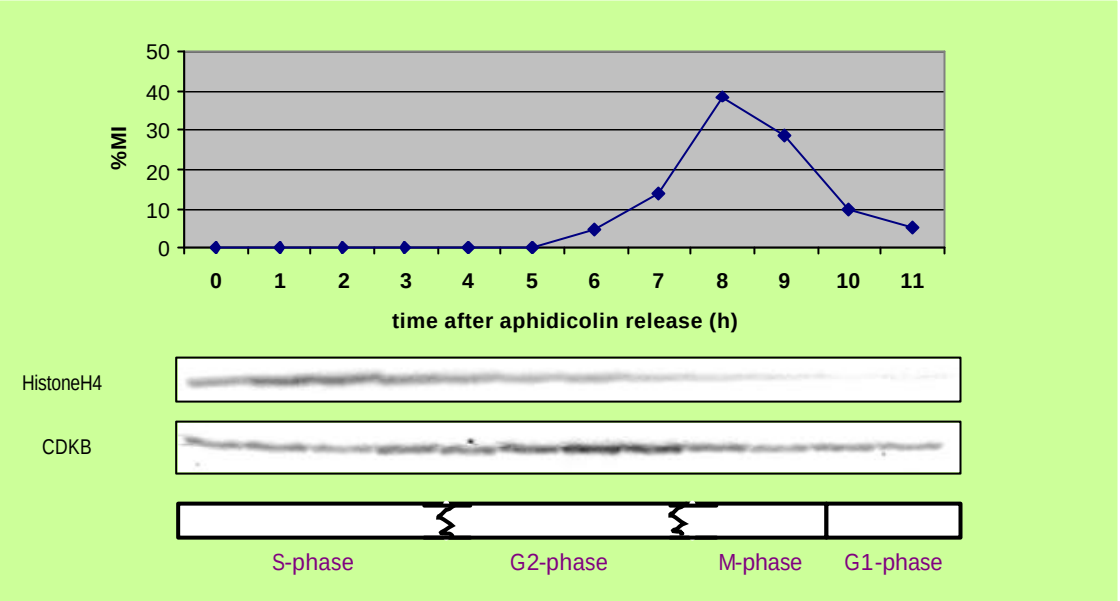


Figure 1: Definition of the different cell cycle phases in the harvested plant material used for the cDNA-AFLP expression profiling experiment
The graph represents the determination of the mitotic index (MI) by fluorescence microscopy. The mitotic peak is observed 8 hours after aphidicolin release. The expression patterns of well-known cell cycle markers (of which histone H4 and CDKB are shown here) additionally helped to determine the different phases

3.3. Results

3.3.1. Whole-genome expression profiling reveals the existence of several hundreds of genes with a cell cycle modulated expression pattern

In order to screen for cell cycle modulated gene expression, a synchronized tobacco BY-2 cell culture was established by arresting the cells for 24 hours with aphidicolin, a DNA-polymerase inhibiting drug. Sampling was done every hour after release of the cells from the blocking agent, starting from time point 0 until 11 hours after removal of the blocking agent, covering cell cycle progress from early S-phase until slightly past the M/G1 transition (figure 1). Further sampling, in order to obtain material covering the G1 phase and subsequent G1/S transition, was not done since the synchrony level of the cell culture becomes rapidly unacceptably low once the cells have passed the M-phase. By flow cytometry and microscopical determination of the mitotic index of the cells in all harvested samples, the peak of mitosis (i.c. 40%) was observed at 8 hours after release from aphidicolin. Exit from Sphase was estimated

approximately at time point 4, while G2/M transition is assumed to occur at time point 7.

The cDNA-AFLP-based expression profiling experiments were carried out in two separate screening rounds. In a first part of the experiments, abundant messengers, such as histones and tubulins, were detected by performing the selective amplifications using primers with 1 or 2 additional selective nucleotides. In a second round, we were able to detect the more scarce RNA messengers by the use of primers with 2 or 3 additional nucleotides. Among these transcript derived fragments, cyclins and CDKs could be isolated (see also section 3.3.4). Additionally, the experiments that used primers with 3 additional nucleotides were performed in a complementary fashion by using +1 selective nucleotide for the BstYI primer and +2 for the MseI counterpart and vice versa.

In summary, all possible primer combinations were tested and the resulting gels were scored both by eye and the Quantar Pro™ software package used for quantitative analysis. Only the significant differences occurring in at least two successive time points were considered for further analysis, since a differential band in one discrete point of the time course mostly corresponds to an artefact produced during the PCR amplification process. The Quantar-Pro™ software enabled us to calculate quantified levels of expression for a given gene in each sampled time point (see chapter 4 for more details). As a measure for differential expression, a statistical coefficient of variance (CV) was calculated for each expression profile. A CV value of 0.25 was determined to be the minimal threshold value. Profiles with a lower CV were considered to have a constitutive expression pattern. This resulted in the removal of 43 tags from the data set, which were previously scored by eye. In total, 2667 fragments were judged to exhibit a differential expression pattern during the sampled time course.

3.3.2. Characterization of the isolated cell cycle modulated transcript-derived fragments

All bands corresponding to differentially expressed genes were cut out from the dried gels for further characterization. For about 65% of the gene fragments that have been subjected to sequence analysis up till now, the direct sequencing approach of reamplified PCR products resulted in a sequence of good quality. As a consequence, the sequence of the remaining tags has to be retrieved by the sequencing of cloned fragments. Therefore, 6 individual clones were randomly selected. Mostly (in 70% of the cases), this results in the generation of different sequences corresponding to one fragment, thus requiring additional experiments to discover the true identity of these gene fragments. Only 43% of the cloned fragments have been characterized by a sequence yet (13% of the fragments could additionally be defined by the presence of a redundant complementary counterpart as indicated by a selfblast homology search of all sequenced fragments).

At present, we can conclude that for almost 70% of all detected differentially expressed genes, the sequence has been determined by the methods that were applied for sequencing. By performing a selfblast homology search, the redundancy among these sequences, as a consequence of the complimentary design of the majority of the AFLP experiments, could be eliminated. It is estimated that our AFLP tag

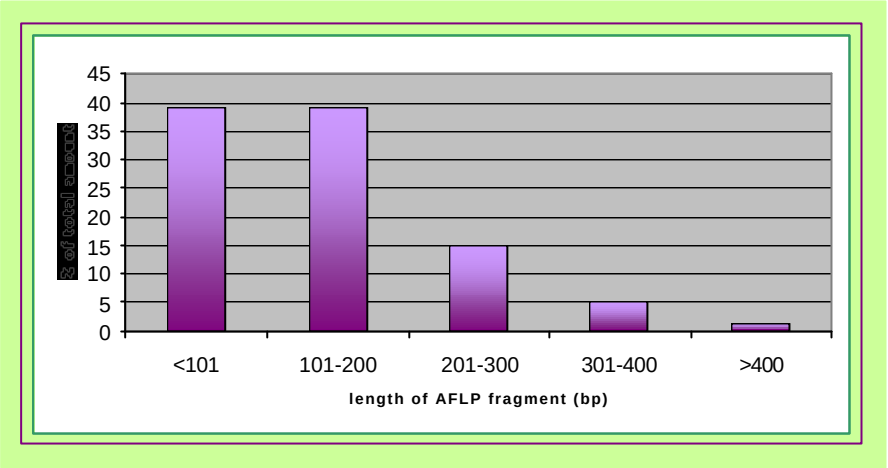


Figure 2: Length distribution of the isolated AFLP tags for which no functional annotation could be made by sequence homology searching by the blast algorithm

collection corresponds to approximately 1500 different genes.

Further characterization of the fragments was done by homology searching using the blast algorithm (Altschul *et al*., 1997). Therefore, the nucleotide sequence was compared with those present in DNA sequence databases (Blastn), with protein sequence databases (after translation in all 6 possible frames: Blastx) and with a translated sequence database in 6 frames (tBlastx). A significant similarity with genes with a characterized function was found for approximately 33% of the sequenced tags (a more detailed analysis is presented in section 3.3.4.). This means that for the majority of the sequences (67%), no functional annotation could be made. In 15% of the cases, significant matches were retrieved with a gene of unknown function, such as ESTs, cDNAs, genomic clones or so-called putative proteins. The EST and cDNA sequences are mostly derived from other *Solanaceae*, such as tomato and potato. The remaining 52% may represent true novel genes, since no significant homology or even no hits (14% of the cases) was found. However, this could be a consequence of the fact that the produced AFLP tags are too short (figure 2 shows the length distribution of all tags for which no functional annotation could be made). Another explanation that might contribute to this high level of unknown genes is the fact that the AFLP tags originate from the 3' end region of the transcripts. Tags that are derived from the 3' untranslated region of a gene might be too diverged to find homologous sequences.

By *in silico* analysis of AFLP fragments, it was revealed that the majority of the tags at least partially reside within the protein encoding regions (see chapter two). Furthermore, extrapolation of the results from the sequence analysis of 66 selected cDNA clones after screening of a tobacco cDNA library with some of our isolated AFLP fragments, indicates that 10 to 20% of all the cell cycle modulated transcript tags are possibly derived from novel genes (see chapter two).

The fact that only a small percentage of the tags corresponds to the 3' untranslated region, also means that those gene fragments, for which a highly significant homology with a

EST clone was observed, could be replaced by this EST sequence (when taking some severe criteria into account). Thus, the lack of sequence information for small tags could be partially compensated. Approximately 200 tags were substituted in this way. In general, the homology searches with cDNAs confirmed the results previously obtained with the AFLP tags. As a consequence, for the cases where the tags could not be annotated, the additional sequence information provided by the EST only resulted in new information and annotation for 10% of the cases, hence keeping the amount of uncharacterized genes in our screen high. Results, obtained with approximately 160 full-length sequences, which were picked up by screening a BY-2 cDNA library with isolated tags, also point out that many genes that were visualized in our cDNA-AFLP-based transcript profiling screen are novel. Here, around 40% of the full-length sequences, corresponding to AFLP tags that might be unknown due to a lack of sequence information, lead to the isolation of a homologous counterpart and subsequent functional annotation.

3.3.3. cDNA-AFLP-based transcript profiling allows the discrete detection of paralogues

One of the main characteristics of the cDNA-AFLP technology is its discriminatory strength. As previously mentioned in chapter 2, even if two genes are highly homologous and only slightly different in size, they are both to be separately detected by this technique. Regarding our screen for genes with a function in the plant cell cycle, this aspect is clearly demonstrated by the fact that many different paralogues of histones and tubulins were isolated in our approach, genes which are known to be encoded by multigene families (Snustad *et al.,* 1992; Chaubet and Gigot, 1998; McKinney and Meagher, 1998; Meshi *et al*., 2000). Figure 3 demonstrates the high similarity of some histone H2A and H2B homologous AFLP fragments by multiple alignment analysis. In summary, almost 50 different histone paralogues were found of all different subtypes (H1 (3 tags), H2A (9 tags), H2B (15 tags), H3 (15 tags) and H4(7 tags)) as well as 11 a-tubulins and 10 ß-tubulins.

3.3.4. An overview of the different functional groups of isolated cell cycle modulated gene fragments

By homology searching it was possible to determine the specific function of approximately 500 AFLP fragments that were judged to be cell cycle modulated in tobacco BY-2 cells. They were subdivided in several functional groups and will be discussed in the next sections of the text. Several additional members for already existing plant cell cycle gene classes were isolated. Furthermore, homologies to some genes that have not been isolated from plants before were found and suggest the existence of certain pathways in plants in analogy to other eukaryotic species. The related expression profiles of the tags presented here and the deduced significance of the corresponding genes in a certain phase or process during cell division will be discussed thoroughly in chapter four.

TABLE 1: Overview of AFLP fragments homologous to the core cell cycle machinery

Annotation	Tagname	Length (bp)	Significant homology to
A-type cyclins			
	Bstt3-11-120	54	Nicta;CycA1;1
	Bstc1-43-160	101	Nicta;CycA2;1
	Bstc1-44-180	129	Nicta;CycA3;1
	Bstt32-22-175	134	A2-type *L. esculentum*
	Bstc1-13-95	74	A2-type *L. esculentum*
B-type cyclins			
	Bstt14-41-125	70	Nicta;CycB1;2
	Bstt4-22-570	598	Nicta;CycB1;1
	MBT14-160	130	Nicta;CycB1;2 (E_{value} 1e-9)
	Bstt4-23-380	319	B2-type *L. esculentum*
	Bstt4-34-225	185	B2-type *L. esculentum*
	Bstc4-21-340	295	Clb1p yeast (28% homology)
D-type cyclins			
	Bstc21-4-220	160	Nicta;CycD2;1
CDKs			
	Bstt12-3-410	362	CDKB1;2 *N. tabacum*
	Bstt2-21-410	372	both CDKB1;1 and B1;2 over stretch of 90 bp (=low homology)
	MBC23-120	98	Medsa;CDKB1;1
	Bstt34-145	86	B2-type CDK *L. esculentum*
	Bstc2-31-138	86	CDKB1;2 *N. tabacum*
	Bstt2-42-225	198	CDKC-class alfalfa
Various			
	Bstc2-34-240	202	CDK5-interactor
	Bstt2-42-520	440	prohibitin
	Bstc21-24-265	**218**	MS5-like protein
	Bstt3-11-330	264	MS5-like protein (low homology)
	Bstt3-24-430	368	FtsJ
	Bstt3-12-150	110	tumor-related protein
	Bstt41-1-260	215	tumor-related protein
	Bstc33-2-200*	161	CCR4-associated protein
	Bstt23-3-400*	347	dual-specific phosphatase

***** are mentioned here because of their importance for cell cycle regulation, but are discussed in other parts of section 3.3.4. (respectively "transcription factors" and "signal transduction")

Core cell cycle machinery

Several tags corresponding to the basic components of the cell cycle regulation machinery were identified in our screen and are summarized in table 1. Two tobacco cyclinB1 homologues were detected, which previously have been isolated in BY-2 cell suspensions (Setiady *et al*., 1995; Qin *et al*., 1995). A third one (significantly homologous to *Nicta;CycB1;2*) is probably a new member of this gene class. Additionally, 2 homologues with the tomato cyclinB2 proteins published by Joubès *et al.* (2000a) were found. Concerning the A-type cyclins, the tobacco A1-, A2- and A3-types were visualized by the cDNA-AFLP method (Setiady *et al*., 1995; Reichheld *et al*., 1996), as well as 2 homologues with tomato cyclins of the A2-group (Joubès *et al*., 2000a). Furthermore, a tag was isolated, that shows homology with the yeast mitotic cyclin CLB1p. However, the expression pattern does not correspond to the G2-specific expression reported for the genes encoding these cyclins. Finally, the results of our experiments confirm the differential expression behavior of *cyclinD2;1* in BY-2 cell suspension, as reported by Sorrell and coworkers (1999).

We were able to detect 6 CDC2-kinase homologues. Five of them are situated in the plant-specific CDKB-class of cyclin-dependent kinases: The tobacco CDKB1 and –B2 subtypes were found in addition to some homologues to tomato and alfalfa CDKB1 cyclin-dependent kinases (Magyar *et al.,* 1997; Joubès *et al*., 1999; Sorrell *et al*., 2001). Moreover, a homologue to an alfalfa CDK of the C class was isolated (Magyar *et al*., 1997). This was unexpected, since the genes encoding cyclin-dependent kinases of this type, isolated in other plant species, do not have an oscillating expression profile during cell cycle progress (Joubès *et al*., 2000b).

Next to the basic components of the cell cycle control machinery discussed above, homologous tags to some related proteins were found. Significant homology was found to a CDK5-interactor binding protein of *Rattus norvegicus* and the *A. thaliana* homologue of this protein. However, it has not been demonstrated that the complex of this animal cyclin-dependent kinases with its interactor (p35) is implicated in basic cell cycle control (Nigg, 1995). Instead, the importance of this protein complex in differentiated vertebrate neuronal cells is well documented in literature (Homayouni and Curran, 2000). The homologous interactor-binding protein is known to become

phosphorylated by the CDK5/p35 complex, but in contrast it is not exclusively expressed in neuronal cells (Ching *et al*., 2000). Although present in plant species, a possible function for the CDK5-interactor binding protein homologues has not been postulated yet. Furthermore, a M-phase-specific tag has been isolated, which is homologous to prohibitin. In the mammalian cell cycle, this protein represses E2F-mediated transcription via interaction with the retinoblastoma protein, thereby blocking cellular proliferation (Wang *et al.,* 1999).

Finally, two homologous fragments to the "male sterility" proteins critical for male meiosis (*A. thaliana*) were found, one of which shows almost complete homology. These MS5 proteins belong to a small gene family highly conserved amongst plant species and are found to be significantly similar to a *Xenopus* Suc1/Cks type protein, which is involved in regulation of cyclin/CDK activity (Glover *et al*., 1998; Patra and Dunphy, 1996; Yang and Sundaresan, 2000) and consistent with the phenotype in the corresponding MS5 mutant where the microsporocytes undergo extra cytokinesis after meiosis I. However, our isolated tag does not show any similarity to such genes, but instead, a weak homology to a CDC16 protein homologue of human and *S. cerevisiae* has been found, which has been demonstrated to be a component of the anaphase promoting complex (Deshaies, 1997; Townsley and Ruderman, 1998). It is possible that the genes corresponding to these tags encode a component of the core cell cycle machinery but obviously, more sequence information has to be generated to annotate a clear function for these fragments.

DNA replication, modification and repair

As already mentioned earlier in this chapter, a big collection of histone genes accumulate temporally when BY-2 cell progress the S-phase of the cell division cycle, needed to wind up the freshly duplicated DNA. Several fragments derived from genes that encode proteins involved into chromatin assembly were isolated, such as an AFLP fragment encoding the anti-silencing function 1 (Bstt34-185) as well as some encoding the large subunit p150 of the human chromatin assembly factor 1 (Bstc4-11-150 and Bstt43-4-215; Quivy *et al.*, 2001). Four tags were found that match with genes encoding high mobility group proteins, known to interact with the nucleosomes after chromatin assembly has been finished (Bstc41-2-370, MBC42-280, Bstc41-1-470 and Bstt32-3-400). Next to these genes, other transcripts for essential components of the basic DNA replication apparatus were identified to be upregulated in this phase, including DNA polymerase a (Bstt44-3-230), replication protein A1 (Bstt4-43-220), PCNA (Bstt3-33-600), replication factor C (Bstt3-33-202) and a member of the replication origin activating proteins, MCM3 (Bstt31-1-110). Interestingly, we also identified a homologue to a bacterial replication initiating protein of *E. coli*, DnaA (Bstt2-23-420).

Several tags derived from genes of importance for DNA modification were detected to be differentially expressed as well. Among them, we distinguish a homologue to the cytosine-5-methyltransferase gene (Bstt42-3-170) and two tags similar to the gene encoding the enzyme S-adenosyl methionine (SAM; Bstc4-14-270 and Bstc4-21-270) synthetase that synthesizes its cofactor and . Five AFLP fragments were found, derived from methionine synthase genes providing the substrate for SAM synthetase (Bstc1-13-195, Bstc12-1-450, Bstc14-

2-190, Bstt3-33-200 and Bstt2-43-230). In addition, a tag was identified corresponding to a putative gene for a nucleotide binding protein that could also be involved in modification of the DNA (Bstc4-21-187).

Chromatin remodeling is an important aspect in the regulation of gene expression. It has already been demonstrated that some genes responsible for these processes are cell cycle regulated. Therefore, it is not surprising that tags corresponding to such genes were isolated in our screening. A histone deacetylase, a member of the SNF2 subfamily of chromodomain DNA-binding proteins and a chromodomain helicase were classified in this group (respectively Bstc2-32-145, Bstc23-2-163 and Bstt43-3-350). Interestingly, we were also able to visualize a tag corresponding to a mammalian inhibitor of growth 1 (p33-ING1 (Bstt12-3-155); Helbing *et al*., 1997). In humans, this protein has been demonstrated to have DNA-binding capacities and is suspected to have a role in chromatin-mediated transcriptional regulation during S-phase (Cheung and Li, 2001; Garkavtsev and Riabowol, 1997). This is in agreement with the expression profile observed in our screening. Moreover, yeast homologues of ING1 are reported to be components of the histone acetyltransferase complex and show similarity to the retinoblastoma binding protein 2 (Loewith *et al*., 2000). In addition, a fragment (Bstt2-23-274) homologous to the *Arabidopsis* WD-40 repeat protein MSI3 was isolated, belonging to a conserved family of WD-40 proteins, known to bind the Retinoblastoma protein in both animals and plants (Ach *et al*., 1998). They act to regulate histone acetylation and deacetylation (Ach *et al*., 1997).

Before the onset of mitosis, the replicated DNA needs to be checked and if appropriate, defects in the DNA structure need to be repaired. In this respect, transcripts of a homologue to a member of the RAD26 subfamily of SNF2 proteins, impaired in chromatin remodeling for the DNA repair process, accumulate in BY2-cells slightly before mitosis. (Bstt41-1-350) Furthermore, a putative DNA repair protein and a protein involved in DNA damage sensing were isolated. Four different transcripts, homologous to ribonucleotide reductase (RNR) genes were found, but accumulate at different moments during cell cycle progress (Bstc11-4-170, Bstc1-43-177, Bstt23-4-170, Bstt4-32-280). RNR proteins are able to convert ribonucleotides into deoxyribonucleotides and therefore are important for DNA-repair. Lastly, tags matching with the gene encoding the DNA topoisomerase II were found to have a peak in expression before mitosis (MBC41-120 and Bstt42-135). Although the functions of this kind of topoisomerase are very diverse and important in all aspects of DNA metabolism, these expression profiles suggest involvement in DNA repair process.

Cytoskeletal structure-related genes

Multiple paralogous genes encoding the basic constituents a- and ß-tubulins of the cytoskeleton were demonstrated to be highly cell cycle modulated in BY-2 cells (cfr. 3.3.3.). In contrast, no fluctuation in ?-tubulin transcript levels was found. However, in literature no indications are found that ?-tubulin genes might be differentially expressed during cell cycle progress (Stoppin-Mellet *et al*., 2000). Additionally, lamin expression was shown to have a fluctuating expression profile, next to three actin homologues and one actin-related protein. Twelve homologues to kinesin-like

proteins (KLPs) were identified. Among them, we found two gene fragments corresponding to the tobacco TKRP125, a plus-end directed motor, two tags matching with the *Kat* genes, that show minus-end directed motor activity and the phragmoplast-associated kinesin KRP1 (Vantard *et al*., 2000). The majority of the recognized KLP homologues are similar to putative kinesin-like proteins of *Arabidopsis*. A mammalian chromokinesin homologue was identified by our screening. Chromokinesins transport DNA as a cargo and play a role in chromosome segregation and metaphase alignment. The collection of motor proteins was further extended by three myosins. Two microtubuli-associated proteins were isolated, as well as a protein associated with the spindle pole body. One of the microtubuli-associated proteins, was already isolated from tobacco and serves to make cross bridges between microtubuli. The other one is homologous to a similar protein from *Drosophila*. Finally, two tags were identified with significant homology to so-called MFP attachment factors, which are responsible for the attachment of higher order chromatin to the nuclear matrix.

Two tags are derived from genes related to the formation of kinetochores, needed to attach the chromosomes to the mitotic spindle. One encodes a centromeric protein, whereas the other one is a gene for a SKP1-interacting factor. SKP1 is a constituent of the protein complex that makes up the kinetochore (Warburton, 2001).

To finish, one gene fragment shows significant homology *Arabidopsis* orthologue the *Vaccinia* virus complement control protein (VCP)/ the yeast CDC48 protein, an important factor for the execution of cytokinesis. In *Arabidopsis*, this protein localizes to the phragmoplast area and may function with KNOLLE in docking of vesicles to their target at the cell plate (Sylvester, 2000).

An overview of the isolated AFLP fragments with homology to the motor proteins and the other constituents of the cytoskeleton mentioned is given in table 2.

TABLE 2: Overview of the isolated AFLP fragments with an annotated function related to the cytoskeleton

Annotation Tagname	Significant homology to
Basic components*	
Bstc12-3-115	actin
Bstc2-32-250	toc64 (actin)
Bstc34-4-350	actin
Bstt21-1-270	actin
Bstt23-3-215	actin
Bstc1-23-200	lamin
Motor proteins	
Bstc4-12-160	kinesin-like protein
Bstc33-2-100	probable kinesin
Bstc1-32-250	kinesin-like *A. thaliana*
Bstc44-3-125	kinesin-like *A. thaliana*
MBC32-110	kinesin-like *A. thaliana*
Bstt12-1-230	kinesin-like *A. thaliana*
Bstt1-34-80	TKRP125
Bstt14-44-190	TKRP125
Bstc33-3-163	Kat-protein *A. thaliana*
Bstt24-4-200	Kat-protein *A. thaliana*
Bstt2-31-215	KRP-1 *A. thaliana*
Bstt34-3-470	chromokinesin
Bstt21-1-550	myosin heavy chain
Bstt42-4-240	myosin
Bstc34-3-263	myosin
Chromosome capture	
Bstc23-4-300	SKP1-interacting partner 6
MBT22-425	centromeric protein
Varia	
Bstt2-11-580	MT-associated protein
Bstt2-44-390	MT-associated protein
Bstc31-3-445	spindle pole body ass. protein
Bstc1-23-125	MFP attachment factor
Bstt34-3-315	MFP attachment factor
Bstt42-3-300	nucleolin
Bstt14-410	cdc48/VCP-like protein
Bstt44-2-320	T-complex 1 chaperonine

***** as a consequence of the multitude of AFLP fragments isolated, which are homologous to a and ß-tubulins (approx. 20), they are not included in this overview

Proteolysis

Protein degradation mediated by ubiquitin-dependent proteolysis is an important aspect in the destruction of important cell cycle regulators, such as cyclins, at two key events of the cell cycle, namely the exit from mitosis and transition to S-phase. Although there is little evidence for cell cycle modulated expression of the genes constituting the components of the

proteasome complex and ubiquitin, since they are also occupied with the degradation of proteins involved in cell cycle independent processes (del Pozo and Estelle, 2000), we found four tags encoding proteins of both the core structure and the regulatory subunit, as well as genes for oligopeptidases, ubiquitin, and some proteins involved in ubiquitin metabolism. Similar observations were made in the study of the human cell cycle, suggesting the fact that a separate proteolytic machinery is exclusively used for the degradation of cell cycle regulators (Cho *et al*., 2001). The expression profiles of all our isolated genes involved in ubiquitin-dependent protein degradation, suggest that they function in the exit from mitosis. Furthermore, five AFLP fragments derived from genes with homology to those encoding UBC (ubiquitin conjugating enzyme) were found. Here we see differences in expression patterns: a part peaks during mitosis, whereas some of them become expressed later. The tags of the latter group are homologous to UBC1 and –2 encoding genes, of which the tobacco counterparts are known to interact with G1/S phase transition protein 1 (Koyama *et al.*, 1999). Therefore, it might be likely that some of our isolated UBC homologues could be involved in the degradation of G1 phase regulators. In addition, a homologous AFLP fragment to an *Arabidopsis* protein with a F-box domain was detected. These kinds of proteins are known to be a part of the SCF (Jackson *et al*., 2000). The exact homologies of the identified fragments with a predicted function in ubiquitin-dependent proteolysis are summarized in table 3.

Other genes involved in protein degradation were also found to be differentially expressed during the cell cycle progress. They are mostly involved in common metabolic processes in the cell (see table 3). Interestingly, a tag was identified which is similar to an *Arabidopsis* homologue of the bacterial ClpP-protease of *Caulobacter crescentus*. This ATP-dependent protease is involved in the cell-cycle dependent degradation of CtrA, a response regulator with a pivotal role in orchestrating multiple cell cycle events in this bacterium (Jenal, 2000).

TABLE 3: AFLP tags involved in protein degradation

Annotation Tagname	Most significant homology
Ubiquitin-dependent proteolysis	
Bstt32-3-190	ubiquitin
Bstc31-1-280	put. ubiquitin carboxylase
Bstc34-2-155	UBC6 homologue
Bstt4-11-240	UBC1 and UBC2
Bstc4-23-270	UBC4 and UBC9
Bstc2-43-135	NtUBC1 and-2
Bstc2-14-130	NtUBC1 and –2
Bstc2-14-140	proteasomeA-type subunit
Bstc2-14-90	proteasome component
Bstt34-4-310	26S proteasome subunit-like prot.
Bstt3-44-170	proteasone regulatory subunit S2
Other proteolytic mechanisms	
Bstc13-2-290	serine protease
Bstt41-3-130	serine protease
Bstc3-34-140	subtilisin-like serine protease
Bstt41-260	LON2 protease
Bstt4-42-310	protease
Bstc34-2-155	GUT8-28 protein tobacco
Bstc1-21-190	cathepsin B-like cysteine protease
Bstc2-32-155	oligopeptidase
Bstc2-42-280	oligopeptidase
Bstc2-23-235	caseinolytic protease
Bstc4-11-260	glutamyl amino peptidase

Hormone response

Although it is known that auxin together with cytokinin play an essential role in the progress from plant cells at both G2/M and G1/S transitions and influence the expression of cell cycle genes such as A-type CDKs and D3-type cyclins (Stals and Inzé, 2001), there were no data about the cell cycle modulated expression of auxin-induced genes. In our approach, we were able to detect differences in the expression pattern of a number of auxin-induced genes.

A first group of genes is induced at the moment that cell are released into S-phase, and

consists of the *parA*, *parB* and *parC* genes, encoding auxin-inducible and/or -binding gluthatione-S-transferases (respectively Bstt14-1-205, Bstt2-24-190 and Bstt1-34-340). However, most often the expression of such genes is observed under stress conditions (Abel and Theologis, 1996). Combined with the rapid accumulation of their transcripts after release of the aphidicolin block, this might indicate a stress reaction rather than a cell cycle dependent auxin response. Only the parB gene has been truly demonstrated to be implicated in the cell cycle. In tobacco BY-2 suspensions, it has been shown that gluthationine and associated parB expression are necessary for the cells to pass G1/S transition (Vernoux *et al*., 2000). Apart from this link, there is no evidence for a role of the *par* genes in cell division.

More interesting is the second group of gene fragments, which accumulate around G2/M transition and of which some are impaired in the auxin signaling pathway. A homologue to an AUX1-like permease was found (Bstt33-215), which serves for the import of IAA into the plant cell, next to a member of the early auxin response AUX/IAA gene family (Bstc14-3-155) and a tobacco gene identified in a screen for auxin-induced genes (Bstt43-2-180). Interestingly, a homologue to the gene coding for the auxin response factor 1 (ARF1; Bstt14-1-420) was isolated, known to bind a consensus sequence (the auxin response element) in promoters of genes and to interact with members of the AUX/IAA protein family (Walker and Estelle, 1998). Additionally, we found an *AX22* gene homologue, encoding an IAA-induced transcription factor, which is known to interact with ARF proteins (Bstt41-3-160). Finally, one tag was found to be homologous to an IAA-alanine resistance protein (IAR1; Bstc21-100), an integral membrane protein with a role in both auxin response and metabolism. To conclude, these results obtained in our screening suggest the existence of a cell cycle regulated signal pathway linking auxin responses to the basic cell cycle machinery.

Another gene fragment that was isolated is homologous to an abscissic acid upregulated gene. This hormone is a known antagonist of cell cycle progression (Stals and Inzé, 2001; Bstc14-2-102).

Stress and cell death

During cell cycle progress of our sampled BY-2 suspension, a group of genes known to be involved in stress and cell death were tagged for differential expression. The group of stress-related protein homologues includes several proteins involved in oxidative stress responses, heat shock proteins, metallothoneins and factors involved in responses induced as a consequence of ethylene accumulation. Possibly, a part of them is induced as a result of the blocking by aphidicolin.

Two gene fragments were identified with a significant homology to mammalian genes, which encode elements of the tumor necrosis factor (TNF) signaling complex. One of them shows homology to a gene encoding a TRAF-interacting protein (TRIP; Bstc4-12-245). TRIP was first acknowledged as a signaling component of the TNFR super family, which promotes cell death when complexed with TRAF (Lee *et al*., 1997). The second AFLP tag (and substituting EST and full length sequence) shows homology to the RING-finger domain of the inhibitor of apoptosis proteins (IAPs; Bstt4-34-240). In literature, it has been proposed that IAPs

are important for the prevention of TRAF-mediated cell death when recruited to the TNFR-TRAF complex (Lee *et al*., 1997). These AFLP tags are intriguing candidates for a further characterization in order to reveal the true nature of their function in plants. Their induced expression during S-phase could possibly indicate the existence of a signaling pathway, which rather stimulates cell division instead of cell death.

Signal transduction

Considering the multitude of processes involved in cell cycle control and the basic mechanisms of DNA replication, mitosis and subsequent cytokinesis, it is no surprise that a large collection of signal transduction components were found, such as receptors and associated proteins and kinases/phosphatases.

Intriguingly, among the isolated phosphatases, there is one AFLP fragment, peaking in mitosis, with a significant homology to a dual-specific phosphatase (Bstt23-3-400). In animals and yeast, a phosphatase of this type (CDC25) is important for the regulation of the activity of cyclin/CDK complexes essential for cell cycle progression. Furthermore, two gene fragments similar to serine/threonine protein phosphatase PP2A were isolated, which are important for the dephosphorylation of targets of cyclin/CDK complexes (Bstt33-42-370 and Bstt3-23-125). In addition, we were able to identify a phosphatase 2A inhibitor (Bstc2-21-175). In the large group of kinases, which were picked up by our screen, four were found with a potential function in cell cycle progress. The first one is a homologue to *ayk1*, a mammalian gene related to the *Drosophila* AURORA centrosome separation kinase, crucial for centrosome separation and chromosome segregation (Bischoff and Plowman, 1999; MBC42-90). Another tag is weakly homologous to another kinase of the AURORA family, ARK2 (Bstt32-4-500). A third fragment has homology with a myosin heavy chain kinase of *Dictostelium* (Bstc3-41-360). The last one is derived from a tobacco gene for MAPKK. This protein becomes phosphorylated by the MAPKKK NPK1, which has been demonstrated to have a role in cell plate formation during plant cytokines is, most likely in the outward redistribution of phragmoplast microtubules (Bstc23-4-160; Nishihama *et al.,* 2001).

Interestingly, three homologues to ankyrin proteins were found to have a cell cycle modulated expression pattern (Bstt3-23-140, Bstt3-42-175 and Bstt33-2-222). Ankyrins constitute a family of large, membrane-associated proteins that mediate the linkage of the cytoskeleton to a variety of membrane transport and receptor proteins. Furthermore, The conserved domain of ankyrin-repeats has been identified in proteins involved with cell cycle control (Devarajan *et al*., 1996; Bennett and Chen, 2001).

Transcription factors

Several tags were identified that are derived from genes encoding transcription factors of different well-known families in plants, such as AP2, bZIP, MADS and HTH and C2H2 Zn-finger. Additionally, some gene fragments were scored which have homology to transcription factors linked to specific cell cycle processes and the regulation machinery. Three tags were found to be homologous to MYB transcription factors, of which one belongs to a specific class named 3R-MYB (Bstt14-3-210; Stracke *et al*., 2001). These

type of Myb proteins are more related to the mammalian c-Myb-like oncogenes, known to be involved in the regulation of CDC2 at G1/S transition, than to other plant Myb proteins. Recently, it has been shown by Ito and coworkers (2001) that a 3R-MYB is involved in the activation of B-type cyclins and other proteins, including kinesins, through a conserved M-phase specific activator motif in their promoter sequence. Another fragment shows similarity to CCR4 associated-like proteins (CAF; Bstc33-2-200). This protein forms a complex together with the CCR4 and DBF2 proteins, resulting in a transcriptional activator, which in yeast has been demonstrated to be impaired in G1/S transition (Liu *et al.*, 1997). A homologue to a DIP protein of *A. thaliana* was identified as well (Bstt43-3-540). These kind of proteins seem to interact with the PARP Zn finger protein, which is implicated in DNA repair and chromatin structure modulation (Storozhenko *et al*., 2001). Finally, a full-length sequence of an AFLP fragment revealed that it corresponds to a gene encoding a Myc-like protein (Bstt21-4-270). In mammalians, this transcription factor is an important element in the promotion of cell proliferation: When adding growth factor to quiescent cells, a pathway through Myc, working parallel of Rb and E2F is induced, leading to G1/S transition (Bartek and Lucas, 2001). In addition, we found a sf21 homologue, which is a downstream target gene of Myc (Bstt2-21-305).

Cell wall synthesis

Almost 30 gene fragments were identified, for which further characterization showed that they are derived from genes involved in cell wall synthesis or modification. In general, their expression levels peak at the end of mitosis, as expected.

Common metabolism, basic transcriptional, RNA processing and translational processes

A significant amount of the tags showing oscillations in expression during cell cycle progress, match with genes coding for enzymes involved in the basic metabolism of the cell. This is quite evident, since the formation of two daughter cells requires cell growth and thus the synthesis of new molecules. Approximately sixty fragments are derived from genes encoding enzymes occupied with the synthesis and breakdown of sugars, lipids and other household molecules. Furthermore, several proteins were found to be impaired with secretion and transport of material in the cell. Among them, we found some homologues to proteins involved in intracellular vesicle flow and the transport of membrane proteins, such as the signal recognition particle (SRP) receptor, a bacterial homologue of the SRP (FtsX), ß- and ?-subunits of COP, Sec61p and PITP. Another 65 homologues to basic constituents of RNA synthesis, -processing and translation were identified. Remarkable is the fact that a large assortment of ribosomal proteins, belonging to approximately 20 subfamilies, seems to be cell cycle modulated. In addition, the S-phase specific expression of the cyc07 protein, previously reported in *Catharantus roseus* cell suspensions (Bstt21-2-300; Ito *et al*., 1991) and involved in protein synthesis at the level of the ribosome, was confirmed in our experiments. For some of the isolated ribosomal protein, like L9 and L27a, transcriptional regulation has been demonstrated in plants (Lee *et al*., 1999; Moran,

2000). Moreover, the transcript levels of these genes are especially high in dividing cells or meristems and low in differentiated tissues. Our results could indicate that this cell growth induced transcriptional activation of ribosomal proteins can be extended to other types of ribosomal proteins. Moreover, it might be possible that the combination of the high amount of ribosomal transcripts in combination with those related to the translational machinery mirrors the regulation of translational processes common in mammals. In these species, it has been known already for quite some time that stimulation of mitogenesis leads to the phosphorylation of the ribosomal protein S6 through the action of c-Myc (Jefferies and Thomas, 1995; Prober and Edgar, 2001). This protein plays an important role in the selective translation of mRNAs that possess a 5' terminal oligopyrimidine tract (5'TOP). This type of mRNAs encodes many components of the translational machinery, such as the ribosomal proteins. A similar pathway might be active in plants, since in *Arabidopsis* cell cultures, auxins and cytokinins lead to the phosphorylation of S6 (Bailey-Serres, 1999). Our results could indicate the additional involvement of transcriptional regulation in this process.

Lastly, some interesting homologues to genes involved in RNA-processing of other eukaryotes were found. A gene tag corresponds to the HuB and –C members of the mammalian ELAV family of RNA-binding proteins involved in alternative splicing in neuronal cells (Bstc13-1-190). Studies indicated that mutations in these genes lead to encephalomyelitis and diseases associated with tumor development. In tumors, a heightened transcript level of these genes was observed (Posner and Dalmau, 1997). It was found that these proteins bind to both AU-rich elements in the 3' UTR and the poly-A tail of some RNA-species of genes, which regulate cell proliferation, such as c-Fos and c-Myc. By this mechanism, selective RNA-degradation is achieved (Akamatsu *et al*., 1999; Kasashima *et al*., 1999). Dominant-negative mutations block differentiation of neurons and ultimately lead to neoplastic tumor formation. Another tag shows homology to a *Drosophila* gene encoding the *pitchoune* DEAD-box helicase (Bstt4-34-280). Recently, this helicase has been shown to be involved in the genetic control of cell size and -form as a downstream target of Myc (Zaffran *et al*., 1998; Stocker and Hafen, 2000).

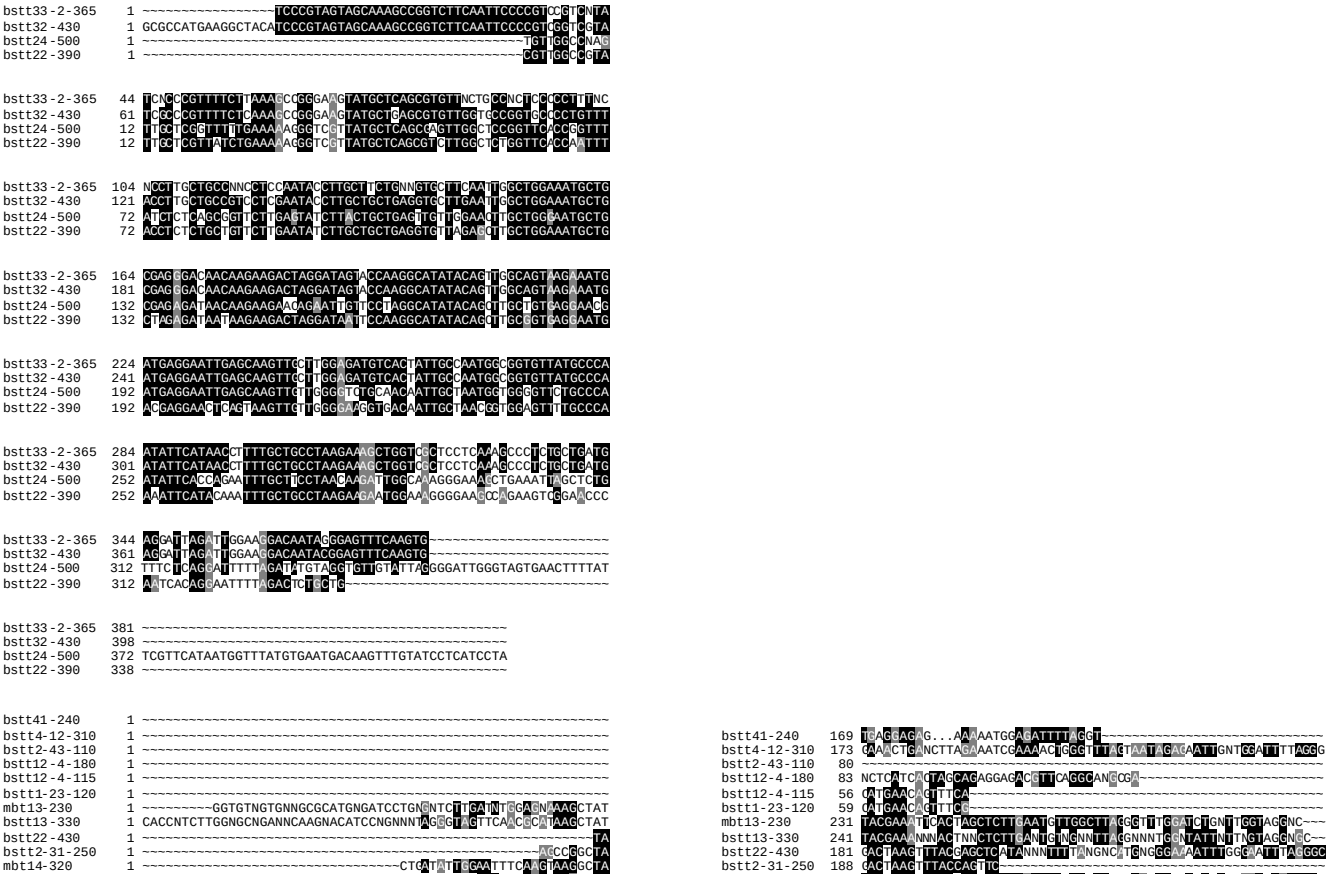


Figure 3: The identification of cell cycle modulated, paralogous genes by cDNA-AFLP transcript profiling

The upper part of this figure represents the alignment of four isolated AFLP tags, which were found to be homologous to histone H2A. The lower part shows the alignment of 13 tags homologous to histone H2B. As indicated by the dark boxes, corresponding to identical (black) or similar (grey) nucleotides, these tags are highly paralogues. This demonstrates the fact that by the cDNA-AFLP technique large gene families can be seperately visualized by small differences in their length

3.4. Discussion

By choosing an approach in which the use of synchronized tobacco BY-2 suspension cells is combined with the cDNA-AFLP-based transcript profiling technique, we were able to build the first comprehensive inventory of plant genes with cell cycle modulated expression. Our collection comprises approximately 1500 unique transcript fragments corresponding to genes with fluctuating expression levels during cell cycle progress. One third of the tagged fragments show significant homology to genes with a known function. Among these, we find many components of processes that are related to the execution of cell division in eukaryotes. Some of them have not been isolated in plants before and therefore are interesting candidates for further characterization.

When comparing the size of our collection (1500 genes) to the results obtained in the cell cycle screenings in yeast (800 genes, Spellman *et al.*, 1998) and human (700 genes, Cho *et al*., 2001), it might appear like that specific processes during plant cell division require many additional genes. Probably this is not the case, since for a part (20%), no *Arabidopsis* homologue could be found. Therefore, it is necessary to realize that a significant part of the gene fragments which show a differential expression pattern during cell cycle progress, might not be part of the essential, conserved processes of the plant cell cycle, but are only distantly related and are involved in more species-specific processes. In addition, the fact that tobacco is an amphidiploid plant species, most likely also results in the isolation of more than one orthologue for the same gene, hereby reducing the size of our isolated gene collection to a certain degree.

As cDNA-AFLP is not reliant on any prior sequence information, it makes it ideally suited for the discovery of new genes. The obtained results of our screening clearly reflect this statement, since 65% of the AFLP fragments shows no homology at all with a known sequence or does match with a sequence without an assigned function. This might be due to the fact that the produced fragments are too short to reveal any significant homology. However, preliminary data obtained with isolated full-length cDNAs showed that around 20% of the genes remain novel. Most interestingly, as will be highlighted in the next chapter, the majority of these tags are upregulated during M-phase. As the principal differences in cell cycle events occur during mitosis, it is tempting to speculate that several of these transcripts correspond to as yet uncharacterized plant-specific genes which trigger these particular mitotic events.

In our setup, we were able to detect all the core cell cycle regulatory genes, which were reported in literature to have a cell cycle dependent expression behavior. The only exceptions are the regulators of G1/S transition, such as the Rb protein and the E2F transcription factor. This is most probably due to the fact that our sampling did not cover the G1-phase of the tobacco BY-2 cell cycle. Further support for this hypothesis is provided by the finding that transcripts generated by early targets of E2F are already present at high levels in the beginning of

the time course. Further studies, using cells which have been blocked in M-phase for synchronization, will be needed to screen for cell cycle modulated expression in order to identify the plant regulators of G1/S transition.

The cDNA-AFLP-based transcript profiling proved to be sensitive enough to detect scarcely expressed genes, such as transcription factors. Among these classes of genes, we found homologues to 3R-MYB, which recently have been shown to be key factors in the transcriptional control of some cell cycle regulators (Ito *et al*., 2001). Another potentially interesting candidate to be a cell cycle regulator in plants is the corresponding homology of one of the tags with a dual-specificity phosphatase in *Arabidopsis*. CDC25, which has an important role in the activation of cyclin/CDK complexes in animals and yeast, belongs to this type of proteins. To date, no plant orthologue has been found, although experimental evidence suggest the necessity of dephosphorylation on Thr/Tyr residues in order to activate cyclin-CDK complexes. However, our gene doesn't show any homology at all with *cdc25* genes and is found to accumulate after G2/M transition. Even though the phosphatase identified in our screen is most probably not the plant CDC25 orthologue, it still might be involved in the control of the cell cycle in another phase.

Some findings were quite unexpected and shed new light on the execution of the plant cell cycle. The identification of some transcript tags with homology to some animal genes involved in regulatory networks controlling cell growth and/or apoptosis (ING1, IAP, TRIP and *pitchoune*), indicate that comparable pathways trigger these processes in plants. Especially ING1 is intriguing, since in mammalians it has been demonstrated that this protein has many functional properties in common with the tumor suppressor p53 and both are known to cooperate in a number of processes such as cell cycle arrest and apoptosis (Garkavtsev *et al*., 1998). Plant homologues of p53 haven not been identified yet and although ING1 can function independently of p53, it will be interesting to identify possible interactors of this plant homologue.

Some other novel information was retrieved from the fact that several genes induced by the plant hormone auxin show a cell cycle modulated expression profile. Some of them encode short-lived proteins involved in transcriptional regulation (Walker and Estelle, 1998). The specific accumulation of these transcripts at certain moments during cell division further strengthens the idea that auxin has an important function in cell division.

Taken together, the data presented in this chapter confirm earlier reported observations on the plant cell cycle, while some novel findings were done. A more thorough characterization of the transcript tags matching with putative novel genes or genes with unknown function will further allow the unraveling of control mechanisms and regulatory networks underlying the plant cell cycle.

Acknowledgments

The enormous amount of sequencing reactions were performed by Caroline Buysschaert, Rebecca Declercq, Jan Gielen and Raimundo 'Ninootje' Villarroel, who are greatly appreciated for their continuous working efforts during the past 2 years. Klaas Vandepoele, Jeroen Raes and Stephane Rombauts helped us a great deal with their bioinformatical expertise in interpreting some sequencing data. Rudy Vanderhaeghen, Raimundo Villarroel and Bernard Cannoot invested a lot in trying to retrieve some full-length sequences, hereby providing us with additional, useful information. Finally, the stimulating discussions with the 'experts' Lieven De Veylder, Frank Van Breusegem, Gilbert Engler and Dirk Inzé were a good guide to find our way in the resulting gene collection.

Chapter Four

Analysis of the importance of transcriptional regulation for plant cell division through clustering of cell cycle modulated gene expression data

Abstract

The comprehensive catalog of tobacco BY-2 genes, which showed significant differential expression behavior upon cell cycle progress, was analyzed in more detail by means of clustering of the quantified expression data for these gene fragments. The results obtained by cluster analysis strongly underline the importance of transcriptional regulation for the execution of mitosis and subsequent cytokinesis. Moreover, progress through M-phase seems to involve many unknown genes and processes, which could be specific for plants. In addition, the expression of some functional gene classes involved in important cell cycle processes was detected to be specific for some cell cycle phases. Comparison of the expression profiles of known cell cycle genes reveal that in general, they are alike their counterparts in yeast and human. However, some clear differences were observed. On the way towards a more thorough understanding of the plant cell cycle, the global expression analysis of the cell division regulated transcriptome in BY-2 cells opens intriguing perspectives for further elucidation of both conserved and more plant-specific pathways essential for proper cell propagation.

The results of this chapter will be submitted to **Nature Genetics** by **Breyne*, Dreesen*, De Veylder, Van Breusegem, Callewaert, Vandepoele, Rombauts, Raes, Engler, Inzé, and Zabeau** under the title **"Functional analysis of the transcriptome during cell division in plants"**

***** these authors contributed equally to this work

A part of the results was orally presented at the VIB seminar, March 2001

4.1. Introduction

The cell division cycle represents a collection of processes of fundamental importance for growth and persistence of all living beings. Mechanisms for both the correct execution and follow-up of all essential events are highly conserved among higher eukaryotes and can be regulated in different ways (Amon, 1998; Leatherwood, 1998). In this respect, many critical aspects are known to be governed at the level of transcriptional activity of genes with a key function. As a consequence, many genes have been reported to express fluctuating amounts of mRNA accumulating at specific moments during cell cycle progress (Dynlacht, 1997).

The availability of high-throughput technologies to study biological processes on a whole-genome scale created the chance to perform landmark gene expression studies on cell division. Very recently, reports have been published describing cell cycle-regulated gene expression in bacteria, yeast and human (Spellman *et al.*, 1998; Laub *et al.*, 2000; Cho *et al.*, 2001). In order to create a comprehensive catalog of gene expression profiles in relation to the cell division process in plants, we performed a whole-genome expression analysis study using tobacco BY-2 cell suspensions as a model system. This resulted in a collection of approximately 1500 gene fragments with significant periodical variation in their transcript levels during cell cycle progression (as described in Chapter three).

In order to gain more insights in the different processes of importance for plant cell division, which are either highly similar to other eukaryotes or more specific for the plant kingdom, we performed a cluster analysis on our data set. The major objective of this approach was to detect groups with highly similar expression profiles, since co-expression of genes is a strong indication of their involvement in the same processes. By these means, we classified all our gene fragments according to their expression profiles and were able to perform a systematic analysis of functional gene groups in relation to the plant cell cycle. The obtained results are presented and thoroughly discussed in this chapter. Moreover, comparisons are made with the observations in whole-genome studies performed in other eukaryotes.

4.2. Experimental procedures

4.2.1. Set-up and execution of the whole-genome expression analysis experiment

Experiments were performed on a tobacco BY-2 cell suspension culture, synchronized by blocking with aphidicolin at G1/S transition. The culturing conditions of these cells,

as well as the synchronization protocol are described in the experimental procedures sections of both Chapter two and three. The preparation of the cDNA template and the cDNA-AFLP-based transcription technique are thoroughly described in Chapter two (sections 2.2.3 and 2.2.4). For protocols concerning the recuperation of AFLP fragments, its sequencing and characterization by homology searching, the reader is referred to Chapter three, sections 3.2.5 and 3.2.6.

4.2.2. Quantitative measurements of the expression profiles and data analysis

Dried cDNA-AFLP gels were scanned by means of the Phosphor Imager™ technology (Molecular Dynamics). Subsequent quantitation of the expression profiles was done using the Quantar-Pro™ image analysis software (Keygene). By this procedure all visible AFLP fragments were scored and individual band intensities were measured per gel lane. In this way, the quantitative expression profile of each transcript could be determined. Raw data were corrected to eliminate differences in total lane intensities, after which each individual gene expression profile was variance normalized (according to Tavazoie *et al.*, 1999; more detailed instructions are provided in sections 2.2.6 and 3.2.4).

4.2.3. Cluster analysis

Cluster analysis of the expression data was done by two methods. In the first method, a hierarchical, average linkage clustering was performed using the Cluster software developed by Michael Eisen and coworkers (1998). For the visualization of the results, the Treeview software (Eisen *et al.*, 1998) was applied. Both software packages (if to be used for scientific purposes) can be downloaded for free at http://www.microarrays.org/software.html. The resulting cluster shows a hierarchical tree, in which each line represents a gene. By clicking on a node, the corresponding cluster can be viewed in more detail. The expression values are presented with color-codes for each measured time point, where bright red stands for the peak expression value and bright green stands for the lowest expression value. Intermediate values are presented as mixtures of either green or red with black. Black is considered to be the mean expression value of a given expression profile. Missing data are represented in gray. The second method is called Adaptive quality-based clustering (De Smet *et al.*, submitted) and is accessible for use on http://www.esat.kuleuven.ac.be/~thijs/Work/Clustering.html. As an output, genes are grouped in different clusters and are presented in graphs where their average expression pattern is shown in function of time. Both methods require the input expression data to be submitted in a text-format (.txt), where a given row indicates data for one single gene and each column represents an expression value for a time point.

4.2.4. Statistical analysis

Almost 500 gene fragments, for which a clear annotation could be made, were categorized in 16 functional classes by reviewing information from Medline and other publicly available information sources. If the published information strongly supported the placement of a gene into a category, this assignment was made. Gene fragments for which no annotation

was possible (because of no significant homology with any gene in the screened databases by Blast analysis) were classified under the term 'unknown'. To test for significant over-representation of genes of a given functional class in a certain phase of the cell division cycle, the binomial distribution function was applied. A *P* value was calculated based on the frequency of occurrence of genes with a biological function in each phase. If the number of genes with an expression profile peaking in a given cell cycle phase is less or equal to the number expected by random chance (as was the case for all our functional classes), then the *P* value was calculated using the formula:

$$P=[n!/(n-x)!x!)]p^{x}(1-p)^{n-x}$$

where n is the number of genes in a given phase, x the observed number of genes found to peak in that phase and a certain functional category and p the overall frequency at which genes of a given functional category are found in the genes represented in our study.

TABLE1: Biological function enrichments in cell-cycle-regulated expression clusters

Biological function	S-phase (361)	G2-phase (263)	G2/M transition (183)	M-phase (473)	Early G1-phase (73)
DNA replication (59)	**97.05090366**	0.502633519	2.299905112	0.431312297	-
DNA modification and repair (28)	4.945142134	**11.97669802**	**11.95928267**	**7.42048656**	3.465555678
cytoskeleton (45)	2.150196588	**7.543381422**	**27.13586114**	**20.75812185**	1.084013674
signal transduction (43)	**16.80127372**	3.897975655	**9.340062892**	**22.48902581**	2.95502132
excretion/vesicle transport (17)	2.931791208	1.482432422	1.482432422	**12.51411173**	6.173574252
transport of molecules (17)	2.931791208	1.482432422	5.276573522	**8.219037677**	**13.91219933**
proteolysis (10)	-	-	6.661487925	**14.76795024**	-
proteolysis-ubiquitin-mediated (12)	1.629352804	3.58709066	1.925598383	**14.76795024**	1.964008555
stress (28)	**15.93144773**	3.721992672	2.502191575	**18.42445128**	1.564573787
transcription factors (22)	3.756930732	**17.02614841**	**9.089113989**	5.070225749	1.667136578
core cell cycle machinery (30)	6.020750988	**14.46246084**	**13.44436352**	**8.449866521**	1.535479587
hormone response (8)	6.355677656	2.099951595	2.099951595	6.355677656	-
cell wall synthesis (33)	4.351254226	**8.390267515**	**9.128132798**	**26.63628589**	-
common metabolism (56)	**10.19204263**	**7.790280414**	2.31472825	**29.5724827**	**14.30602983**
RNAprocessing (18)	1.173999412	1.291093391	**7.322331013**	**18.38665785**	3.822260758
translation (47)	**18.11810532**	6.023586442	6.023586442	**25.56837743**	3.925249243
unknown (880)	**11.52870829**	**15.47755577**	3.655607726	**17.51570016**	1.199282922

Enrichments are calculated based on the binomial distribution function in sets of cell-cycle regulated expression clusters. The total number of genes in each functional category and in each temporal expression group is shown in parentheses. Negative logarithms of P values are listed. P values less than $1x10^{-7}$ are considered to indicate significant enrichment and therefore are represented in bold.

4.3. Results

4.3.1. General results of the cluster analysis

Screening for cell cycle modulated gene expression in tobacco BY-2 cell cultured yielded a collection of 2667 genes with a significant fluctuating expression profile during cell cycle progression from G1/S to early G1 phase (12 time points). From this initial dataset, 2440 quantified expression profiles were subjected to clustering. 227 expression profiles were excluded because of their insufficient quality for cluster analysis (approximately 50 had missing data for more than 3 time points) or the fact that they are derived from the same gene (this is related to the complementary nature of our experiments, see also section 3.3.2).

In a first approach hierarchical, average linkage clustering was done according to Eisen *et al.* (1998). Because of the large amount of data,

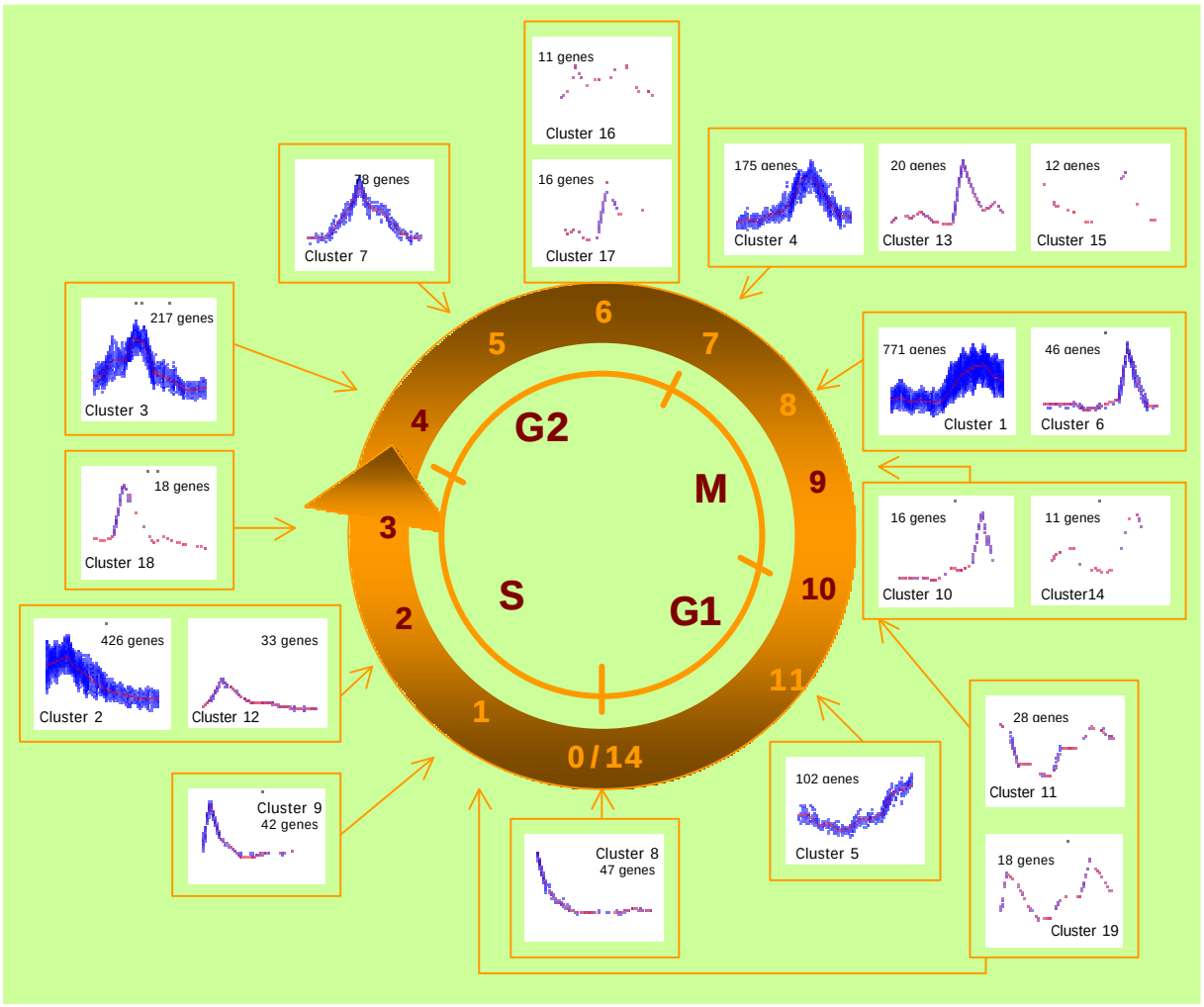


Figure 1: Overview of the results obtained with the adaptive quality based clustering approach
The 19 biggest clusters were shown (out of 58), representing approximately 85.5% of all clustered profiles

it is not possible to insert a comprehensible overview picture of the result in the text. Therefore, the result is available on the CD-ROM presented together with this thesis for a more close inspection of the resulting cluster (file clusterallgenes.cdt to be opened with the treeview software provided) . The more close the genes are sorted to each other, the more similar their expression pattern is and by selecting different subnodes of the cluster, groups of co-expressed genes can be assigned. Inspection of the resulting cluster reveals that the majority of the genes becomes expressed during M-phase. Another large group shows S-phase-specific expression behavior.

Additionally, a second clustering method was applied on our dataset, named adaptive quality-based clustering (De Smet *et al.*, submitted). When compared to the clustering algorithm of Eisen and coworkers (1998), this method clearly defines separate groups of co-expressed genes in such a way that only the significantly co-expressed genes are included. Unlike the similar K-means clustering method, one does not need to specify the number of groups *a priori*. As a result, certain specific expression patterns are easily discriminated. When applying a minimum of 5 genes in one cluster, 37 clusters were generated, excluding approximately 60 expression profiles. With a minimum limit of 2 genes, all 2440 genes became sorted into 58 different clusters. 85,6% of the 2440 clustered expression profiles fall into the 20 biggest clusters (figure 1). The results obtained by this clustering method are similar as observed with hierarchical, average linkage clustering and also stress the importance of transcriptional regulation for both the DNA replication process and the mechanisms needed for chromosome segregation and cytokinesis.

4.3.2. Systematic functional analysis of gene expression profiles in relation to the plant cell cycle

We also wanted to analyze the coordinated upregulation of transcripts involved in basic processes involved in the plant's cell cycle. To this purpose, we restricted our expression profile collection to the amount of genes for which the sequence already has been determined. The genes that show no significant homology to any gene of known function in the database were termed as 'unknown'. The other genes, for which a specific annotation was possible, were divided in several functional groups. Placement of a gene fragments in a given functional category was done based on knowledge retrieved out of all available literature. Over-representation of genes of a certain functional class in a given cell cycle phase might reveal global pathway activation at this moment. By use of the binomial distribution function, P values were calculated based on the frequency of occurrence of genes with common biological function in a given phase. This P value can be regarded as a measure of over-representation of a specific functional gene class in a certain cell cycle phase.

The results are shown in table 1, where statistically significant representations for specific functions are presented in bold. The most significant enrichments are found during M-phase (at time points 8 and 9 in our experiment), in correspondence with the earlier observed amount of genes residing in M-phase-specific clusters.

Coordinated expression of genes involved in DNA replication was observed at S-phase, as expected. Later on, during G2 and

especially at G2/M transition, the genes involved in chromatin modeling and DNA-modifications are over-represented. In the phases preceding mitosis, the different components of the cytoskeleton are accumulating, as well as in the early M-phase. Most of these genes are expressed immediately before onset of mitosis at the G2/M transition. AFLP tags derived from genes necessary for cell wall synthesis are gradually accumulating starting from G2-phase until M-phase. Another interesting observation is the fact that vesicle transport and formation is stimulated during M-phase. These findings are most probably related to the formation of membranes in the newly formed daughter cells. A subdivision was made for those genes involved in protein degradation, i.c. ubiquitin-mediated proteolysis and other proteolytic mechanisms. For both groups, significant enrichments are found during M-phase. This result further substantializes the suspected existence of an APC in plants. Moreover, the fact that also other proteases seem to be significantly involved in the breakdown of proteins at this moment, suggests the importance of other mechanisms in plants that operate additional to the ubiquitin-mediated proteolysis mechanism. Expectedly, components of the core cell cycle machinery become most significantly expressed in the time points preceding to the G2/M checkpoint.

The significant appearance of stress-related genes in the S-phase is most probably due to the synchronization procedure. Striking is also that genes involved in RNA-processing and translation processes are massively expressed in certain phases of the cell cycle. The observation that translation mechanisms are most activated in M-phase, might point to the fact that in plants a similar mechanism exist as seen in mammalians, where translation of important cell cycle regulators, such as c-Myc, is boosted in a cell cycle-dependent fashion (Bailey and Serres, 1999; Prober and Edgar, 2001).

Obviously, the large amount of novel genes or genes with unknown function leads to an over-representation of this group of genes during nearly the complete cell cycle. Again, this is most striking during M-phase and might be a representation of the fact that chromosome segregation and cytokinesis in plants involves processes of which the components have not been identified and characterized before.

4.3.3. An overview of cell-cycle controlled mechanisms essential for plant cell division

In order to get a better overview of the expression analysis results, additional hierarchical clustering was done on a simplified dataset consisting of the profiles of the genes with a known function (figure 2; file clusterannotatedgenes.cdt on CD-ROM). In the next sections, different processes of importance for cell division in plants will be discussed in more detail, based on the different expression patterns of the genes involved.

DNA replication and modification

In agreement with studies performed on yeast and human fibroblasts, transcripts corresponding to genes involved in DNA replication accumulate during S-phase and exhibit rather broad expression profiles (figure 2 and 3). At the moment that the cells are released from the aphidicolin blocking, high expression levels are already registered for the AFLP tags corresponding to genes encoding replication factors, DNA polymerize a as well as some

histones H3 and H4. This reflects the fact that their expression already has been induced prior to the aphidicolin-induced cell cycle arrest. Upon further progress into S-phase, the majority of the histones becomes expressed, as well as other factors involved in the duplication of the DNA content of the cell. The fact that in general, histones H1, H2A and H2B become slightly later expressed than the types H3 and H4 is an interesting manifestation, reflecting that the latter become deposited in a later stage in the nucleosomes than the first group. Moreover, the expression profile of the anti-silencing function 1 protein, which is alike the histones H3 and H4, is coherent with the actuality that these three proteins are part of the replication-coupling complex mediating chromatin assembly (Tyler *et al.*, 1999). As a conclusion, the generated clusters clearly demonstrate that the genes involved in the initiation and execution of DNA replication are induced in late G1-phase, whereas the genes involved in chromatin assembly are only expressed at the moment when they are needed. These results correspond to the observations made in the studies of the yeast cell cycle (Spellman *et al.*,1998).

Later on in S-phase, genes involved in irreversible DNA-modification are activated, such as S-adenosyl methionine synthase and cytosine-5-methyl transferase. The genes encoding methionine synthase exhibit a peak of expression during late M-phase, which is in contrast to their yeast counterparts that are expressed in late S-phase.

At G2/M transition, the tags corresponding to high mobility group proteins (also called 98b proteins), known to interact with nucleosomes after chromatin assembly become expressed. Genes involved in chromatin remodeling, related to the modulation of gene transcription, follow more diverse expression patterns. A histone deacetylase is accumulating during G2-phase, indicating that it might function to activate genes needed at the G2/M transition. Another gene fragment, homologous to SNF2 chromodomain proteins, is expressed precisely at the G2/M checkpoint, suggesting a similar role. Interestingly, a topoisomerase follows an exactly similar expression profile. A third gene tag is expressed slightly later, and is homologous to the MSI3 proteins, involved in histone acetylation or deacetylation as well as chromatin formation. In addition, we also isolated an AFLP fragment corresponding to acyl-CoAsynthetase, catalyzing the last step to the formation of the cofactor (acylCoA) of histone acetyltransferase. This tag has a rather complex expression pattern, peaking in both S-phase and late M-phase.

Interestingly, a tag homologous to the mammalian p33-ING DNA-binding protein is already present at the first sampled time point. In combination with the fact that yeast homologues of this protein are involved in a histone acetyltransferase complex and show similarity to Rb-binding protein 2, this might suggest that our isolated tag could be involved in Rb-mediated transcriptional regulation of the core cell cycle machinery for progress through G1/S.

The expression profiles of the isolated ribonucleotide reductase (RNR) genes have a more complex expression pattern, probably related to their diverse functions in both DNA replication and repair. A first one is solely expressed in early S-phase, indicating its specialized function in DNA duplication. Other RNR gene homologues have a sharp peak of expression in S-phase, but also have accumulating transcripts at G2/M phase transition, suggesting a dual role in both replication and repair. The last one is exclusively

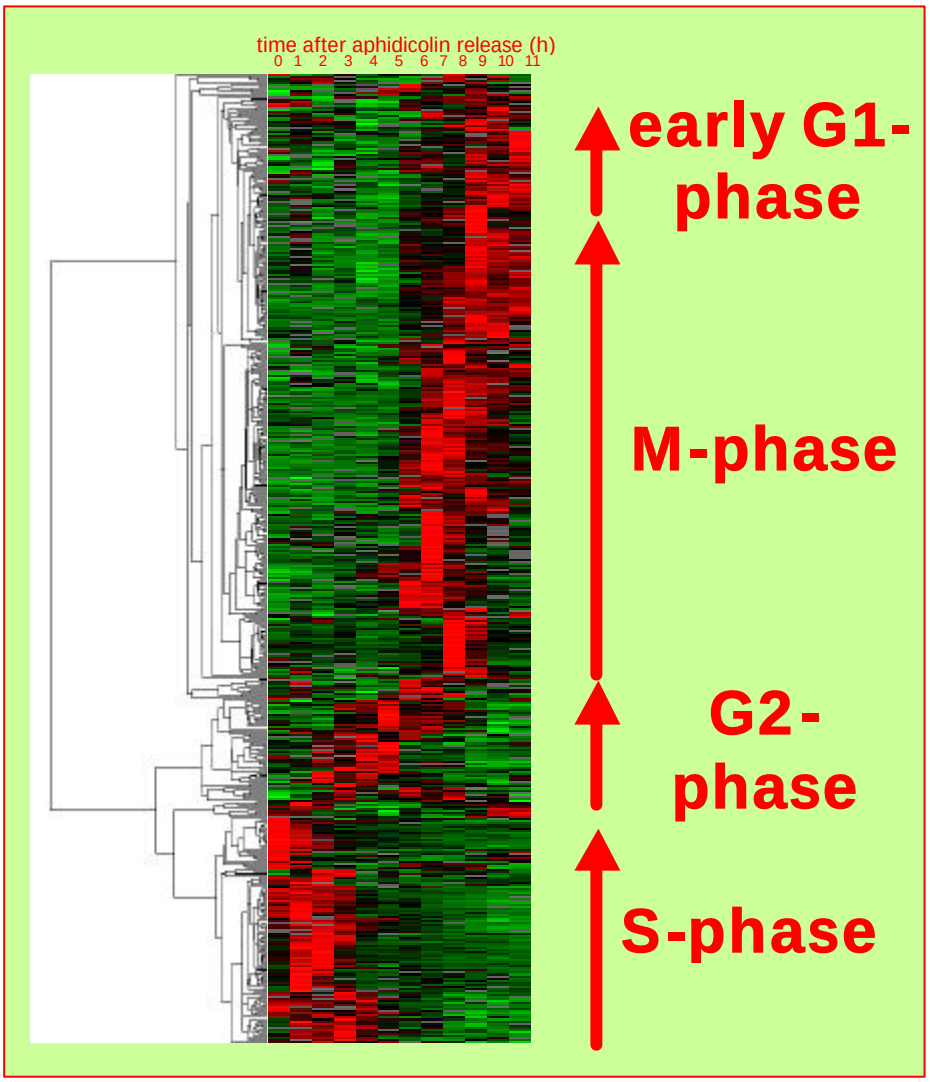


Figure 2: Hierarchical clustering of 473 annotated isolated AFLP tags by the method of Eisen and coworkers (1998)
The representation of the different cell cycle phases in the cluster is roughly indicated by the arrows. The result can be observed in more detail on the CD-ROM presented together with this thesis (filename clusterannotatedgenes.cdt)

expressed during M-phase. This kind of expression behavior has recently also been described for a RNR gene isolated from *Xenopus*, where it seems to be involved in the nucleation of microtubules (Takada *et al.*, 2000).

Core cell cycle machinery

In agreement with earlier observations in plants, AFLP fragments corresponding to the mitotic B1- and B2-type cyclins accumulate in early mitosis, hereby exhibiting a very narrow expression profile (Mironov *et al.*, 1999; figure 2 and 4). Moreover, the mitotic expression profile of cyclinD2.1, as reported by Sorrell *et al.* (1999), was confirmed by our experiment and is similar as that of the B-type cyclins. The A-type cyclins picked up in our screen, fall into three groups, which appear in a sequential fashion during cell cycle progress. Transcripts of the group of A3 cyclins are already present at onset of S-phase and decline gradually until their disappearance

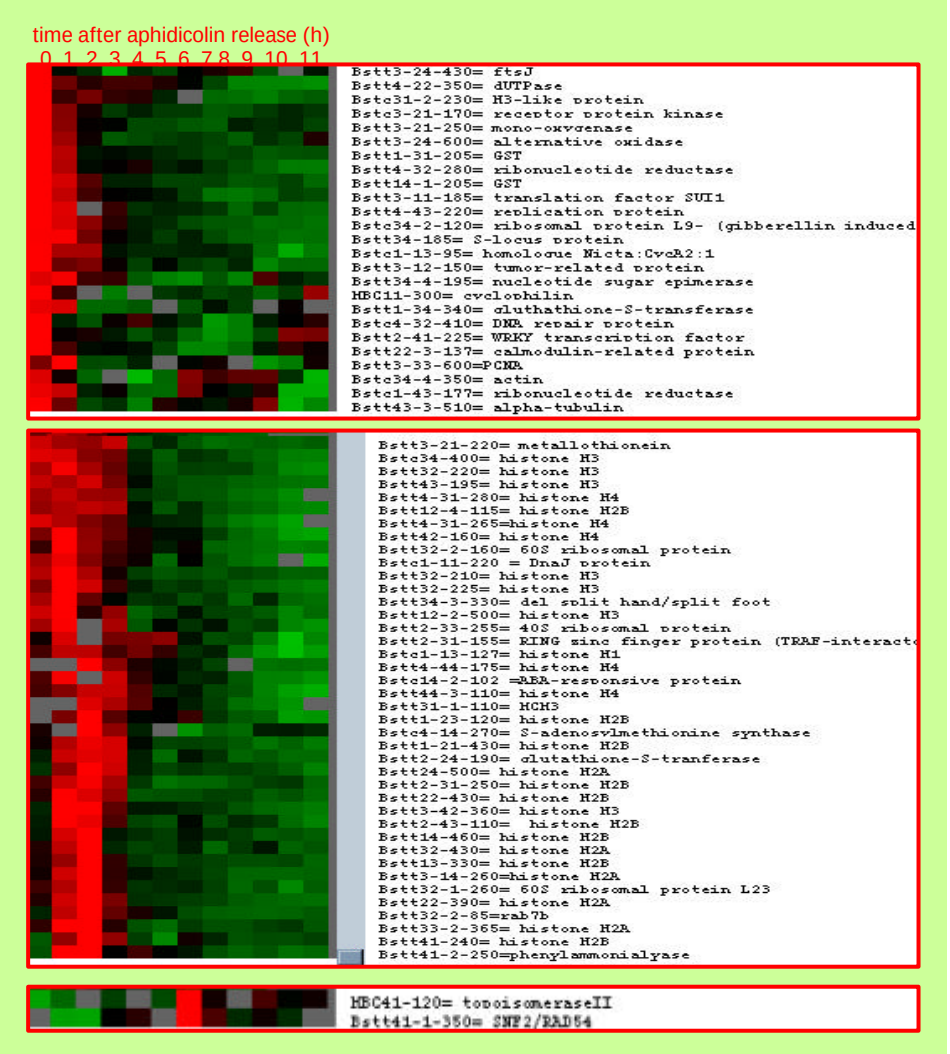


Figure 3: Subclusters with genes involved in DNA replication and modification

early in the subsequent G2-phase. A2-type cyclins come up in late S-phase and stay expressed until M-phase. Lastly, the A1-type cyclins follow the same expression pattern as the B- and D-type cyclins that were isolated. Such expression patterns are similar as reported by Reichheld and coworkers (1996).

Genes encoding the CDKs CDKB1 and CDKB2 are expressed slightly before the mitotic cyclins as described by Porceddu *et al.* (2001). Additionally, cell cycle modulated expression patterns were observed for an alfalfa CDKC-homologue. This is in contrast to what has been observed for this type of CDK in partially synchronized alfalfa cultures (Magyar *et al.*, 1997). Transcripts of this gene are detected from late mitosis on and are also present during early S-phase, suggesting a possible function for the regulation of progress through G1-phase.

The AFLP tag with homology to prohibitin becomes specifically expressed during M-phase. This expression profile suggests a similar role as reported for the prohibitins involved in the mammalian cell cycle, where its

Figure 4: subclusters with some core cell cycle genes

interaction with Rb is impaired in the repression of E2F transcription (Wang *et al.*, 1999). By these means, too early G1/S transition is prevented.

Among the collection of isolated transcription factors, kinases and phosphatases expressed before or at the G2/M checkpoint, we find some with a known or putative role in cell cycle control. One tag, homologous to the plant 3R-MYB-class is found to peak sharply just before the mitotic cyclins. This is in analogy to a very recent report, in which this type of transcription factors have been shown to activate the expression of B-type cyclins and some other genes through interaction with a so-called M-phase activator domain (Ito *et al.*, 2001). Another tag, also corresponding to a myb-like transcription factor, shows the same expression pattern. The transcript, which is homologous to a myc-like transcription factor, becomes expressed in early G1-phase. This further supports the vision that G1/S transition in plants depends on similar mechanisms as occur in animals. The homologue to the CCR4-associated protein CAF peaks in M-phase, in correspondence with its reported role in cell wall integrity, methionine biosynthesis and M/G1 transition. Among the M-specific kinases and phosphatases, a dual-specific phosphatase is found. A phosphatase of this type, CDC25, is known to play a crucial role in cell cycle control in yeast and animals (G2/M

Figure 5: subclusters with components of the cytoskeleton

checkpoint; Nigg, 1995). However, the observed expression profile of the isolated plant homologue contradicts the possibility of a similar function.

Organization of the mitotic cytoskeleton and cytokinesis

The high amount of tubulin a and ß encoding paralogues appear in general prior to or early in mitosis. Their expression is highly cell cycle modulated (figure 5). The majority of the isolated kinesins and myosins fall into the same cluster as the tubulins that peak before mitosis, except for two kinesin homologues. Most interestingly, these two AFLP fragments correspond to the plant-specific phragmoplast-associated type of kinesin, PAKRP1 (Lee and Liu, 2000).

Three of the kinase homologues, which fall into a M-phase-specific cluster, were unambiguously recognized to play a role in cytokinesis. One is an AURORA-type protein kinase not yet described in plants, which is known to be of importance for controlling chromosome segregation, centrosome separation and cytokinesis in yeast and animals (Bischoff and Plowman, 1999). Another is a NPK1 MAPKKK, which is most likely involved in outward redistribution of phragmoplast microtubules (Nishishama *et al.*, 2001). Finally, a member of the multifunctional group of casein kinases type I, accumulates late in mitosis, suggesting its involvement in cytokinesis or vesicular trafficking, as reported in yeast (Gross

Figure 6: subclusters, containing genes involved in hormone responses
The expression profile of the IAR homologue is not to be found back, since the quality of its expression profile was not sufficient for cluster analysis

and Anderson, 1998). The homologue to the VCP/cdc48 protein, important for cytokinesis, accumulates already in G2-phase.

As mentioned before, significant enrichment of transcripts involved in vesicle formation and transport occurs during M-phase. Among them we find several components of COP I-coated vesicles, many GTP-binding proteins known to mediate vesicle targeting and membrane modeling and fusion and proteins involved in the docking of the vesicle with its target membrane. This observation is clearly linked to the centrifugal extension of the plant phragmoplast during cytokinesis. Around the same time, the majority of the genes involved in cell wall synthesis have their peak of expression.

Hormones and plant cell regulation

Among the gene fragments isolated that are linked to hormone response, we find some interesting homologues involved in auxin signaling or responding to this plant hormone (figure 6). Notably, with exception of the auxin-inducible parB protein, they are all expressed at G2/M transition and early M-phase. This further substantializes the results of earlier studies, where the importance of auxin for G2/M transition was indicated (Stals and Inzé, 2001). Recent reports indicate that the MAPKKK protein NPK1, of which we find accumulating transcripts during mitosis, is involved in the subsequent inactivation of this signal and hereby guaranteeing the short-living of auxin-induced transcripts (Bögre *et al.*, 2000).

The gene fragments, whose transcriptional upregulation is linked to abscissic acid and gibberellin action, are expressed during S-phase.

4.4. Discussion

The study presented in this chapter further demonstrates the importance of transcriptional regulation of key processes involved in the execution of cell division. Systematic expression analysis of the isolated genes by means of the cDNA-AFLP-based transcript profiling experiment revealed both known and unexpected results.

The most striking observation made by cluster analysis is the fact that the majority of the genes are expressed during M-phase. Given the fact that during M-phase, the chromatin becomes densely packed into chromosomes and hereby prevents transcription, this seems rather unusual. Moreover, RNA polymerase transcription is known to be repressed during mitosis (Heix *et al.*, 1998), what is confirmed by the deduced expression profiles of two isolated AFLP tags in our screen, which are annotated as RNA polymerase II. The determination of the mitotic index in our samples, showed a peak of 40% at 8 hours after release of the aphidicolin drug. This indicates that the remaining 60% has not yet reached this phase or already progressed to G1. Therefore, elevated expression levels at the time point equivalent to the early M-phase are most likely derived from retarded cells still in G2 phase, whereas later on in mitosis a significant part of the cell population already entered G1-phase. This is clearly demonstrated for a part of the genes implied in the establishment of the mitotic spindle, such as tubulins and kinesins and myosins, of which transcript accumulation peaks at early M-phase. For a proper organization of mitosis, they should already be expressed at G2/M transition. However, the fact that our analysis is based on the presence of polyA+-RNA, could imply that it's possible that many transcripts only become processed to mRNA at onset of mitosis. This thesis is supported by the significant overrepresentation of the functional class harboring RNA processing genes at this stage.

Many of the isolated unknown genes are also expressed during M-phase. This indicates that mitosis and subsequent cytokinesis in plants involves processes of which the components have not been characterized to date. This is not surprising, since especially for events occurring at mitosis, it was expected that plants have developed some unique mechanisms controlling karyo- and cytokinesis. A typical plant cell is surrounded by a rigid wall and can as such not divide by constriction. Moreover, a typical plant cytoskeletal structure has been observed, termed the phragmoplast and the structure of the mitotic spindle is unlike animals, since the absence of a centrosome prevents the tight focusing of this structure at the poles.

Several of the genes that could be characterized by their deduced sequence, showed transcript accumulation profiles that fit with the functional properties of the corresponding genes and with knowledge out of previous studies. This is a clear demonstration of the reliability of the experimental setup and the

technology used. Genes involved in replication and core cell cycle regulatory genes showed a temporal expression pattern similar as concluded from studies in yeast and human (Spellman *et al*., 1998; Cho *et al*., 2001). However, for some functional classes we observed some differences compared to other eukaryotes. For example, kinesins and tubulins peak during Sphase in yeast, whereas in tobacco this occurs in G2- and early M-phase. Moreover, tubulins are only known to have moderate fluctuations in their transcript levels in yeast, while in plants they tend to be highly cell cycle modulated. Also for methionine biosynthesis, there is a discrepancy between yeast and tobacco. Although no clear explanation can be given for these observations, it could be related to differences in cell cycle progression between both eukaryotes. For example, the G2-phase of *S. cerevisiae* is known to be short. As a consequence, it might be required that cytoskeletal genes already need to be expressed during S-phase in order to build up the necessary quantities. Alternatively, differences in RNA-turnover and translation processes could provide an explanation for the detected temporal differences.

When our data are compared to previous studies executed in plants, we mostly confirm these data. However, this was not the case for the CDKC-type protein, homologous to the one isolated from alfalfa (Magyar *et al*., 1997), which is constitutively expressed in cycling alfalfa cells. In our study, we observed a gradual accumulation from late M-phase until our last sampled time point in early G1-phase, indicating a regulating function at the G1/S checkpoint. Most likely, this observation is coherent on the model system used. Tobacco BY-2 cells are well known for their extreme competence to divide at a fast rate (Nagata *a*and Kumagai, 1999). Therefore, the fluctuating expression of the CDKC-homologue could be related to the specific adaptation of these cell line for fast division, as is thought for the differential expression pattern of cyclinD2;1 (Sorrell *et al*., 1999).

Our data further stress the importance of the ubiquitin-dependent proteolytic machinery for the degradation of cell cycle regulators during mitosis, as observed in humans (Cho *et al.* 2001). Moreover, transcripts of other proteolytic enzymes tend to accumulate at this moment, hereby suggesting that other mechanisms might be of importance to perform this task. However, the lack of sampling after early G1-phase prevented to check the eventual presence of the SCF proteolytic apparatus around G1/S transition.

All together, our whole-genome expression analysis study of the plant cell cycle not only provided us with many new candidates with a linked function, but also enabled us to situate the processes occurring during plant cell division and compare them with other eukaryotes. This underlined once more the highly conserved features of cell division, but also revealed some particular differences that could be related to some plant-specific processes.

Acknowledgments

We wish to thank Marnik Vuylsteke for useful advice with the statistical analysis of the obtained data. The people from ESAT -SISTA (Leuven) were greatly appreciated for allowing us to profit of their unpublished clustering method.

Chapter Five

Conclusions and Perspectives

Conclusions and perspectives

The adaptation of the cDNA-AFLP method for genome-wide expression analysis allowed us to perform a detailed study of the transcriptome during plant cell propagation. The obtained expression profiles and sequence data from the resulting comprehensive gene collection gave us some hints about the global regulation of processes related to plant cell division. Moreover, the inventory includes several new candidate regulatory genes, such as transcription factors or components of signal transduction pathways, as well as some first plant homologues of known cell cycle-regulated eukaryotic genes. In addition, many putative novel genes were isolated. Our resulting data set provides a solid basis for supplementary studies towards the further unraveling of highly conserved eukaryotic pathways in plants. In addition, it creates a unique opportunity to characterize some plant-specific processes essential for cell cycle control and the execution of mitosis and cytokinesis.

One of the main drawbacks of cDNA-AFLP is that the generated tags are rather short and not informative enough for the identification of known homologues. The fact that more than half of the tags remain uncharacterized after sequencing and homology searches clearly demonstrates this assumption. To be able to further characterize these tags and the corresponding genes, isolation of longer cDNA clones is necessary. In case of BY-2, this turned out to be a difficult task, most probably because of the amphidiploid nature of the species and the high level of gene redundancy. As a consequence, several homologous cDNAs are isolated which cannot be directly assigned to a specific tag. Although these homologues can help in the functional characterization of the tag, the link with the quantitative expression profile cannot be established, as different homologues may have different expression patterns. A related problem encountered was the fact that AFLP bands may consist of co-migrating fragments of different sequence, impeding unique characterization.

Nevertheless, based on the preliminary set of cDNAs isolated, it became clear that around 20% of the cell cycle modulated genes in the tobacco BY-2 cell line do not have an *Arabidopsis* homologue. This is intriguing in view of the fact that the cell cycle is a highly conserved process and indicates that at certain points different plant species have evolved different regulation mechanisms or that several genes involved in non-cell cycle related processes also display cell cycle modulated expression. Further analysis of these genes will have to reveal what their exact function is, but these findings demonstrate that several genes are not present in the model species *Arabidopsis*.

Our study has shown that cDNA-AFLP is a valid tool for gene discovery and genome-wide expression analysis and is complementary to microarrays. Although the microarray technology will be further improved with respect to sensitivity, specificity and transcript coverage, full genome arrays can only be made for species of which the entire genome has been sequenced and carefully annotated. In this respect, cDNA-AFLP will remain a good alternative for transcriptome studies involving non-model species.

However, some recommendations can be made towards a further improvement of the established gene collection. First and foremost, as the cDNA-AFLP- based transcript profiling technique gives rise to rather small gene fragments, the characterization through sequence analysis is mostly impeded and results in insignificant homologies or no hits at all. Therefore, the isolation of full-length clones is necessary for a proper gene annotation and will additionally allow us to estimate the amount of putative novel genes. Furthermore, the experimental setup of the experiment only permitted sampling until early G1-phase. As such, many interesting genes involved in the regulation of G1/S transition are missed. In order to investigate gene expression in the G1-fase, as well as G1/S transition, an experiment needs to be performed with another cell cycle blocker. Preferably, this synchronization experiment should allow us to block the cells in M-phase.

Part II

Addendum

Molecular and physiological studies on the interaction of the phytopathogen *Rhodococcus fascians* with its host plants

Chapter Six

An introduction to the specific characteristics of the phytopathogenic conduct exerted by *Rhodococcus fascians*

In the most basical definition, *Rhodococcus fascians* can be described as a Gram-positive bacterium, which is gifted to induce hyperplasias on a large collection of plant species. The upcoming sections deal with the most important features of this phytopathogen including the bacterial behavior upon infection as well as the consequences for its interacting partner.

6.1. Description and taxonomical classification of *R. fascians*

A report published in the twenties, describing the witches' broom phenomenon on sweet peas, is the first time that one acknowledged this type of fasciation to be of bacterial origin (Brown, 1927). Although the actual causal agent could not be identified at the time, it was thought to be *Agrobacterium radiobacter* subsp. *tumefaciens*. Almost one decennium later, Tilford (1936) isolated a Gram-positive bacterium that proved to be responsible for the disease symptoms and named it *Phytomonas fascians.* In the years to come, scientific discussion on the most appropriate taxonomical classification lead to switches from *Bacterium fascians* (Lacey, 1939) to *Corynebacterium fascians* (Dowson, 1942). Finally, *Corynebacterium fascians* was reclassified as *Rhodococcus fascians* by the action of Goodfellow (1984). This conclusion was based on the presence of phosphatidylethanolamine and tuberculostearic acid in the cell wall and on a DNA GC content of about 61 to 67.6%, combined with the fact that in numerical phenetic surveys this bacterium clusters with other bacteria belonging to the genus.

R. fascians is a Gram-positive, non-motile, and soil-born aerobic bacterium that, upon cultivation under defined conditions, grows yellow to orange colonies, which can be either rough or smooth. The bacterium requires thiamine for successful growth. Since the *Rhodococcus* genus belongs to the nocardioform actinomycetes, a growth cycle is followed ranging from cocci and short rods to more complex growth phases where hyphae filaments are formed. Ultimately, these structures will fragment into cocci and rods again (Tilford, 1936, Lacey, 1936, Elia *et al*., 1983, 1984; Goodfellow, 1984*). R. fascians* is known to show additional pleomorphic behavior since sometimes U- and V-forms as well as Chinese-character structures were visualized by microscopical analysis (Cornelis, 2000; Goodfellow, 1984). The most odd pleomorphic appearance until now was reported by Lacey (1961). In her experiment, viable filter-passing particles extracted from gall-tissue could be cultivated on a gelatine-containing medium. Initially, granules without a cell wall and of variable size were observed, but in a part of the examined cultures, further development took place: motile, Gram-negative diplococci appeared, occasionally completely regenerating into normal *R. fascians*-type rods. Interestingly, although isolated from filtrate capable of inducing gall formation, both the isolated cocci and the resulting rods were shown to be non-pathogenic. It was stated that these filter-passing elements corresponded to the development of the so-called L-phase of bacteria, representing the form in which the bacterium inhabits the internal tissue of infected plants (Lacey, 1961; Lelliot, 1966). According to Kleinberger-Nobel (1951), this L-form is part of a regeneration procedure probably equivalent to a sexual process in higher organisms.

6.2. Host range of *R. fascians* and colonizing behavior on plants

R. fascians can be considered as an all-round phytopathogen, since it is capable to infect a wide variety of plants. At present, the known host range encompasses 39 families and 86 genera of dicotyledonous as well as monocotyledonous plants and this list is continuously becoming larger (Vereecke *et al*., 2000; Vereecke, personal communication). Several papers report on economical losses in ornamental crops, since infection results in a decrease of flower production or the development of malformed flowers (Miller *et al*., 1980; Oduro, 1975; Zutra *et al*., 1994). In addition to spreading by seeds or infected material for plant propagation, high inoculum-levels of *R. fascians* may be largely responsible for outbreaks of the disease (Tilford, 1936; Lacey, 1939; Miller *et al*., 1980; Oduro, 1975).

On plants *R. fascians* behaves like an epiphyte and is detected on the surface of infected plant tissues, appearing along the leaf edges, epidermal junctions and in or proximal to stomata (Lacey, 1936; Baker, 1950; Rousseaux, 1965; Vereecke *et al*., 1997; Cornelis *et al*., 2001). Scanning electron microscopical images revealed the appearance of pleiomorphic forms next to the common rods or coccal shapes and the bacteria appear to be embedded in a slime layer (Cornelis *et al*., 2001). However, such successful colonization of the phylloplane is observed for both pathogenic and non-pathogenic *R. fascians* strains (Cornelis *et al*., 2001).

As mentioned before, the occurrence of non-pathogenic *R. fascians* in internal tissues was reported for the first time some 40 years ago (Lacey, 1961). This result proved to be difficult to reproduce and for that reason became contested (Faivre-Amiot, 1967). Nevertheless, in 1979 Van Hoof and coworkers successfully retrieved virulent *Rhodococci* from the inner tissue of an isolated gall. Recently, the use of a fluorescence *in situ* hybridization technique with a probe directed to a conserved region in the bacterial 16S rRNA as well as microscopical observations of *uid*A-labelled bacteria, confirmed the existence of *R. fascians* cells in the internal parts of the infected plants (Cornelis *et al*., 2001; Cornelis *et al*., in press). Ingression sites appear to be the principal doorway to invade the plant interior. Once inside, bacteria localize preferably at the intercellular spaces, although there are indications that they can reside intracellularly (Cornelis *et al*., 2001; Cornelis, 2000). Regarding the temporal aspects of infection, it has also been observed that the onset of symptom development takes place before the internal colonization of the plant. This suggests the relative importance of bacteria at the exterior of the plant to direct symptom development (Cornelis *et al*., in press; see also further in this chapter).

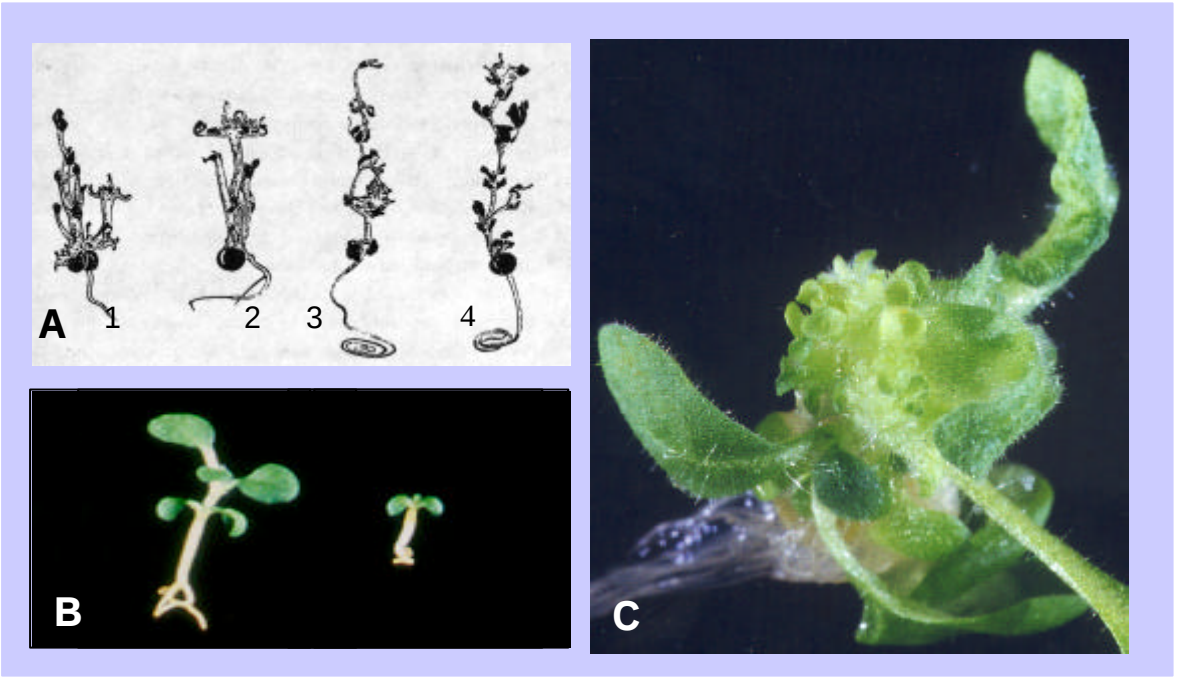


Figure 1: Fenotypes of plants infected with *R. fascians*
A. Development of witches' broom (1), fasciation (2), and leafy galls (3 and 4) on pea seedlings (Rousseaux, 165)
B. Complete growth inhibition of a tobacco seedling infected with *R. fascians* (right), compared with an uninfected seedling (left)
C. Leafy gall on tobacco

6.3. Symptom development associated with infection

As mentioned above, *R. fascians* behaves like a hyperplasia-inducing phytopathogen upon interaction with its host plant. Besides to the plant species and the bacterial strain (Vereecke *et al.*, 2000), the features and the severity of the resulting symptoms are also determined by the age of the plant (Rousseaux, 1965; Faivre-Amiot, 1967), the growth conditions of the bacterium (Eason *et al.*, 1995) and the inoculation method used (Faivre-Amiot, 1967). In order to successfully induce a disease phenotype, the bacterium does not depend on wounding of the plant (Lacey, 1939; Rousseaux, 1965; Vereecke *et al.*, 2000; Manes *et al.*, 2001), but the degree and efficiency of symptom development is enhanced when this situation is encountered by the infecting bacteria (Vereecke *et al.*, 2000).

The morphology of the hyperplasias provoked by *R. fascians* is diverse (figure 1). Witches' brooms were thoroughly described by Brown (1927) on sweet pea and consist of a bunch of short, fleshy shoots associated with malformed or even aborted leaves. They typically originate at the crown of the stem. The term 'fasciation' is derived from the Latin word 'fasces' (bundle of wooden sticks, used as by Roman soldiers) and is used to term the occurrence of flattened bundles of multiple fused shoots. They carry small leaves with thickened petioles and veins (Rousseaux, 1965; Faivre-Amiot, 1967). The most dramatically symptom is the so-called

leafy gall, first reported by Tilford (1936). This structure is best described as a mass of proliferating meristematic tissue, covered with little shoots that are inhibited in their further outgrowth (Lacey, 1936; Vereecke *et al*., 2000). Biochemical analysis of leafy gall tissue demonstrated their distinct composition from other plant tissues. Moreover, leafy galls show delayed senescence compared to the rest of the plant (Vereecke *et al*., 1997). However, they cannot be regarded as autonomous plant structures, since they require the persistence of the pathogen to retain this specific state (Rousseaux, 1965). Inhibition or killing *R. fascians* cells inside the leafy gall allows the shoots to elongate and to develop into normal plants. This feature, combined with the broad host range, was successfully adapted and used for application in *in vitro* plant propagation. The leafy gall-based approach can offer advantages compared to other methods, since some plant species, which show recalcitrant behavior when applying classical techniques, can become readily infected by this phytopathogen (Vereecke *et al*., 2000).

Initially, it was assumed that *R. fascians* could only affect buds and existing meristematic tissues (Lacey, 1936; Rousseaux, 1965; Lelliot, 1966). Recently, this view was extended by the observations that leafy galls can also originate from tissues with competence to re-enter the cell cycle, like the leaf margins and veins (Vereecke, 2000) and from *de novo* induced meristems in the sub-epidermal cortical cells (Manes *et al*., 2001). The development of the root system becomes only affected under severe infection conditions and is associated with the formation of an abnormally thick main root in addition to the absence of any secondary roots (Lacey *et al*., 1936; Tilford, 1936; Rousseaux, 1965; Faivre-Amiot, 1967). Examination of the expression levels of some important marker genes for cell division indicated that upon infection with *R. fascians,* there is a significant decrease of meristematic activity in the roots of *Arabidopsis thaliana* plants (Vereecke *et al*., 2000). On the same plant species, there was a reduction of the number and length of roots, as well as an induction of lateral root formation. The infection of germinating seedlings causes a general inhibition of growth in the shoot as well as the root, next to the thickening of the hypocotyl (Vereecke *et al*., 2000).

It is beyond question that the phenotypical alterations provoked by *R. fascians* are the result of perturbations of the internal hormonal balance of the plant. The exact nature of the events that take place and the essential signaling actions that occur between the two interacting partners are still on the way to be resolved. Studying the etiology of a leafy gall learned that it originates in the close proximity of the inoculation site. Consequently, the responsible signals do not diffuse over a long distance. Moreover, the establishment of a leafy gall and the maintenance of this structure necessitate a persistent presence of signaling induced by the bacterium (Vereecke *et al*., 2000).

Cytokinins are thought to attribute to many aspects of the infection phenotype because of the appearance of serrated or wrinkled leaves, the massive amplification of shoots, the inhibition of root development and the delayed process of senescence. Some of these symptoms can be mimicked by the exogenous application of cytokinins (Klämbt *et al*., 1966). *R. fascians* and cytokinins exert similar effects on shoot meristems and the expression of cell cycle markers albeit the consequences of cytokinin application are less pronounced (Vereecke *et al*.,

2000). It seems therefore likely that the bacterium is responsible for an infection phenotype by the secretion of this hormone. Indeed, different cytokinins could be recuperated from the culture supernatant (Klämbt *et al*., 1966; Scarbrough *et al*., 1973; Armstrong *et al*., 1976; Eason *et al*., 1996) and a positive correlation between cytokinin production and the pathogenicity of a given strain has been made by Murai *et al.* (1980). However, the latter has been contested by other authors since the cytokinin production by virulent and non-pathogenic strains hardly showed any difference in cytokinin production (Crespi *et al*., 1992; Eason *et al*., 1996). Moreover, the amount of secreted cytokinins by virulent *R. fascians* cultures is negligible compared to those of other hyperplasia-inducing bacteria such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi* pv. *savastanoi* (Powell *et al*., 1988; Murai *et al*., 1980). How can one explain the obvious cytokinin-like effects? Crespi *et al.* (1992) suggested either a direct targeting of the cytokinins by the bacterium to the plant cells or an enhancement of cytokinin production once the pathogen reaches the vicinity of it's host plant. Another possibility is that, upon infection, the plant itself becomes stimulated to enhance the production of cytokinins (Thimann and Sachs, 1966). A straightforward approach to test these alternatives is to measure a clear difference in the levels of cytokinins in infected tissue compared to uninfected plants. In *Pelargonium zonale*, more cytokinins could be extracted from infected plants than from the control batch of plants (Balázs and Sziráki, 1974). However, these findings were not confirmed by experiments performed by Eason *et al.* (1996) or Vereecke and coworkers (2000).

Obviously the strategy virulent *R. fascians* strains use to infect plants is more complex. Some reports indicate the capacity of *R. fascians* to degrade auxins (Rousseaux, 1965; Kemp, 1978). It was thought that in combination with the estimated increase of cytokinin levels, this action could provoke a shift of the hormonal balance in favor of cytokinin effects (Balázs and Sziráki, 1974). However, again contradictory observations were made in plants carrying a leafy gall, higher levels of IAA were detected (Vereecke *et al*., 2000). Indeed, typical auxin effects could be observed on plants upon infection with *R. fascians*, such as the swelling of cells, the induction of lateral roots and the secondary differentiation of vascular tissue. Moreover, it has now been established that *R. fascians* is capable to produce auxins in culture conditions (M. Jaziri, personal communication).

In addition to the contribution of cytokinins and auxins to symptom development, there is reason to presume the involvement of other phytohormones. Studies on pea showed a counteracting effect of gibberellic acid on the symptoms resulting from *R. fascians* infection. In *Aracea*, the activity of fungicides that block the functioning of cytochrome P450, which are involved in the biosynthesis of biological active gibberellins, in combination with cytokinins produce a phenotype reminiscent of *R. fascians* action (Werbrouck *et al*., 1996). However, on second sight the situation proves to be more complex, since gibberellin treatment of *Arabidopsis thaliana* mutants defective in gibberellin production or wild-type plants, both infected by *R. fascians,* could not able to influence the formation of the typical *R. fascians* symptoms induced (Ritsema *et al*., in preparation).

Observations of *A. thaliana* hormone mutants (defective in cytokinin, auxin, ethylene, gibberellic acid, and abscissic acid production) after infection by a highly virulent *R. fascians* strain indicate no significant variations in the establishment of a disease phenotype. There are also no clear differences to be reported when infected *Arabidopsis* plants are treated with hormones or hormone inhibitors (Ritsema *et al.*, in preparation). Based on these results, it is concluded that the endogenous hormone levels of the host do not have a critical role in the formation of a leafy gall, but instead there is an autonomous action of the signals originating from the phytopathogen to establish the disease symptoms.

6.4. The molecular basis of phytopathogenicity in *R. fascians*

The implementation of molecular-genetic approaches to reveal the mechanisms leading to pathogenicity in virulent *R. fascians* strains became feasible after the development of molecular utensils to study this bacterium. Desomer and collegues (1988; 1990; 1991; 1992) developed a wide array of tools to achieve this goal, such as methods to isolate plasmids and to conjugate them among different *Rhodococcus* strains (Desomer *et al.*, 1988), an electroporation protocol for successful transformation together with the development of specific cloning vectors (Desomer *et al.*, 1990), a random mutagenesis procedure (Desomer *et al.*, 1991), and finally some useful reporter genes (Desomer *et al.*, 1992).

The presence of circular plasmids associated with virulence has been described for several hyperplasia-inducing bacteria (Morris, 1996; Zaenen *et al.*, 1974) and a similar mechanism was suggested for *R. fascians* (Gross *et al.*, 1979; Murai *et al.*, 1980). However, this assumption was soon denied by the observation that the occurrence of circular plasmids was widespread among both virulent and non-virulent strains (Lawson *et al.*, 1982). Nevertheless, Crespi *et al.* (1992) were able to isolate a large, 180 kb linear plasmid (pFiD188, where Fi stands for Fasciation inducing and D188 for the highly virulent strain used) that is indispensable for virulence. Indeed, a strain cured from this plasmid, lost the leafy gall-inducing capacity. Moreover, using illegitimate integration of non-replicating plasmids, mutations were introduced into pFiD188 and *R. fascians* strains were isolated with and altered virulence behavior. This resulted in the identification of three different loci (*fas, att,* and *hyp*; figure 2) that are important in the establishment of phytopathogenicity. When the *fas*-locus is inactivated, *R. fascians* turns into a non-virulent strain. Insertion mutagenesis of the *att*- and *hyp*-

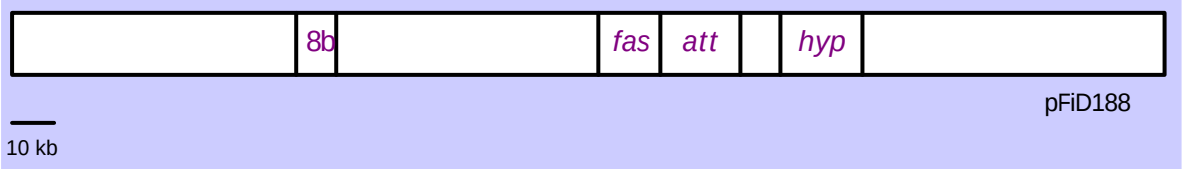


Figure 2: Organization of the virulence loci on pFiD188

region leads to an altered degree of virulence, yielding moderately virulent bacteria in the first case and hypervirulent ones in the latter (Crespi *et al.*, 1992).

6.4.1. The *fas*-locus is essential for virulence

The *fas* locus (figure 3) contains an operon consisting of 6 open reading frames (ORFs). Localization and sequencing of the isolated mutation, responsible for virulence loss lead to the identification of an isopentenyltransferase (*ipt*) gene (Crespi *et al.*, 1992). This discovery was a breakthrough in *R. fascians* research, since Murai *et al.* (1980) already suggested the existence of an alternative route via isopentenyltransferase for cytokinin biosynthesis in *R. fascians* in addition to the tRNA as a source for cytokinins (Matsubara *et al.*, 1968). Although the overall sequence homology with known isopentenyltransferases from *A. tumefaciens* and *P. savastanoi* pv. *savastanoi* is low, *in vitro* isopentenyltransferase activity has been detected for this gene product (Crespi *et al.*, 1992). The relevance of the *ipt* gene for virulence has been further supported by Stange and coworkers (1996), who demonstrated the presence of this gene in every virulent strain tested. The insertion leading to the *fas* mutant was shown to be in the fourth ORF. A role for the other ORFs has been proposed based on their sequence homology. The proteins encoded by the first 2 ORFs are homologous with P450 cytochromes and ancillary ferredoxins respectively. In addition, ORF2 has been found to contain a carboxy-terminal domain similar to the a-subunit of pyruvate dehydrogenases, whereas ORF3 encodes the ß-subunit of these kinds of enzymes. Finally, ORF5 and 6 are homologous to genes encoding a cytokinin oxidase and a lysine decarboxylase respectively (Crespi *et al.*, 1994; Temmerman, 2000). Temmerman (2000) proposed a model based on these sequence data where the *ipt* gene is the key factor in the synthesis of a cytokinin-like structure that becomes additionally modified by the action of the gene products from the other ORFs (figure 4). The first 3 ORFs would be responsible for a transport system of high-energy electrons, which, upon delivery, are used by cytochrome P450 to modify the cytokinin-like compound. The protein encoded by ORF5 would be involved in the activation of the N^6-atom of the formed cytokinin, where a polyamine or derived structure could be linked through the action of the gene product of ORF6. An insertion mutant in ORF1 was found to be completely non-virulent as is the mutant in the *ipt* gene. However, an isolated mutant in the fifth ORF retained their full virulence under all tested conditions except on older plants. There, they produced leafy galls in which the shoots are not inhibited in their elongation. A credible explanation for this result can be that the *fas*-derived compounds are involved in the shoot initiation process and inhibition of the formed shoots. For the latter case, the presence of intact ORF5 and/or 6 proves to be critical (Temmerman, 2000).

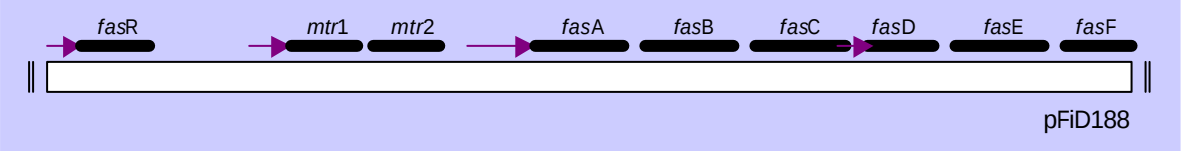


Figure 3: Organization of the *fas*-locus

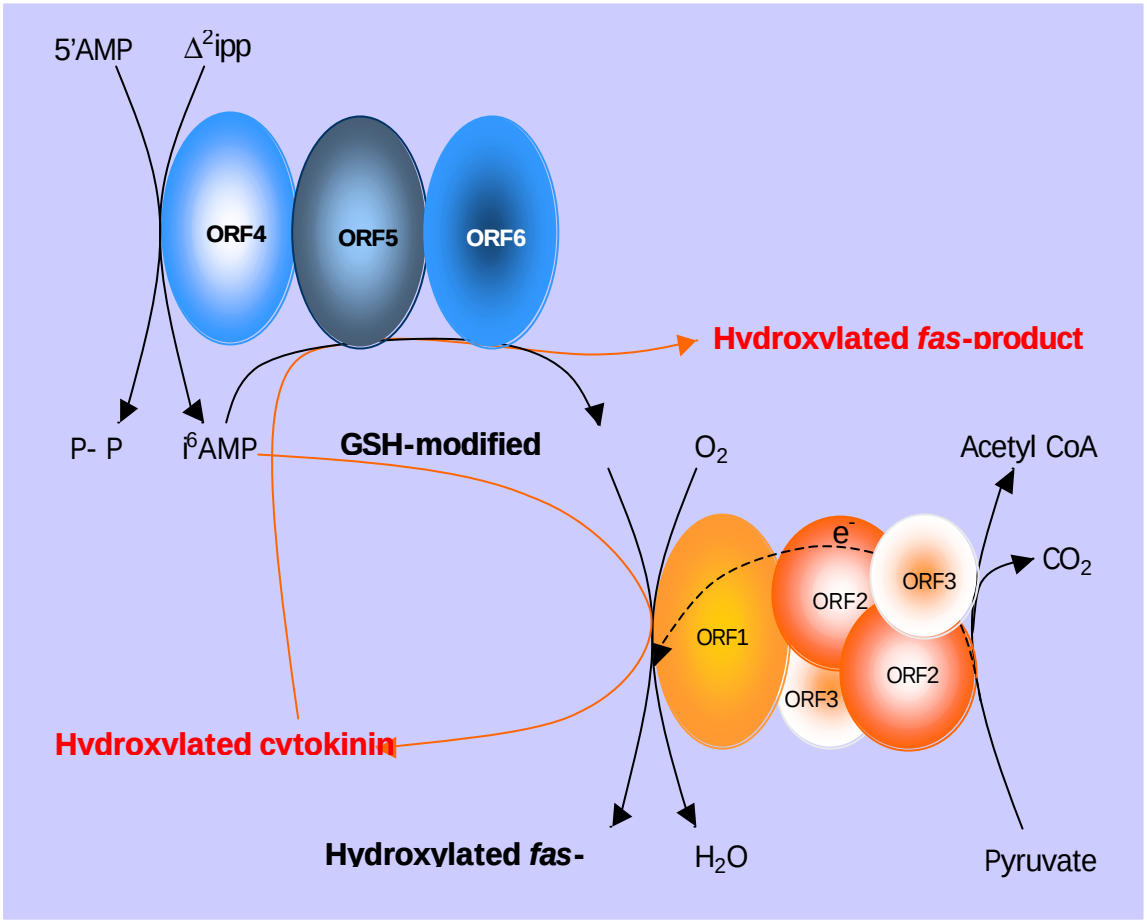


Figure 4: Hypothetical model for the function of the *fas* encoded proteins in the biosynthesis of a cytokinin-like molecule (as described in the text)

Numerous efforts have been done to determine the nature of the *fas*-derived product and to characterize its biological activity. At present, the precise structure of this product is still unknown. Supernatant of D188 strains induced in optimized conditions for *fas*-expression (see Chapter 7 for details) has some biological activities that are not present in the supernatans of *fas*-mutants or non-induced strains. Among these, the capacity to reduce internode length and leaf size of plants, the reduction of leaf and root development of germinating seedlings and the delay of the prophase of mitosis in tobacco BY-2 cell cultures are observed (Temmerman, 2000; Temmerman *et al.*, 2001). Purified *fas*-dependent products, exhibit some cytokinin activities in an *Amaranthus caudatus* bioassay, have a strong de-etiolating effect on *A. thaliana* and induce the formation of lateral roots in the same bio-assay (Temmerman, 2000).

By using *uid*A-fusions, the expression behavior of the *fas*-locus could be followed *in planta*. In the first days after infection, the *fas*-locus was found to be expressed both on the surface and the interior of the plants (around the sites where later on meristematic activity was initiated), but interestingly, as time progressed there was mainly GUS staining in bacteria

localized inside the plant (Cornelis, 2000; Cornelis *et al*, in press).

6.4.2. The *att*- and *hyp*-loci are involved in the establishment of a fully virulent phenotype

When a batch of plant becomes infected with the *att*-mutant, a collection of infection phenotypes can be distinguished. One third of the plant population seems not to be affected, but the majority tends to develop smaller or less developed leafy galls. When seedlings become inoculated with this mutant, they grow to an intermediate length (Crespi *et al*., 1992). The *att*-locus is found to contain 9 ORFs (*att*A to –G, -R and –X; figure 5) homologous to genes involved in the biosynthesis of arginine and the biosynthesis of ß-lactam structures (Maes, 2001; Maes *et al*., 2001). Expression of this locus is tightly regulated through the action of a LysR-type transcriptional regulator (AttR) and is dependent on gall-specific compounds (Maes *et al*., 2001). Since in literature a possibility has been suggested to synthesize the ß-lactam containing molecule clavulanic acid starting from arginine together with 3 carbon metabolites (McGowan *et al*., 1998), these sequencing data could indicate that the ORFs of the *att*-locus are involved in the synthesis of a complex molecule with a ß-lactam ring present in its structure (Maes, 2000; Maes *et al*., 2001).

Results from *in situ* hybridization experiments show that the non-pathogenic strain D188-5 (cured from the linear plasmid) is less efficient in penetrating the interior of tobacco plants compared to the wild type strain (Cornelis *et al*., 2001). However, this result could not be reproduced upon infection of *A. thaliana* plants (Cornelis *et al*., 2001). Interestingly, phenotypical differences in symtom development between D188 and the *att*-mutant are not always registrated upon interaction with this plant species (Maes, 2001). It is hence tempting to speculate that the *att*-locus could be implicated in extension of the host range. Further on, *att*-derived products were found to be involved in cell-cell signaling events acting as auto regulatory compounds. They are responsible for the induction of both *fas*- and *att*-expression (Maes, 2001; Maes *et al*., 2001). This role was substantiated by the visualization of bacteria *in planta* carrying a GUS-fusion with the *att*-locus. GUS-expression was observed at the plant surface prior to the activation of *fas*-expression (Cornelis *et al*., in press) and as mentioned already before, those bacteria are thought to direct symptom development.

A region located in *Bam*HI restriction fragment *8b* of the linear plasmid pFiD188 seems to be involved in bacterial virulence (Vereecke *et al*., unpublished data). Sequence analysis led to the identification of a 6 ORF-containing gene cluster, which probably encodes a nonribosomal peptide synthase (NRPS; Maes, 2001). A deletion mutant in this *8b* region showed an attenuated virulence indistinguishable from the *att*- mutants, suggesting a mutual involvement of these loci in one process (Maes, 2001). Indeed, under *in batch* conditions, the formation of *att*-related compounds is abolished in the *8b* deletion mutant. However, gall extracts of tissues

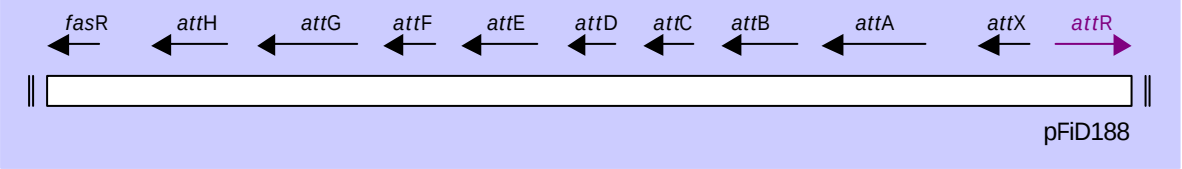


Figure 5: The organization of the *att*-locus of *R. fascians.*

infected with the *8b* deletion mutant do contain a limited amount of inducer. Clearly, more information has to be gathered to elucidate this apparent contradiction.

The *hyp*-mutant induces a hypervirulent phenotype, with leafy galls that tend to be larger than those originating induced by wild type bacteria. One of the ORFs of this locus is homologous to a gene encoding a RNA helicase of the DEAD-box family (Temmerman, 2000; Schmidt and Linder, 1992). Possibly the importance of the *hyp*-locus resides in the post-transcriptional regulation of another locus. A likely candidate herefore is the *fas*-locus (Temmerman, 2000).

6.4.3. Description of the chromosomally encoded bacterial virulence locus *vic*

When trying to comprehend the development of a leafy gall, one is obliged to ask the question why the bacterium is determined to go through the trouble of infecting a plant. A possible clue is given by the study of a locus residing on the chromosome of *R. fascians,* which has been named *vic* (virulence in chromosome).

The isolated *vic*-mutant has a reduced pathogenicity and elicits either no symptoms or only small shoot-like outgrowths or loosely associated leafy galls, which are only moderately inhibited in their outgrowth. Seedlings exhibit an extreme thickening of the hypocotyl. The insertion was found to be in a malate synthase gene encoding the second enzyme in the glyoxylate shunt of the Krebs cycle. This enzyme catalyzes the condensation of acetylCoA and glyoxylate resulting in malate and enables an organism to survive on a diet consisting exclusively of C2-compounds (Kornberg, 1966; Vereecke *et al*., submitted). It is proposed that the *vic*-locus would be involved in the metabolism of nutrients that are specifically present in symptomatic tissue, by detoxifying glyoxylate formed as an intermediate compound during this catabolism. In the *vic*-mutant, malate synthase activity is absent, consequently leading to the accumulation of toxic glyoxylate levels, which inhibit bacterial growth or kill the bacteria. This hypothesis is confirmed by the observation that the *vic*-mutant is significantly deficient in the colonizing of symptomatic but not healthy tissue (Cornelis, 2000). Hence, a leafy gall should be considered as a specific ecological niche. The process leading to the establishment of this condition is called 'metabolic habitat modification' (Vereecke *et al*., submitted).

Chapter Seven

Leafy gall formation is controlled by *fasR*, an AraC-type regulatory gene in *Rhodococcus fascians*

Abstract

Rhodococcus fascians can interact with many plant species and induce the formation of either leafy galls or fasciations. To provoke symptoms, *R. fascians* strain D188 requires pathogenicity genes that are located on a linear plasmid, pFiD188. The *fas* genes are essential for virulence and constitute an operon that encodes, among other functions, a cytokinin synthase gene. Expression of the *fas* genes is induced by extracts of infected plant tissue only. We have isolated an AraC-type regulatory gene, *fasR*, located on pFiD188, which is indispensable for pathogenesis and for *fas* gene expression. The combined results of our experiments show that *in vitro* expression of the *fas* genes in a defined medium is strictly regulated and that several environmental factors (pH, carbon and nitrogen sources, phosphate and oxygen content, and cell density) and regulatory proteins are involved. We further show that expression of the *fas* genes is controlled at both the transcriptional and the translational levels. The complex expression pattern probably reflects the necessity of integrating a multitude of signals and underlines the importance of the *fas* operon in the pathogenicity of *R. fascians*.

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7.1 Introduction

The gram-positive bacterium *Rhodococcus fascians* (Tilford, 1936) infects diverse plant species. Infection of dicotyledonous plants can result in the local proliferation of meristematic tissue, leading to galls that are covered with leaflets, known as leafy galls (Elia *et al.*, 1984, Vereecke *et al.*, 2000). On monocotyledonous plants, such as lilies, *R. fascians* provokes severe malformations of the bulbs and the formation of long side shoots (Miller *et al.*, 1980, Vantomme *et al.*, 1982), resulting in abnormal plants that are unfit for commercial use (Baker, 1950, Faivre-Amiot, 1967). Infection of tobacco seedlings with *R. fascians* strongly inhibits growth, accompanied by arrested root development, thickening and stunting of the hypocotyl, and inhibition of leaf formation (Crespi *et al.*, 1992).

In 1966, the production of cytokinins was inferred as a major virulence determinant of *R. fascians* (Klämbt *et al.*, 1966, Thimann and Sachs, 1966). In our laboratory, in *R. fascians* strain D188, genes involved in pathogenicity were shown to be located on a large, conjugative, linear, fasciation-inducing plasmid (pFiD188) (Crespi *et al.*, 1992). Random mutagenesis of pFiD188 led to the identification of three virulence loci, of which the best characterized is the essential *fas* locus. This locus consists of an operon of six genes, of which the most important are a cytochrome P450 homologue gene (ORF1) and an isopentenyl transferase (*ipt*) gene (ORF4) homologous to *ipt* genes of other phytopathogens (Crespi *et al.*, 1992, Crespi *et al.*, 1994). The *ipt* genes are typically involved in the biosynthesis of isopentenyl AMP (i6AMP), a general precursor of several cytokinins (Kaminek, 1992). However, the chemical structure of the compound resulting from the action of the *fas* gene products remains to be determined. Two other pFiD188-located virulence loci, *hyp* and *att*, are necessary for balanced virulence because mutations in these regions result in hypervirulence and attenuated virulence, respectively (Crespi *et al.*, 1992).

Expression of the *fas* genes is induced by extracts of infected plant tissues and not of uninfected plants (Crespi *et al.*, 1992). In many other pathogens, induction of a whole battery of virulence genes follows sensing of signals from the environment (Brandl and Lindow, 1997, Clough *et al.*, 1994, Miller *et al.*, 1989, Schell, 1996, Skorupski and Taylor, 1997, Winans, 1990). This environmentally modulated expression is often mediated by a single pleiotropic regulatory protein (Gallegos *et al.*, 1993, Huang *et al.*, 1998) or by a two-component regulatory system (Hoch and Silhavy, 1995, Soncini and Groisman, 1996).

Here, we report on the isolation and characterization of a new virulence gene located on pFiD188 that codes for a regulatory protein belonging to the AraC family (Gallegos *et al.*, 1993, Ramos *et al.*, 1990). We present data on the significance of this gene for *R. fascians* pathogenesis on tobacco and reveal its involvement in the complex regulation of *fas* gene expression.

7.2 Experimental procedures

7.2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used are listed in Tables 1 and 2. *Escherichia coli* strains were grown at 37°C in Luria broth (Sambrook *et al*., 1989), whereas *R. fascians* strains were grown at 28°C in yeast extract broth (YEB) (Miller, 1972). For determining *fas* gene expression levels, *R. fascians* strains were grown in MinA medium [6.4 mM KH_2PO_4, 33.6 mM K_2HPO_4, 0.1% $(NH4)_2SO_4$, 0.05% sodium citrate, 0.025% $MgSO_4$, 0.001% thiamine, and 20 mM carbon source of interest]. When appropriate, media were supplemented with carbenicillin (200 µg/ml), chloramphenicol (25 µg/ml), or phleomycin (1µg/ml).

7.2.2. DNA sequencing and analysis

The DNA sequence of both strands was determined by using automated dideoxy-sequencing systems (A.L.F. DNA Sequencer [Pharmacia, Uppsala, Sweden] and ABI377 DNA Sequencer [Applied Biosystems, Foster City, Calif.]). Computer-assisted interpretation of the sequence was performed by the Genetics Computer Group (Madison, Wis.) sequence analysis software package (version 9.1). Homology searches with the Swiss-Prot (release 35), Unique-PIR (release 53), and EMBL (release 53) databases were done using the FASTA algorithm (Pearson and Lipman, 1988). Alignments were done using PILEUP.

7.2.3. Deletion mutagenesis

The *fasR* deletion mutant was isolated via double homologous recombination. For this purpose, plasmid pUCDV3 was constructed; it carries the chloramphenicol resistance (*cmr*) gene (Desomer *et al*., 1992) and the DNA region containing *fasR*, in which a deletion was generated (Fig. 1). Because pUCDV3 cannot replicate in *R. fascians*, electroporation into strain D188 and plating on chloramphenicol-containing medium resulted in the isolation of single recombinants. Growth of these clones without selective pressure allowed a second recombination event, and after screening was performed for Cm^R transformants, a deletion mutant was isolated. First and second recombinations were verified by Southern hybridization analysis (Sambrook *et al*., 1989).

7.2.4. Virulence tests

Sterile *Nicotiana tabacum* (L.) W38 seeds were germinated on half-strength MS medium (Murashige and Skoog, 1962) supplemented with 0.001% thiamine, 1% sucrose, and 0.8% agar. For the virulence assays, after 2 days of germination, when the radicle emerged, 20 µl of a concentrated *R. fascians* culture was added to the seedlings, or the plants were decapitated and infected with a saturated *R. fascians* culture 6 to 7 weeks after germination. Phenotypes were scored after 2 to 4 weeks.

7.2.5. Inductions and GUS assays

For the in planta expression analysis, 3- to 4-week-old sterile *N. tabacum* W38 plants were immersed in a culture of the test strain resuspended in MinA medium, and submitted to a vacuum generated by a water pump for 2 min. After being washed with MinA medium, the plants were replanted in half-strength MS medium, and after 3 days they were used for extraction and b-glucuronidase (GUS) measurements. Extracts were prepared by extensively crushing the plants or leafy galls excised from the infected plants with a pestle in an Eppendorf tube. After centrifugation and filter sterilization, a 50- to 70-µl extract was obtained from 100 mg of tissue. For the in planta expression assay, 1 ml of MUG buffer (50 mM $NaPO_4$ [pH 7.0], 10 mM b-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium dodecyl sulfate, and 0.1% Triton X-100) was added to 200 mg of crushed plant tissue. The substrate 4-methylumbelliferyl-b-D-glucuronide (0.1 mM) was added, the reaction mixtures were kept at 37°C, and the reactions were stopped after 1 h by adding a 50-µl sample to 200 µl of 0.2 M Na_2CO_3. GUS activity was determined by excitation at 365 nm and measurement of emissions at 460 nm and is calculated as the measured emission X 1,000/time (in minutes). Every assay was performed on the same amount (fresh weight) of plant material, leading to relative and comparable data.

For *fas* and *fasR* gene expression, cells were grown for 2 days in YEB, diluted 10-fold in YEB, and allowed to grow overnight. After growth on YEB, the cells were collected by centrifugation, washed, and diluted to the desired optical density at 600 nm (OD600) in MinA medium. The pH of the MinA medium was adjusted to 6.5 and 5.7 by changing the KH_2PO_4/K_2HPO_4 ratio and to 3.0, 4.0, 5.0, and 5.7 by using citric acid and sodium citrate as a buffer system (10 mM). GUS activity was measured after the cells were incubated overnight with gall extracts (20 µl/ml), tobacco plant extracts (40 µl/ml), different carbon sources (20mM), and/or amino acids (5mM). For the GUS assay, the cells were collected by centrifugation and resuspended in 1 ml of MUG buffer, and the GUS activity was measured as described above and calculated as the measured emission X (1,000/OD600) X time (in minutes).

7.2.6 Other methods

Plasmid isolation and DNA cloning were performed according to the methods of Sambrook et al. (1989), and *R. fascians* transformation was done as described before (Desomer *et al.*, 1990).

TABLE 1. **Bacterial strains**

Strain	Description	Reference
E. coli		
MC1061	*ara? 139 (?ara leu)7697 ? lacX74 galU galK hsrR hsrM1 rpsL* 1 StrA	Casadaban and Cohen, 1980
DH5a	F2 f80d*lacZ*? M15? (*lacZ4A argF)U169 recA1 endA1 hsdR17* (rK1 mK1) *supE44* l2 *thi gyrA relA1*	Hanahan, 1983
R. fascians		
D188	Wild type; virulent	Desomer *et al.*, 1988
D188-5	Plasmid-free strain; nonpathogenic	Desomer *et al.*, 198813
D188? *fasR*	Deletion mutant; nonpathogenic	This work

TABLE 2. **Plasmids**

Plasmid	Marker gene(s)	Relevant characteristics	Reference
pRF37	Phleo^r Ap^r	Shuttle cloning vector replicating in *R. fascians* and *E. coli*	Desomer *et al.*, 1990
pJGV131	Ap^r	Shuttle cloning vector replicating in *R. fascians* and *E. coli*	Crespi *et al.*, 1994
pUCDV1	Cm^r Ap^r	*Bam*HI clone containing a 4.9-kb upstream region of mutant *fas6* and the *cmr* gene as a 2.5-kb *Xba*I fragment in pUC18	Crespi *et al.*, 1994
pUCDV3	Cm^r Ap^r	Clone derived from pUCDV1 by deleting a 912-bp *Acc*I-*Nco*I fragment in the *fasR* gene, resulting in a suicide plasmid for the generation of D188D*fasR*	This work
pRFDV2	Phleo^r Ap^r	Clone of a 1.7-kb *Xho*I fragment of pFiD188 derived from *Bam*HI fragment 1 and containing the *fasR* gene in pRF37, resulting in a complementation construct for D188?*fasR*	Crespi *et al.*, 1994; this work
pJBDV1	Ap^r	Clone of the 0.9-kb *Xho*I fragment of pFiD188 *Bam*HI fragment 1 containing the *fasR* promoter and the 1.9-kb *Sal*I-*Sac*I fragment of pRG960sd containing *uidA* in pJB66, resulting in a transcriptional fusion between *fasR* and *uidA*	Botterman and Zabeau, 1987, Van den Eede *et al.*, 1992; this work
pRFDV6	Phleo^r Ap^r	Clone of the 2.8-kb *Xba*I fragment of pJBDV1 in pRF37, replicating transcriptional *fasR-uidA* fusion	This work
pJDGV2	Ap^r	Clone of the 1.5-kb *Stu*I-*Sno*I fragment of pFiD188 *Bam*HI fragment 1 in pGUS1, resulting in a translational fusion between the 111 amino-terminal amino acids ofORF1 of the *fas* operon and *uidA*	Crespi *et al.*, 1994
pJDGV3	Cm^r Ap^r	Clone of the 4.0-kb *Hin*dIII-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	Crespi *et al.*, 1994
pJDGV4	Cm^r Ap^r	Clone of the 2.9-kb *Asc*I-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	Crespi *et al.*, 1994
pJDGV5	Cm^r Ap^r	Clone of the 3.3-kb *Sal*I-*Xba*I fragment of pJDGV2 in pJGV131 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, replicating translational ORF1-*uidA* fusion	This work
pUCWT1	Cm^r Ap^r	Clone of the 4.0-kb *Hin*dIII-*Xba*I fragment of pJDGV3 in pUC18 with the 2.5-kb *Xba*I fragment containing the *cmr* gene of *R. fascians* NCPPB1675, integrating translational ORF1-*uidA* fusion	Norrander *et al.*, 1983;this work
pSPWT1	Ap^R	Clone of the 0.6-kb *Sal*I-*Sac*I fragment of pFiD188 *Bam*HI fragment 1 containing the *fas* promoter with the 2.1-kb *Sma*I-*Eco*RI fragment of pRG960sd containing the *uidA* gene in pSP72 (Promega, Madison, Wis.), resulting in a transcriptional fusion between ORF1 of the *fas* operon and *uidA*	Van den Eede *et al.*, 1992;this work
pJBWT1	Ap^r	Clone of the 2.5-kb *Pvu*II-*Bgl*II fragment of pSPWT1 in pJB66, integrating transcriptional ORF1-*uidA* fusion	Botterman and Zabeau, 1987; this work
pRFWT11	Phleo^r Ap^r	Clone of the 2.5-kb *Xba*I-*Hin*dIII fragment of pJBWT1 in pRF37, replicating transcriptional ORF1-*uidA* fusion	This work
pJBWT2	Phleo^r Ap^r	Clone of the 3-kb *Bam*HI fragment of pMSA4 containing the Phleor gene in pJBWT1, integrating transcriptional ORF1-*uidA* fusion	Sugiyama *et al.*, 1994;this work

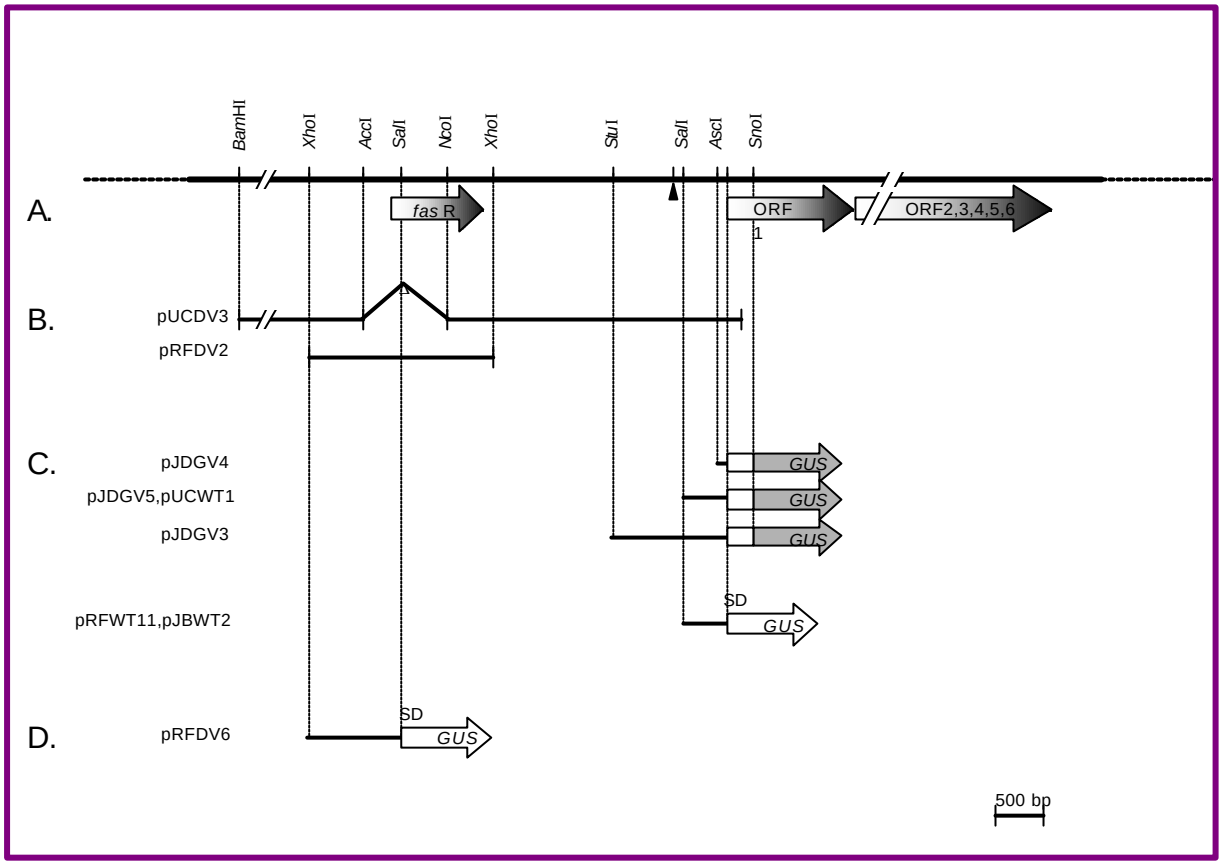

Figure 1: Physical map of the relevant region of pFiD188
A. Physical map of the region of pFiD188 spanning *fasR* and the *fas* operon . ORFs and relevant restriction sites are shown. The arrowhead indicates the previously determined 5' border of the *fas* operon
B. pUCDV3, suicide plasmid carrying a 912bp *Acc*I/*Nco*I deletion (?) in the *fasR* region used to generate D188? *fasR*. pRFDV2 is a fragment used for the complementation analysis
C. Fragments used for the *fas-gus* fusions. The shaded and open arrows represent translational and transcriptional GUS fusions, respectively
D. Fragment used for the transcriptional *fasR-gus* fusion. SD, Shine-Dalgarno sequence

7.3. Results

7.3.1. An AraC-type regulatory gene, *fasR*, is essential for virulence

Determination of the DNA sequence between the linked *fas* and *att* locus (Crespi *et al*., 1992) revealed an open reading frame (ORF) of 834 bp (potentially encoding a protein of 277 amino acids) located 3,282 bp upstream from ORF1 of the *fas* operon and in the same transcriptional orientation (Fig. 1A). Three base pairs upstream from the ATG start codon, the sequence GAACGACAG, which represents a putative ribosome-binding site of *R. fascians*, is present (Crespi *et al*., 1994). The ORF has a G1C content of 53% and a G+C content at the third position of 50%, both very low for *R. fascians* (G1C, 61 to 68%) (LeChevalier, 1986). All codons are used in this ORF, but remarkably, UUA, which is usually a rare codon in *R. fascians* as well as in *Streptomyces* and corynebacteria

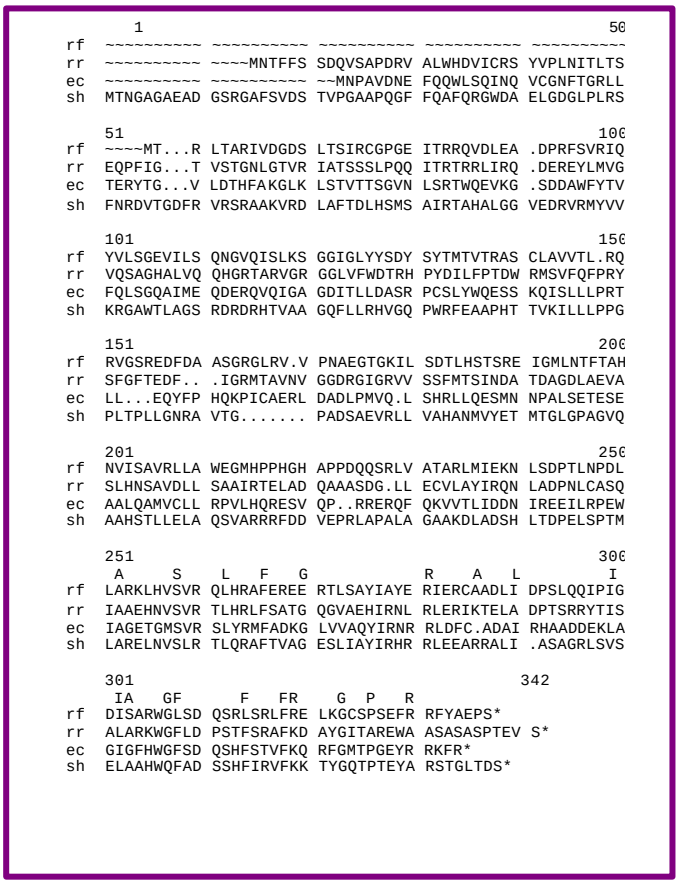

Figure 2: Alignment of AraC-type transcriptional regulators.
R. fascians (rf), *R. rodochrous* (rr), *E. coli* (ec), and *S. hygroscopicus* (sh). Identical and/or similar amino acids are shaded. The AraC-family characteristic motif is indicated above the alignment (Gallegos *et al.*, 1997); with n representing any amino acid, it is as follows: $An_5Sn_3Ln_3Fn_4Gn_{10}Rn_8An_3Ln_8(I/V)n_2(I/V)n_4G(F/Y)n_5Fn_3F(R/K)n_3Gn_2P$. Dots were inserted for optimal alignment and asterisks indicate stop codons.

(Malumbres *et al.,* 1993, Wright and Bibb, 1992), is frequently used for Leu (7 out of 30).

Comparative sequence searches revealed that this ORF potentially encodes a protein that is homologous to different members of the AraC family of transcription regulators (Gallegos *et al*., 1997; Fig. 2). Although the similarity of these proteins is highest in the carboxyl terminus, where the DNA-binding helix-turn-helix motifs are located, the overall similarity is also significant. Over a 100-amino-acid-residue stretch, encompassing the defined AraC family profile (PROSITE database entry PSO1124), the highest similarities are found with an AraC-type regulator involved in rapamycin biosynthesis in *Streptomyces hygroscopicus* (38% identity; 48% similarity) (Molnár *et al*., 1996), with the transcription regulator (NitR) of the nitrilase gene of *Rhodococcus rhodochrous* (34% identity; 43% similarity) (Komeda *et al*., 1996), and with MoaB, a positive regulator of the monoamine oxidase gene of *E. coli* (34% identity; 47% similarity; Yamashita *et al*., 1996) (Fig. 2).

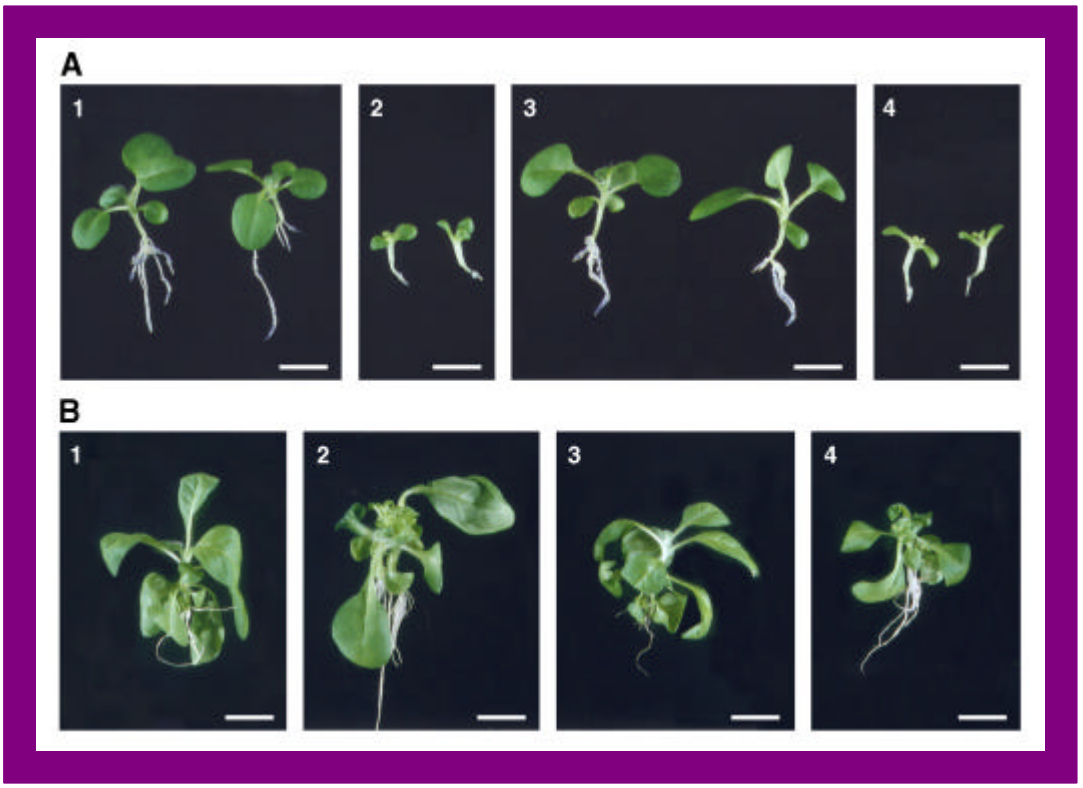

Figure 3: Phenotypes of tobacco inoculated with different *R. fascians* strains.
A. Seedlings infected with D188- 5 (1), D188 (2), D188Δ*fas*R (3), and D188Δ*fas*R[pRFDV2] (4); B. Inoculation after decapitation of the apical meristem (1) without bacteria, (2) with strain D188, a leafy gall forms at the cutting site and no axillary shoot meristem can grow out, (3) with strain D188Δ*fas*R phenotype as in panel 1, and (4) with strain D188Δ*fas*R[pRFDV2] phenotype as in panel 2. A: Bar = 0.5 cm; B: Bar = 2 cm.

Because the ORF is located between two pathogenicity loci, *fas* and *att*, the possible role of this gene in the virulence of *R. fascians* was examined by deleting part of the ORF in pFiD188. For this purpose, plasmid pUCDV3 (Fig. 1B), which carried a 912-bp *Acc*I/*Nco*I deletion in the region, was introduced into the wild-type strain D188. Because this plasmid could not replicate in D188, selection for chloramphenicol resistance (Cmr) followed by a subsequent screening for the loss of the vector-located marker gene (*cmr*) resulted in the isolation of homogenotes that carried a deletion in pFiD188, as judged by Southern hybridization analysis (data not shown). Inoculation of such a deletion mutant on tobacco seedlings and on decapitated tobacco plants showed that it was not pathogenic (Fig. 3). This phenotype was identical to the described *fas* phenotype (Crespi *et al*., 1992), suggesting that the new ORF could control *fas* gene expression. Because of the infection phenotype and the relation of the ORF to a family of regulatory genes, the ORF was named *fasR* (for fasciation regulator), and the corresponding mutant strain was called D188?*fasR*. Introduction of a replicating plasmid, pRFDV2, covering *fasR* (Fig. 1B) in strain D188?*fasR* restored virulence (Fig. 3).

7.2.2. Expression of the *fas* locus is induced during interaction with the plant

Three replicating plasmids, pJDGV3, pJDGV4, and pJDGV5, that carry translational *uidA* (*gus*) fusions to the regions of the cytochrome P450 gene encoding the 111 aminoterminal amino acids (ORF1) and different lengths of the upstream region (Fig. 1C) were introduced into strain D188 via electroporation. Subsequently, the expression of ORF1 was determined in planta. For this purpose, 3-week-old tobacco plants were infected by vacuum infiltration with cultures of the wild-type strain and of the three recombinant *R. fascians* strains, and 3 days later, the GUS activity of extracts of the infected plants was determined (see experimental procedures). The GUS levels obtained with plasmids pJDGV3 and pJDGV5 were very high (table 3), whereas plasmid pJDGV4 showed no GUS activity (36.4 ± 25.7 compared to 61.7 ± 35.7 when no plasmid was present). These results show that the sequences located between the *Stu*I site of pJDGV3 and the *Sal*I site of pJDGV5 are not required for *fas* gene expression and narrow down the previously determined 59-end border of the *fas* operon (Crespi *et al*., 1994) by 105 bp (Fig. 1A). Because the expression levels of strains D188(pJDGV3) and D188(pJDGV5) were comparable, only plasmid pJDGV5 was used further in this study.

The next step was to monitor *fas* gene expression *in vitro*. Using D188(pJDGV5) as the test strain, the *fas* genes were shown not to be expressed in rich medium (Table 4) or in a defined medium (MinA) (data not shown). Also, the addition of plant extracts to MinA medium did not induce *fas* gene expression (Table 4). However, when leafy gall extracts were added to the medium, a 10-fold induction of expression was obtained. This result was in agreement with previous data showing that *ipt* gene expression was induced by extracts of leafy galls (Crespi *et al*., 1992).

7.2.3. Environmental signals influence *fas* gene expression

To characterize the parameters that affect expression levels *in vitro*, the influence of pH, the presence of phosphate, carbon and nitrogen sources, cell density, and oxygen concentration were examined with and without the addition of leafy gall extract. First, the role of the pH of the MinA medium at the start of the induction was evaluated. The data given in Fig. 4A showed that the induction of ORF1 was much higher at lower pH, with a peak expression level at pH 5.0. For the setting of the desired pH, either phosphate or citrate buffers were used. Thus, it became clear that the expression of the *fas* genes was negatively influenced by the presence of phosphate. Indeed, the addition of different concentrations of phosphate to citrate-buffered MinA medium at pH 5.0 significantly decreased gall-dependent induction (Fig. 4B).

Next, at pH 5.0, the glucose in MinA medium was replaced by other carbon sources (20 mM). The results show that none of the tested compounds alone (data not shown) or in combination with plant extracts led to *fas* gene expression (Table 4). Some carbon sources had no effect on gall-dependent expression (citrate, fructose, fucose, galactose, glucose, maltose, and xylose), while others increased gall expression levels (arabinose, glycerol, isocitrate,

mannitol, mannose, pyruvate, succinate, and sucrose) (Table 4).

As a third parameter, the effect of the nitrogen source was tested. For the starting medium, the optimal conditions so far determined were used (MinA medium, pH 5.0, with 20 mM succinate). Whereas none of the tested amino acids alone could induce ORF1 expression (data not shown), all of them had a negative effect on the gall-dependent induction levels (Table 5). Interestingly, the combination of succinate and histidine gave rise to very high GUS activity.

The addition of plant or gall extracts to histidine and succinate resulted in an important decrease in the induction levels (Table 6). This observation prompted us to test whether these extracts contained compounds that repressed *fas* induction. To remove common plant metabolites, leafy gall extracts were used as a nutritional source for *E. coli*. After overnight growth, the *E. coli* cells were removed by centrifugation, and the extracts were filter sterilized and subsequently used in combination with histidine and succinate. Measurement of *fas* gene expression under these conditions showed that there was indeed a partial relief of the repression of the histidine and succinate induction levels observed with complete-plant and leafy gall extracts (Table 6).

Then, the influence of cell density on *fas* expression was investigated. Cultures were used with different optical densities at 600 nm (OD600) at the start of the induction with histidine and succinate in MinA medium at pH 5.0. The results presented in Fig. 4C show a direct correlation between cell density and expression level. A similar result was obtained when leafy gall extracts were used in MinA medium at pH 5.0 (data not shown). Thus, the optimized conditions for *fas* gene expression are MinA medium at pH 5.0 supplemented with 20 mM succinate and 5 mM histidine and at a starting OD600 of 2.0.

Finally, *fas* expression was monitored under anaerobic and semianaerobic conditions. Optimized cultures (Fig. 4D) and cultures induced with leafy gall extracts (data not shown) were incubated under different oxygen concentrations. The experiment showed that low oxygen concentrations had a negative effect on *fas* expression.

7.2.4. The expression of *fasR* is constitutive

Because AraC-type transcriptional regulators are often autoregulatory (Cass and Wilcox, 1988, Hamilton and Lee, 1988), a transcriptional fusion of the upstream region of *fasR* to *uidA* was constructed (pRFDV6) (Fig. 1D). Introduction of the replicating plasmid pRFDV6 into strain D188 and D188?*fasR* and incubation under the different conditions altering *fas* gene expression showed that the overall expression of *fasR* was constitutive and comparable in the two strains (in strains D188 and D188?*fasR*, not induced [178.8 ± 21.1 and 144.8 ± 18.0, respectively] and induced with succinate and histidine [140.8 ± 18.0 and 116.3 ± 8.2, respectively]).

7.2.5. Transcription of the *fas* genes is affected by *fasR* and another pFiD188-encoded regulator

With the optimized induction conditions for *fas* gene expression set (see above), the possible regulatory role of *fasR* could be assessed. Because GUS activity from pJDGV5 is the result of the combined action of transcriptional and translational signals,

transcriptional GUS fusions were constructed. The same upstream region as in pJDGV5 was fused to a *gus* gene carrying its own translational signals, resulting in plasmid pRFWT11 (Fig. 1C). The plasmid was introduced into strains D188, D188?*fasR*, and D188-5, which is a linear plasmid-free strain, and *fas* expression was determined. Under noninduced conditions, D188(pRFWT11) showed a GUS activity level comparable to that of the translational fusion under induced conditions (Table 7). Moreover, the transcriptional activity was not affected by the addition of gall extract, by histidine combined with succinate, by any of the tested carbon and nitrogen sources, or by the pH (data not shown). However, the level of transcription did increase with the cell density (data not shown).

In strain D188-5(pRFWT11), a significant decrease in transcriptional GUS activity was observed compared to the levels measured in D188. However, the transcriptional GUS activity seemed not to be dependent on *fasR*, as shown by the constitutively high *gus* expression level in strain D188?*fasR*(pRFWT11) (Table 7). These results indicate that other regulators involved in *fas* gene expression must be located on the linear plasmid pFiD188.

To evaluate the possible importance of the promoter copy number in regulation, the transcriptional *gus* expression was determined upon integration into the genome. For this purpose, a nonreplicating plasmid was constructed carrying the same GUS fusion as in pRFWT11. This plasmid, pJBWT2 (Fig. 1C), was introduced into D188 and D188?*fasR* via electroporation (Desomer *et al.,* 1991). By Southern hybridization analysis, the plasmid was found integrated into the genome of both strains via illegitimate integration (data not shown). In strain D188::pJBWT2, the measured transcription of the *fas* genes was again constitutive, although the absolute expression level was fivefold lower than that of the replicating transcriptional GUS fusion (Table 7). Furthermore, GUS activity in D188?*fasR*::pJBWT2 was another twofold lower (Table 7), indicating that FasR does affect *fas* gene transcription.

7.2.6. The environmental modulation of *fas* gene expression is translationally controlled and requires *fasR*

Considering the nonpathogenic phenotype of D188?*fasR* and the data described above, we hypothesized that *fasR* would be involved in the translational control of *fas* gene induction. Therefore, plasmid pJDGV5 carrying a translational ORF1-GUS fusion was introduced into strains D188?*fasR* and D188-5. Measurement of the GUS activity showed that the *fas* genes could not be induced in either of the two strains (Table 7). This observation indicates that FasR is essential for regulated *fas* gene expression and that the environmental regulation must be exerted by a translational regulator that is under the control of *fasR*. The possible role of the promoter copy number was assessed with the integrating plasmid pUCWT1 (Fig. 1C) carrying the same GUS fusion as in pJDGV5. The data in Table 6 show that upon integration of the GUS fusion, the translational expression pattern was retained: in strain D188::pUCWT1, succinate combined with histidine led to the induction of the *fas* genes, and no induction could be obtained in strain D188?*fasR*::pUCWT1.

TABLE3 *In planta* expression of ORF 1 of the *fas* operon in strain D188

Plasmid	GUS activity
No plasmid	61.7 ± 35.7
pJDGV3	416.2 ± 112.8
pJDGV5	512.4 ± 9.2
pJDGV4	36.4 ± 25.7

The activity was measured with different constructs containing translational GUS fused to variable upstream regions. The data are tha average of three independent experiments. For details, see Experimental procedures

TABLE 4. **Effects of carbon sources on ORF1 expression**[a]

Carbon source (20 mM)	Growth	GUS activity	
		+ Plant extract (40 µl)	+ Leafy gall extract (20 µl)
YEB	+++	3.6 ± 0.3	3.0 ± 0.2
None	0	3.8 ± 0.5	29.6 ± 7.9
Glucose	+++	2.2 ± 0.4	36.7 ± 2.5
Fructose	+++	4.2 ± 3.9	51.8 ± 16.5
Sucrose	+++	5.4 ± 2.1	97.5 ± 28.7
Maltose	0	3.2 ± 0.9	38.1 ± 4.6
Mannitol	+++	5.9 ± 3.0	86.6 ± 5.8
Glycerol	++	2.6 ± 0.6	75.1 ± 3.7
Galactose	0	3.4 ± 1.0	38.2 ± 18.1
L-Arabinose	+++	2.6 ± 0.2	79.1 ± 12.1
D-Arabinose	0	2.3 ± 0.3	35.3 ± 12.5
Fucose	0	3.1 ± 0.5	36.5 ± 13.1
Mannose	+++	3.2 ± 0.6	96.0 ± 31.2
Xylose	0	2.4 ± 0.3	59.3 ± 24.4
Succinate	0	2.5 ± 0.2	89.6 ± 6.1
Citrate	0	4.1 ± 3.5	43.5 ± 3.1
Isocitrate	0	3.1 ± 0.1	85.4 ± 25.1
Malate	-	2.1 ± 0.1	5.3 ± 0.7
Pyruvate	0	2.5 ± 0.2	53.2 ± 7.6
a-Ketoglutarate	-	4.2 ± 0.9	4.8 ± 0.3
Glucolate	-	4.8 ± 1.0	4.6 ± 0.4
Glyoxylate	-	1.8 ± 0.2	4.3 ± 0.5
Fumarate	-	3.8 ± 0.5	5.6 ± 0.7

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0. For details, Experimental procedures. +, ++, +++, growth relative to the control condition without addition of any carbon source (0); -, negative effect of the added carbon source on bacterial growth. For details, see Experimental procedures.

TABLE 5. **Effects of amino acids on gall-induced ORF1 expression**[a]

Amino acid(s) (5 mM)	GUS activity	
	+ Plant extract (40 ml)	+ Leafy gall extract (20 ml)
None	2.5 ± 0.7	37.4 ± 5.4
Glutamic acid	3.0 ± 1.8	25.0 ± 2.6
Leucine	2.7 ± 1.0	4.4 ± 0.9
Threonine	2.4 ± 0.6	9.6 ± 2.6
Asparagine	2.5 ± 0.6	3.2 ± 1.5
Casamino Acids	1.6 ± 0.4	1.3 ± 0.4
Arginine	2.7 ± 1.0	2.3 ± 1.4
Lysine	2.7 ± 0.7	1.9 ± 0.2
Tyrosine	3.0 ± 1.2	2.0 ± 0.9
Histidine	35.2 ± 16.5	17.9 ± 5.1

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0 supplemented with 20 mM succinate. For details, see Experimental procedures.

TABLE 6. **Presence of repressing compounds in extracts affectingORF1 expression induced by succinate (20 mM) andhistidine (5 mM)a**

Compound	GUS activity
Succinate	1.8 ± 0.5
Succinate + histidine	149.3 ± 34.1
Succinate + histidine + plant extract	35.2 ± 6.5
Succinate + histidine + gall extract	17.9 ± 5.1
Succinate + histidine + depleted gall extract	52.8 ± 8.1

[a] The data are averages of three independent experiments and were measured with test strain D188(pJDGV5) in MinA medium at pH 5.0. For details, see Experimental procedures.

Table 7: Expression of ORF1 of the *fas* operon and *fas*R as measured with different test strains

Strain	pRFDV6 Replicating transcriptional	pRFWT11 Replicating transcriptional	pJBWT2 Integrated transcriptional	pJDGV5 Replicating translational	UCWT1 Integrated translational
D188/S	178.8 ± 21.1	155.3 ± 15.6	31.5 ± 4.0	4.6 ± 0.2	6.0 ± 0.2
D188/SH	140.8 ± 18.0	142.2 ± 25.8	30.8 ± 0.6	158.4 ± 8.0	158.4 ± 8.3
D188ΔfasR/S	144.8 ± 18.0	162.3 ± 29.7	17.3 ± 1.6	4.1 ± 0.2	7.2 ±0.3
D188ΔfasR/SH	116.3 ± 8.2	131.0 ± 28.3	16.7 ± 2.6	3.9 ± 0.5	7.3 ±0.4
D188-5/S	ND	60.9 ± 30.4	ND	6.5 ± 1.5	ND
D188-5/SH	ND	61.1 ± 33.9	ND	6.2 ± 1.0	ND

In MinA medium at pH 5.0 and a start OD_{600} 2.0. /S, not induced condition with the addition of 20 mM succinate; /SH, induced condition with the addition of 20 mM succinate and 5 mM histidine; ND, not determined. pRFDV6 is a *fas*R construct, pRFWT11, pJBWT2, pJDGV5, and pUCWT1 are *fas* constructs.

7.4. Discussion

We have characterized a regulatory gene in *R. fascians*, *fasR*, that belongs to the AraC family of transcription regulators (Fig. 2) (Gallegos *et al*., 1997) and proves to be essential for leafy gall formation (Fig. 3). AraC-type regulators have been shown to regulate virulence genes in the gram-negative phytopathogens *Ralstonia solanacearum* (Genin *et al*., 1992), *Pseudomonas syringae* pv. Phaseolicola (Zhang *et al*., 1997), and *Xanthomonas campestris* (Wengelnik and Bonas, 1996), as well as in several animal pathogens (D'Orazio and Collins, 1995, Kaniga *et al*., 1994, Porter *et al*., 1998, Skorupski and Taylor, 1997, Wattiau and Cornelis, 1994). Typically, the regulatory characteristics exerted by this class of proteins are very complex, with the regulators acting as transcriptional activators or repressors, depending on the growth conditions, their cellular concentrations, the relative positions of their binding sites in the promoters they regulate, and the presence of particular signals. Because a *fasR* deletion mutant, D188D*fasR*, is nonpathogenic and exhibits the same phenotype on plants as a *fas* mutant, it was hypothesized that FasR would regulate *fas* gene expression.

To obtain higher expression levels in batch culture, several parameters had to be adjusted. As a result, *fas* gene expression could be induced upon addition of leafy gall extract, but the highest expression level was obtained in MinA medium at pH 5.0 (Fig. 4A) to which a combination of succinate and histidine was added (Table 6) and with an initial starting OD600 of 2.0 (Fig. 4C). The observed pH optimum is not surprising, because plant fluids are slightly acidic and high *fas* gene expression under these conditions would enable interference with the development of the plant. In other pathogens, virulence gene expression is often correlated with the pH conditions met in the host (Mantis and Winans, 1992, Rahme *et al*., 1992, Singh *et al*., 1997).

Several carbon sources had a positive effect on the galldependent induction levels. Some of these carbon sources (arabinose, fructose, glucose, glycerol, mannitol, mannose, and sucrose) also had a promoting effect on bacterial growth (Table 4). Because there is a positive correlation between cell density and induction level (Fig. 4C), the observed effect of these compounds might be mediated via cell

growth. Nevertheless, other carbon sources (isocitrate, pyruvate, and succinate) had no promoting effect on bacterial growth but still augmented gall-dependent induction levels (Table 4). These carbon sources are Krebs cycle intermediates, which might be related to the function of the *fas*-encoded proteins. In this respect, our working model states that part of the *fas* operon constitutes an electron transport chain that delivers high-energy electrons for the cytochrome P450 reaction (Goethals *et al*., 1995). The presence of the Krebs cycle intermediates might signal that the substrates for P450 activity are available and, in a dual function, lead to the stronger induction of the *fas* operon. Alternatively, in the acetosyringone-mediated induction of the *vir* genes of *Agrobacterium tumefaciens*, several monosaccharides exhibit a synergistic effect (Cangelosi *et al*., 1990, Shimoda *et al*., 1990). A similar observation has been made for the phenolic-induced expression of the *syrB* gene of *P. syringae* pv. syringae (Mo and Gross, 1991). In the case of the leafy gall extract-mediated *fas* gene expression in *R. fascians*, the carbon sources could have an analogous function. The observation that a combination of histidine and succinate also strongly induces *fas* gene expression is puzzling. Possibly, both leafy gall extracts and histidine-succinate provoke a specific metabolic state of the bacteria in which *fas* gene expression is high. In this hypothesis, such conditions would not prevail in plant extracts.

Interestingly, histidine also induces *fas* gene expression in combination with the carbon sources that do not promote bacterial growth but that are synergistic on the leafy gall-dependent induction levels (Table 5). As a corollary, histidine could be hypothesized to be an actual inducing factor present in leafy gall extracts. Preliminary amino acid analysis of uninfected and infected plant tissues did not reveal an apparent increase in histidine levels upon infection with *R. fascians* (data not shown); nevertheless, histidine might be a functional analogue of a putative inducing factor. To date, no further data are available to favor any of these hypotheses.

The higher expression levels obtained at higher cell densities (Fig. 4C) might at first sight resemble quorum sensing. However, *fas* gene expression can also be induced at low cell densities, and the expression levels gradually increase with cell density. These data indicate that the increased expression of the *fas* genes functions via a mechanism that differs from the cell density-dependent expression of LuxR-LuxI-homologous systems, in which a critical cell density is required (Fuqua and Greenberg, 1998, Pierson *et al*., 1998). A possible explanation for our results is that a higher cell density or the presence of some carbon sources alters the metabolism
or the physiological state of the bacteria, rendering them more prone to express the *fas* genes.

Besides the positive effects of carbon sources and cell density, phosphate and amino acids had a negative influence on gall-dependent *fas* gene expression (Fig. 4B and Table 5). In *A. tumefaciens*, a similar, albeit more drastic, effect of phosphate was observed for *virG* expression (Winans, 1990). Because phosphate is often very scarce in nature, its limitation could be a signal for the bacterium to interact with the plant to produce galls that may serve as phosphate sources. Crude plant and leafy gall extracts proved to repress the high induction levels obtained with histidine and succinate. Removal of

general metabolites from leafy gall extracts by depletion with *E. coli* partially relieved this inhibitory effect (Table 6). The inhibitory activity of gall extracts on histidine and succinate induction could be interpreted as a result of catabolite repression. Inhibition of gene expression by nitrogen sources has been reported in *Bacillus subtilis* (Atkinson *et al*., 1990, Fouet *et al*., 1990); in these cases, the mechanism involves regulation of transcription initiation (Wry *et al*., 1994). We have shown that several general amino acids inhibit *fas* gene induction by gall extracts (Tables 5 and 6) or by histidine and succinate (data not shown). Because gall and plant extracts represent a rich mixture of several general metabolites, such catabolite repression could account for the lower expression levels obtained by combining histidine and succinate with these extracts. Following overnight growth of *E. coli* on such extracts, the resulting depletion of metabolites can be assumed to relieve catabolite repression, which could explain the higher *fas* gene expression levels obtained by combining histidine and succinate with such depleted extracts.

To unravel the regulatory circuits controlling the induction of the *fas* genes, translational and transcriptional GUS fusions to ORF1 were constructed, on both replicating and integrating plasmids (Fig. 1C and Table 2), and the expression patterns were determined in strain D188, the plasmid-free strain D188-5, and strain D188D*fasR*. With the replicating transcriptional fusion in strain D188, *fas* expression was constitutive independently of the pH and carbon or nitrogen sources and 30-fold higher than that measured with the translational fusion under noninducing conditions (Table 7). This result shows that under noninducing conditions translation is repressed and that *fas* gene expression is controlled at the translational level. Comparison of the transcription levels in strains D188 and D188-5 further suggested that a second transcriptional regulator besides FasR is located on pFiD188. Integration of the transcriptional fusion into the genome of strains D188 and D188D*fasR* resulted in lower expression levels and showed that FasR also had a positive effect on *fas* gene transcription. The fact that this result was not observed when the replicating plasmid was used suggests that the effect of the regulatory protein is titrated out because of multiple copies of the *fas* promoter. For the translational fusions, similar results were obtained with the replicating and integrated constructs. This observation could be explained by assuming that one or more *trans*-acting factors that are involved in translational regulation are present in limiting amounts only. In strain D188, *fas* gene expression was induced and modulated by environmental factors. However, in strain D188-5 and D188D*fasR*, no induction could be obtained (Table 7). Together, these results indicate that *fas* gene expression is subject to a complex regulatory network incorporating different regulatory loci acting at the transcriptional and translational levels. Thus, the phytopathogen can cope with the variable conditions that it encounters during interaction with its host plant. In this regulatory network, *fasR*, which encodes a transcriptional regulator, plays a crucial role in the induction of *fas* gene expression, which is modulated at the posttranscriptional level. The mechanism of this regulation is currently unknown, but it could be the result of a modulation of RNA or protein stability or of translation initiation. Whatever the mechanism, the factors that control it have to be themselves under control of the *fasR* gene, either directly or indirectly.

Based on the data obtained we can propose a working model for the regulation of *fas* gene expression. In this model, the induction of gene expression is controlled at the translational level and requires FasR. The translational regulator is encoded by the linear plasmid, and its transcription is regulated by FasR. The induction of the *fas* genes is probably mediated by the interaction of one or more inducing compounds present in infected plant tissue with the translational regulatory protein or with FasR. Furthermore, FasR activates *fas* gene transcription. Finally, a second transcriptional activator of the *fas* genes is present on the linear plasmid. Although the majority of regulatory networks, which often control very complex processes in bacteria, consist of only transcriptional regulators (Bibb, 1996, Piggot *et al*., 1994), the interplay of transcriptional and translational regulators that direct the expression of specific pathways has been reported (Klauck *et al*., 1997). The regulation of *fas* gene expression is another example of the latter. Based on the low G+C content of *fasR* and on the apparently superimposed function of FasR on other regulatory pathways, we speculate that *fasR* might have been acquired relatively late during the evolution of *fas* gene regulation in *R. fascians*.

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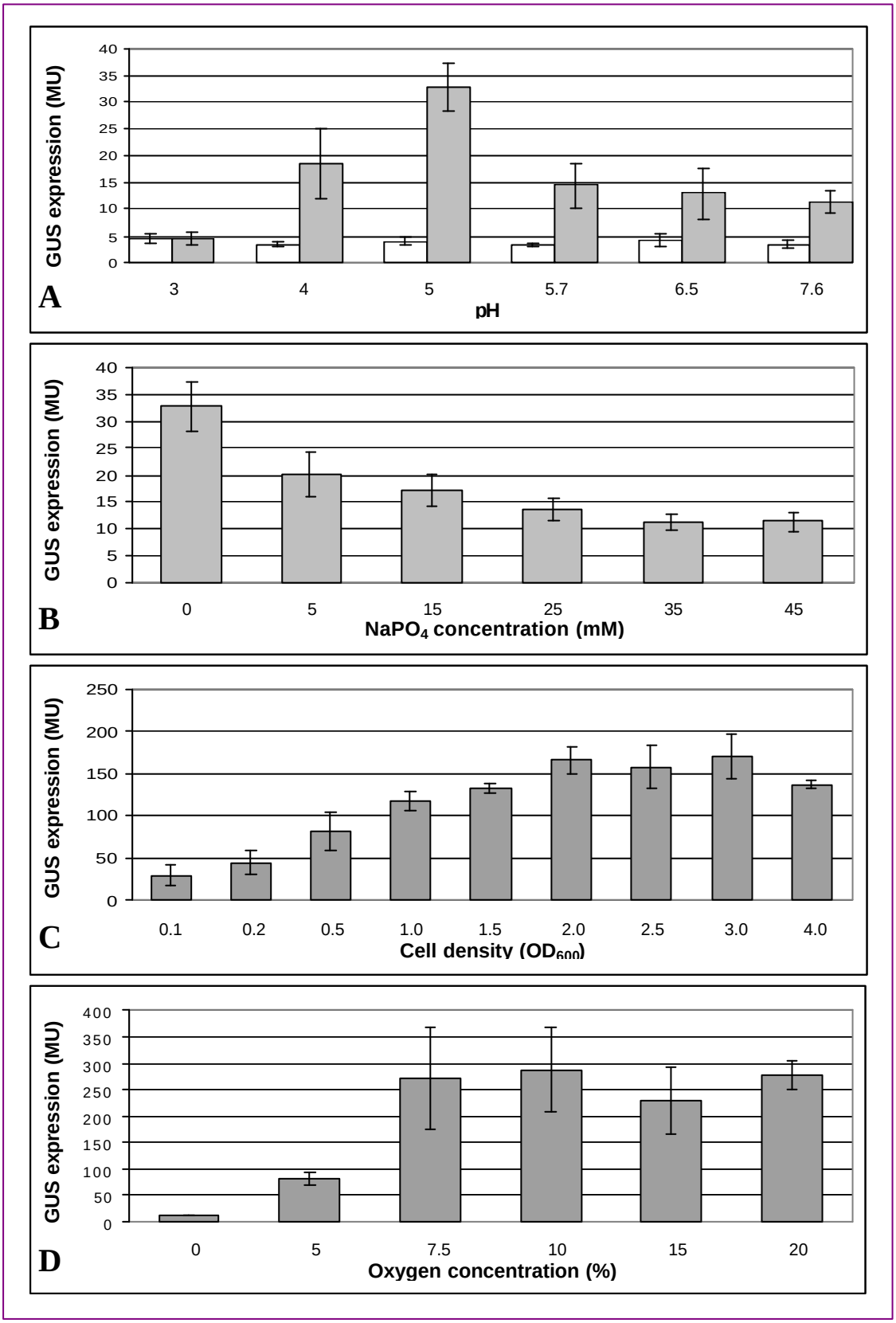


Figure 4: Effect of different conditions on ORF1 expression as measured with test strain D188[pJDGV5].
Effects of pH (A), phosphate (B), cell density (C), and of O_2 concentration (D) in MinA medium with glucose (A and B) and with 5 mM histidine and 20 mM succinate (C and D) at pH 5.0 (B, C, and D) and at OD_{600} (D). With plant extract (white), with leafy gall extract (grey), without extract (hatched).

Chapter Eight

Observation and characterization of some aberrations in the developmental program of *Arabidopsis thaliana* as a consequence of the infection by *Rhodococcus fascians*

Abstract

The interaction between *Rhodococcus fascians* and the crucifer *Arabidopsis thaliana* was studied in more detail. The phenotypes manifesting after bacterial infection on some well-characterized ecotypes were observed and compared. Besides the shooty phenotype, situated in the rosette, when infection occurs while plants are in the vegetative state, *R. fascians* is able to induce severe malformations of the inflorescence and the flowers to be formed. Moreover, interaction between both the non-virulent and the virulent bacterial strain results in precocious onset of flowering. This phenomenon was more closely inspected by use of some late-flowering plant mutants and indicates that the flower-promotive effect is situated at the level of the gibberellin biosynthesis pathway. Our results further substantialize the vision that *R. fascians* is able to cause a redefinition of the plant's hormone balance, hereby giving raise to a wide variation of morphological and developmental changes.

8.1. Introduction

When encountering a potential host plant, the phytopathogenic bacterium *Rhodococcus fascians* is capable to induce some drastic changes in plant development. In general terms, the interaction between the two species leads to the loss of apical dominance and the appearance of numerous, malformed shoots at the site of infection. The most thoroughly characterized symptom up till now is the leafy gall, a mass of proliferating tissue covered with multiple small shoots that are inhibited in their further outgrowth.

Only recently, it has been demonstrated that *Arabidopsis thaliana* belongs to the host range of *R. fascians*, which at present is known to encompass at least 39 families and 86 genera covering both dicotyledonous and monocotyledonous plants (Vereecke *et al*., 2000; Vereecke, personal communication). In agreement with earlier reports on the diversity of symptoms depending on plant family, genus, variety and age as well as the infection method applied (Lacey, 1939; Eason *et al*., 1995; Vereecke *et al*., 2000), the symptoms provoked on *A. thaliana* differ from the typical leafy gall. Instead, the majority of infected *Arabidopsis* plants have a bushy appearance as a consequence of the formation of a multitude of inhibited shoots in the rosette.

The finding that *A. thaliana* is readily infected by *R. fascians* is of considerable value for further experiments towards understanding the etiology of the disease symptoms. In recent years, *A. thaliana* has become the most frequently used model plant for plant molecular biology. As a consequence, the scientific community invests increasingly in research efforts toward an overall characterization of this small weed. There is a continuously growing collection of generated mutants. For the elucidation of the plant processes affected by *R. fascians* infection, hormone mutants are of particular interest, since many phenotypical observations indicate that *R. fascians* influences the plant's hormone balance (as discussed in more detail in section 6.3.).

The study presented in this chapter primarily aimed at a more thorough phenotypical characterization of the symptoms observed on *Arabidopsis* after infection with *R. fascians*. This effort will reveal eventual parallels between the developmental changes induced on *A. thaliana* and on tobacco required for the implementation of *A. thaliana* in the *R. fascians* research. The influence of age and ecotype on the infection phenotype is evaluated and some uncommon symptoms, such as an earlier flowering, are described. In a second part, the effect of *R. fascians* infection of hormone and flowering mutants is analyzed. The results obtained are a demonstration of the influence of this phytopathogenic bacterium on the plant's hormone balance, which consequently will lead to distinct changes in plant development.

8.2. Experimental procedures

8.2.1. Bacterial strains and growth conditions

The *R. fascians* strains used for this study are the wild type strain D188, carrying the linear plasmid pFiD188 necessary for full virulence, and the non-virulent strain D188-5, which has been cured from pFiD188 (Desomer *et al*., 1988). Cultivation of *R. fascians* is optimal at 28°C in Yeast Extract Broth medium (YEB; Miller, 1972).

8.2.2. Plant material and cultivation

All *Arabidopsis* seeds were kindly provided by the Nottingham *Arabidopsis* Stock Center (NASC) and the *Arabidopsis* Biological Resource Center (ABRC). C24, Landsberg *erecta* (Ler) and Columbia were used as wild type ecotypes. The hormone mutants that were studied were all generated in a Ler background, except for *spy1*, which is generated in a Columbia background: the gibberellin responsive dwarf *ga4* (NW61; Koornneef and van der Veen, 1980), the gibberellin responsive dwarf *ga5* (CS62; Koornneef and van der Veen, 1980), the gibberellin insensitive dwarf *gai* (NW63; Koornneef *et al*., 1985), the GA responder *gar2* (CS8078; Wilson and Somerville, 1995) and the mutant *spy1* with increased elongation as a cause of altered GA signal transduction (CS6266; Jacobsen and Olszewski, 1993). The late flowering mutants have the Ler genetic background (Koornneef *et al*., 1991): *co* (NW55), *fca* (NW52) and *fve* (N107). These three mutants flower later with proportionally more rosette leaves

Vernalized seeds were surface sterilized by sequential washings with 70% ethanol (2'), 5% hypochloride solution with 0.5% Tween-20 (10') and 5 times sterile distilled water. Plants were grown *in vitro* on solid medium (0.8% agar) at pH5.7, consisting of the basic Murashige and Skoog salts (1XMS; Duchefa), supplemented with 0.5% MES, 1% sucrose and the vitamins myo-inositol (100µg/ml), nicotinic acid (0.5µg/ml), pyridoxine (0.5µg/ml) and thiamine (1µg/ml). Growth chamber conditions are 22°C and 16 hours of light (long day conditions) or 8 hours of light when performing experiments under a so-called short day regime.

8.2.3. Infection protocols

Infections with *R. fascians* were carried out with actively dividing *R. fascians* cultures of 2 days old (inoculated in 100ml erlenmeyer flasks). After washing away the YEB medium, the bacteria were concentrated 4 times in MinimalA medium (6.4mM KH_2PO_4, 33.6mM K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, 0.025% $MgSO_4$, 0.001% thiamine) at pH5.0.

Two strategies were applied for the infection of *Arabidopsis* plants. In the initial experiments, plants were vacuum infiltrated for 1 minute with a concentrated bacterial suspension. Later on, when it was observed that a simple dipping of the plants is sufficient for successful bacterial infection, this method became commonly used. The plants can easily be manipulated by using cell strainers (Falcon),

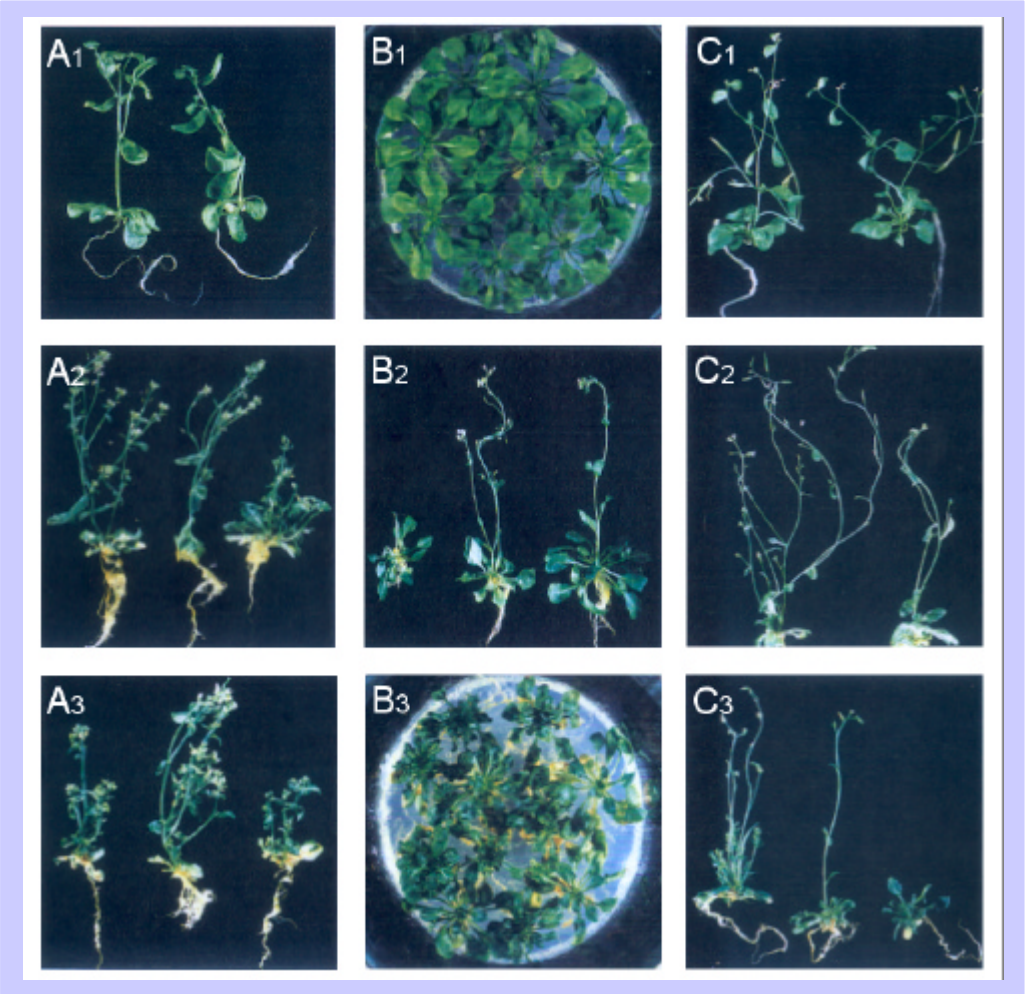


Figure 1: Fenotype observed on 7 week old *A. thaliana* plants infected at 3 weeks post germination
A1-B1-C1: uninfected controls, respectively on ecotypes Landsberg *erecta*, C24 and Columbia
A2-B2-C2: infection with strain D188-5, respectively on ecotypes Landsberg *erecta*, C24 and Columbia
A3-B3-C3: infection by strain D188, respectively on ecotypes Landsberg *erecta*, C24 and Columbia

8.2.4. Hormone synthesis blockers

As a gibberellin biosynthesis blocker, paclobutrazol (Duchefa) was used at a concentration of 0.25mg/l. The ethylene blockers silver ($AgNO_3$) and aminovinylglycine (AVG) were applied at a concentration of 5.10^{-5}M and 5.10^{-6}M respectively.

The plants were subjected to the different treatments immediately after their infection with *R. fascians.* The different products were added to the plant growth medium.

8.2.5. Microscopical analysis

In order to perform a histochemical analysis, fixed plant material was embedded in

paraplast. Staining was done with alcian green safranin as described by Gurr (1965) and the reagents were purchased from Sigma. Slides with sectioned paraplast-embedded plant material were mounted with DePeX-mounting medium (BDH, Poole, UK).

Microscopical observations of selected slides were made using either a Stemi SV11 Apo binocular (Zeiss) or a Diaplan microscope (Leitz, Wetzlar, Germany).

8.3. Results

8.3.1. The establishment of a phenotype on *A. thaliana* upon infection with *R. fascians* depends on the plant ecotype and age

Because *Arabidopsis* is a rosette plant of rather small size, it was practically impossible to decapitate them in the vegetative phase as is commonly practised for the infection of tobacco plants. Hence, the optimalisation of a *bona fide* infection protocol was compulsory in order to generate reproducible phenotypes for *A. thaliana*. Vacuum-infiltration with a dense bacterial suspension, which was already used to induce symptoms on tobacco plants, proved to be a suitable method. The protocol was adapted to infect dozens of plants in one single round under sterile conditions. Later on, it was observed that simple dipping of plants in a bacterial suspension was sufficient to produce similar results.

Additionally, the possible influence of the plant's age and ecotype in the establishment of the infection phenotype were tested. Therefore, vacuum infiltrations or dippings were performed with *Arabidopsis* plants of the 3 most commonly used ecotypes (C24, Landsberg *erecta* and Columbia) at the age of 1, 3 or 5 weeks after sowing.

The phenotypes on plants infiltrated at 1 or 3 weeks after germination are comparable. Under these circumstances, the success-rate of infection is 100% and the symptoms become clearly visible after 2 or 3 weeks of infection. On the other hand, infection at the age of 5 weeks does not guarantee the establishment of a uniform phenotype. A considerable number of plants (30%) do not seem to be affected, whereas on other plants shoot formation is only moderately induced. Only 20% of the plants show typical symptoms similar to those induced on plants infected at a younger age.

Figure 1 shows the symptoms observed on the plants that were 3 weeks old when they were infected 28 days after infiltration. The ecotype C24 seems to be most sensitive to *R. fascians* and experiences a severe reduction of elongation, a loss of apical dominance and the development of multiple inhibited shoots in the axils of the rosette leaves. The massive amplification of shoots and the formation of many young leaves, leads to a characteristic bushy phenotype. Additionally, leaf development is seriously affected, characterized by serrated leave margins, a swollen main vein with no side nerves and a reduced size of the leaf lamina. When development progresses into the generative phase on 5% of the plants, elongation

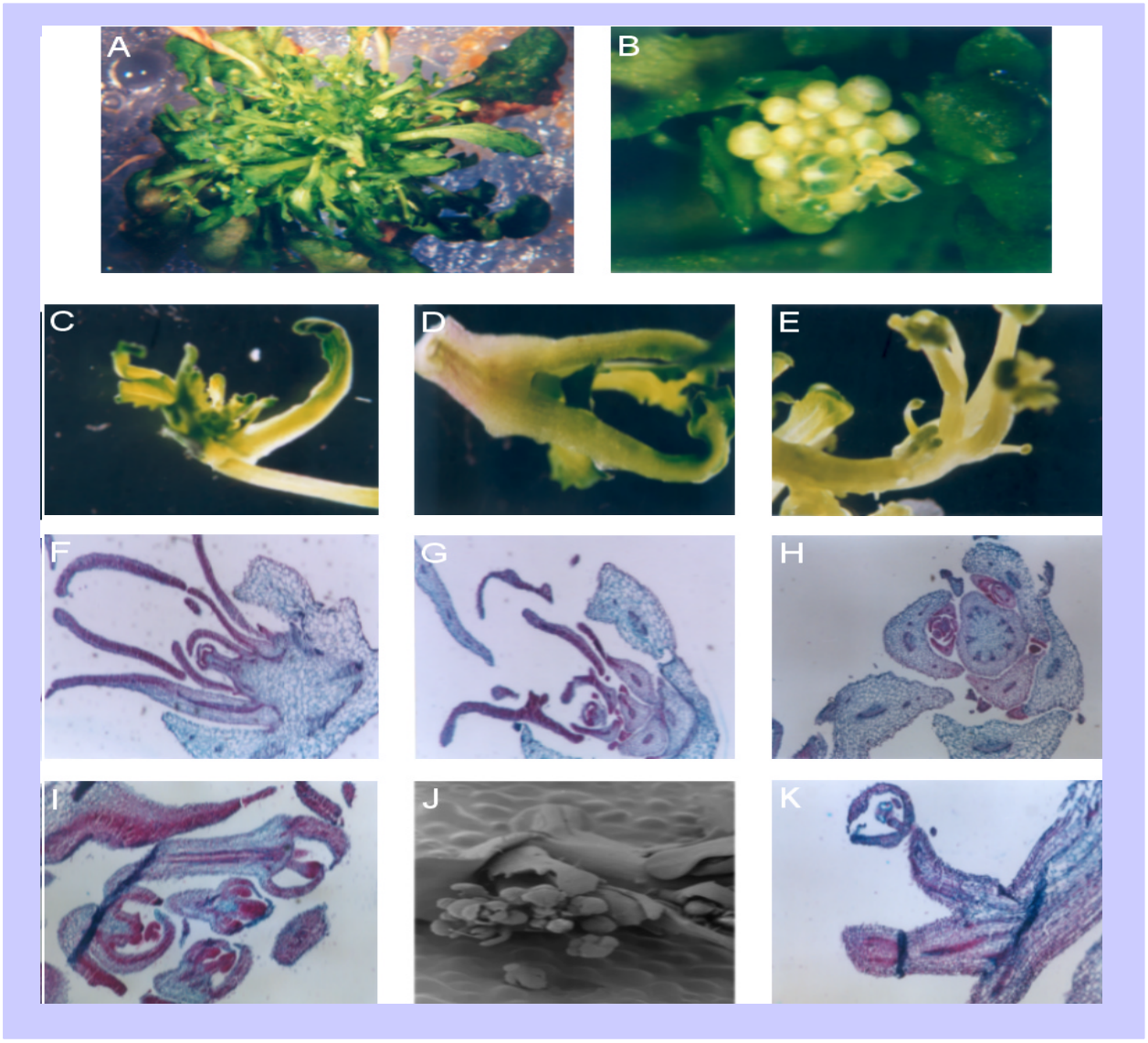


Figure 2: Phenotype observed on C24 plants after infection by the virulent *R. fascians* strain D188

- Overview picture of the plant after 6 weeks of infection,demonstrating their bushy appearance. (B) Close up of flower buds in the rosette. (C) Activated axil in the rosette. (D) serrated leaves with a thickened vein. (E) inhibition of elongation leads to extreme short flower stalks. (F-K) microscopical observations. (F-G) Strongly inhibited elongation of outgrowth. (H) activation of axils in the rosette. (I) section through bunches of flower buds. (J) SEM picture of a bunch of flower buds. (K) flower buds arrested in development
- For the often observed aberrations in flower development, this is similar as observed on Landsberg *erecta* plants (see figure 3)

of an inflorescence is observed. These inflorescences carry both normal flowers as well as abnormal flowers (as discussed in 8.3.2.). The normal flowers produce siliques with viable seeds, whereas abnormal flowers are not capable to produce any seed. On the majority of the plants, the inhibited shoots in the rosette will form bunches of flowers buds. Later on, normal flowers are observed to be formed from these buds. However, a considerable part of the formed flower buds is arrested in further development (section 8.3.2.).

Infections of the ecotypes Landsberg *erecta* and Columbia result in a different phenotype compared to C24. The two ecotypes produce equivalent symptoms, but in general Columbia seems to be less affected by the bacterium. In both ecotypes, the rosette is only

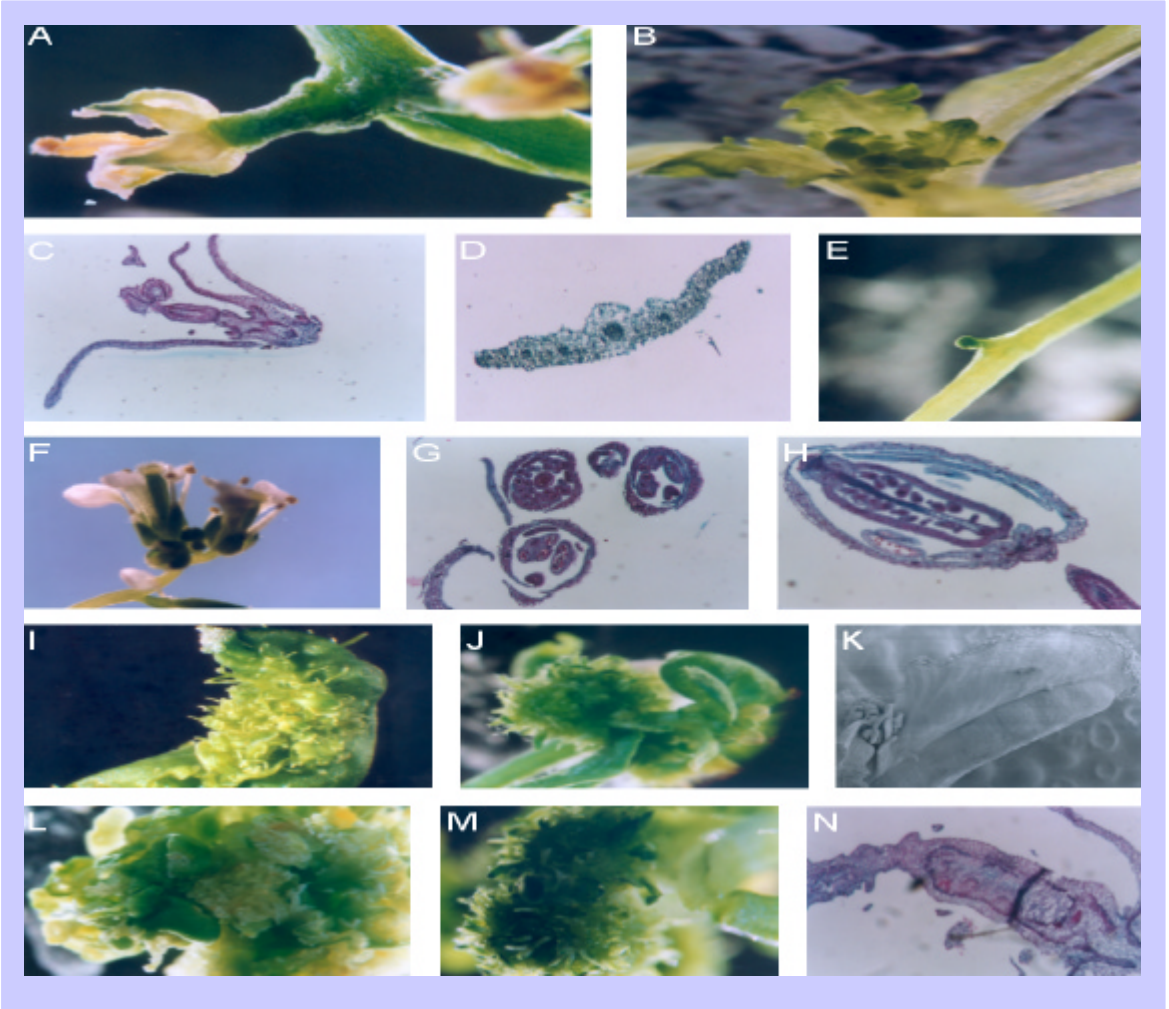


Figure 3: Aberrations observed on Landsberg *erecta* after infection with *R. fascians* strain D188
(A) Close up of a fleshy inflorescence. (B) activated axil of an inflorescence with bunch of flower buds. (C) and (N) microscopical section through a deformed flower, showing that there is no ovule development, but wildgrowth of tissue. (D) section through fasciated flower stalk. (E) aborted flower bud. (F-H) as a comparison, close up and microscopical section of a normal flower. (I) close-up of a deformed flower, with massive development of papillate tissue at the site where the 2 carpls fuse. (J, L, M) flower galls. (K) SEM picture of a deformed flower

slightly affected, there is no massive amplification of shoots and leaves and one or more inflorescences are formed that carry deformed flowers among normal flowers (similar to C24 plants). The development of co-inflorescences is often inhibited, characterized by the hampering of their elongation and the subsequent development of bunches of flower buds. With time, these flower bunches will often evolve to a gall-like structure similar to leafy galls observed on tobacco plants. On both ecotypes, inflorescences can be swollen and fasciated.

A general observation made on all ecotypes tested is the earlier bolting of infected plants compared to the control batch. Interestingly, this phenomenon is most pronounced upon infection with the non-virulent strain D188-5 (as shown in figure 1). In plants infected with the virulent strain D188, this effect is intermediate.

8.3.2. Detailed description of plant developmental changes as a consequence of infection

In order to gain further insights in the developmental changes observed upon infection of *A thaliana* plants with the virulent *R. fascians* strain D188, plants were infected 3 weeks after germination and material was isolated for histochemical analysis after 6 weeks of infection. For a detailed description of the changes observed in the vegetative phase (i.e. the rosette), material was harvested from infected C24 plants. Plants of the ecotype Landsberg *erecta* were considered to be most appropriate for the examination of aberrations in generative development.

8.3.2.1. Infection of C24 plants boosts shoot formation in the rosette

The infection of C24 plants with strain D188 leads to the formation of an extremely bushy plant. This phenotype is the result of an inhibition of elongation combined with the loss of apical dominance, with shoots originating from activated axillary meristems (figure 2). Newly formed leaves mainly consist of a thickened vein with almost no leaf lamina.

After floral transition, the formed inflorescence stalks do not elongate and hence bunches of flower buds arise. Although the flower buds can evolve to normal flowers, a further development is aborted in many cases, giving rise to flowers in which the outermost two whorls are well developed, but the inner whorls, carrying the reproductive organs, stay primordial. Figure 2 illustrates these observations by means of close up pictures with the binocular microscope and microscopical sections through different plant parts.

8.2.3.2. Effects of *R. fascians* on the inflorescence of Landsberg *erecta*

Although the formation of normal flowers occurs, the infection of Landsberg *erecta* plants with *R. fascians* in general leads to the formation of shorter and thicker -sometimes fasciated- inflorescences compared to uninfected control plants. Co-inflorescences become severely affected in their outgrowth, forming bunches of malformed flowers, and the associated cauline leaves appear deformed and serrated. Regarding size and organ number, the first three whorls of the malformed flowers have been developed in a normal fashion, whereas in the fourth whorl, the development of the carpels is dramatically affected, resulting in an extremely large gynoecium. Moreover, no ovules are formed at the margins of the carpels, but instead there is an uncontrolled proliferation of carpel tissue. Additionally, the stigmatic pappillae are not only found on top of the gynoecium, but also along the region where the two carpels fuse. As a consequence, these flowers do not produce normal siliques with viable seeds. In a later stage, in over 50% of the cases the plant organs at the axillary meristems of the inflorescence evolve to a big gall-like structure, which we term a “flower gall” in analogy to the leafy gall. The inner tissue of the structure consists completely of proliferating meristematic cells. From the most external tissue layers, deformed sepals and petals as well as cells with multiple needle-like pappillate cells are formed.

Further along the main inflorescence, many aborted flower buds are observed. On top of the inflorescence, normal flowers, producing

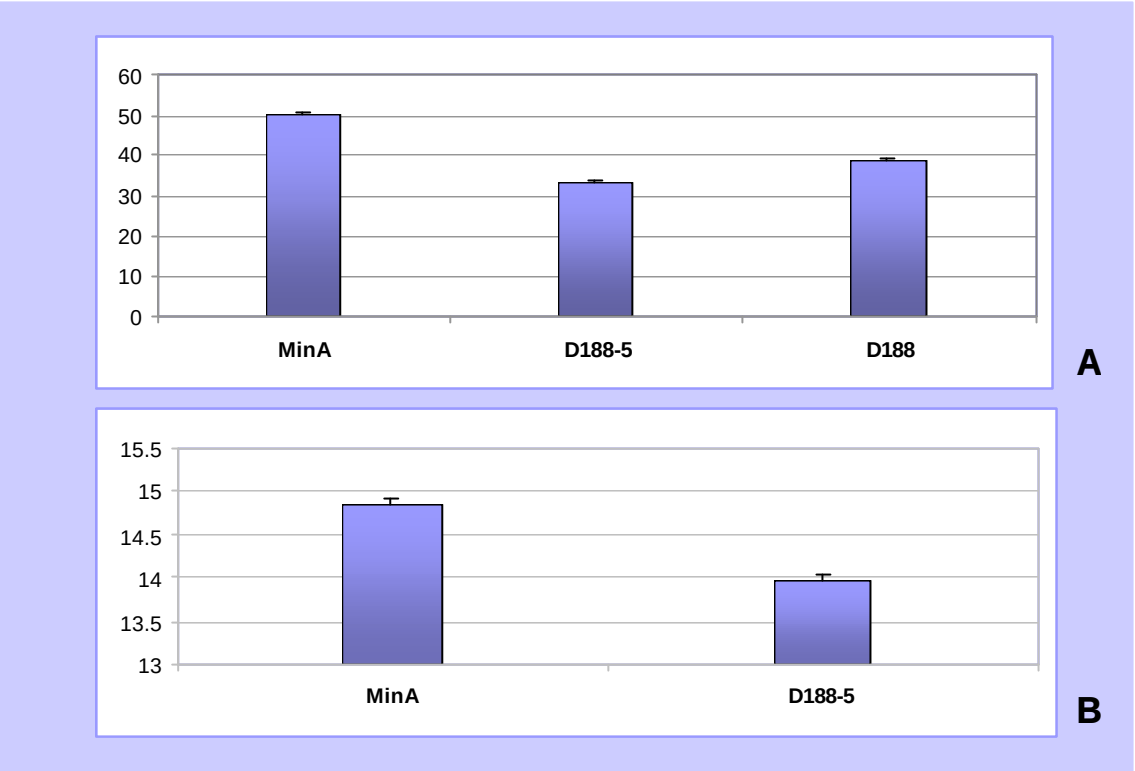


Figure 4: Monitoring transition to flowering in C24 (panel A) and Landsberg *eracta* (panel B)
The data are represented as the average age (in days) of the plants at the moment of flowering

viable seeds, co-exist with deformed flowers as described for the inhibited co-inflorescences.

The phenotypes discussed in these sections are illustrated in figure 3.

8.3.3. Analysis of the flowering-inductive behavior of *R. fascians* on plants

8.3.3.1. The interaction between *R. fascians* and *A. thaliana* leads to premature flowering

To confirm the observed difference in the onset of flowering upon infection with *R. fascians*, another series of infiltrations and dippings were performed with both C24 and Landsberg *erecta* plants. To verify whether these observations could be the result of stress caused by the infection procedure, infected plants were placed on media containing aminovinylglycine (10^{-6}M) or silver (10^{-5}M), blocking respectively the formation and perception of ethylene, known as a precursor of stress symptoms. Under these circumstances a comparable effect was registered, indicating that the early entrance into the generative phase is independent of this stress hormone (data not shown). Figure 4 shows the obtained results, when transition to flowering was monitored on a regular basis after infection. For C24 plants, compared to the controls, the vegetative phase is shortened by approximately 16 days upon infection with the non-virulent strain D188-5 and by 11 days upon infection with the virulent strain D188. For the Landsberg *erecta* plants, a similar phenomenon is observed, although there was only a one day difference between control plants and plants

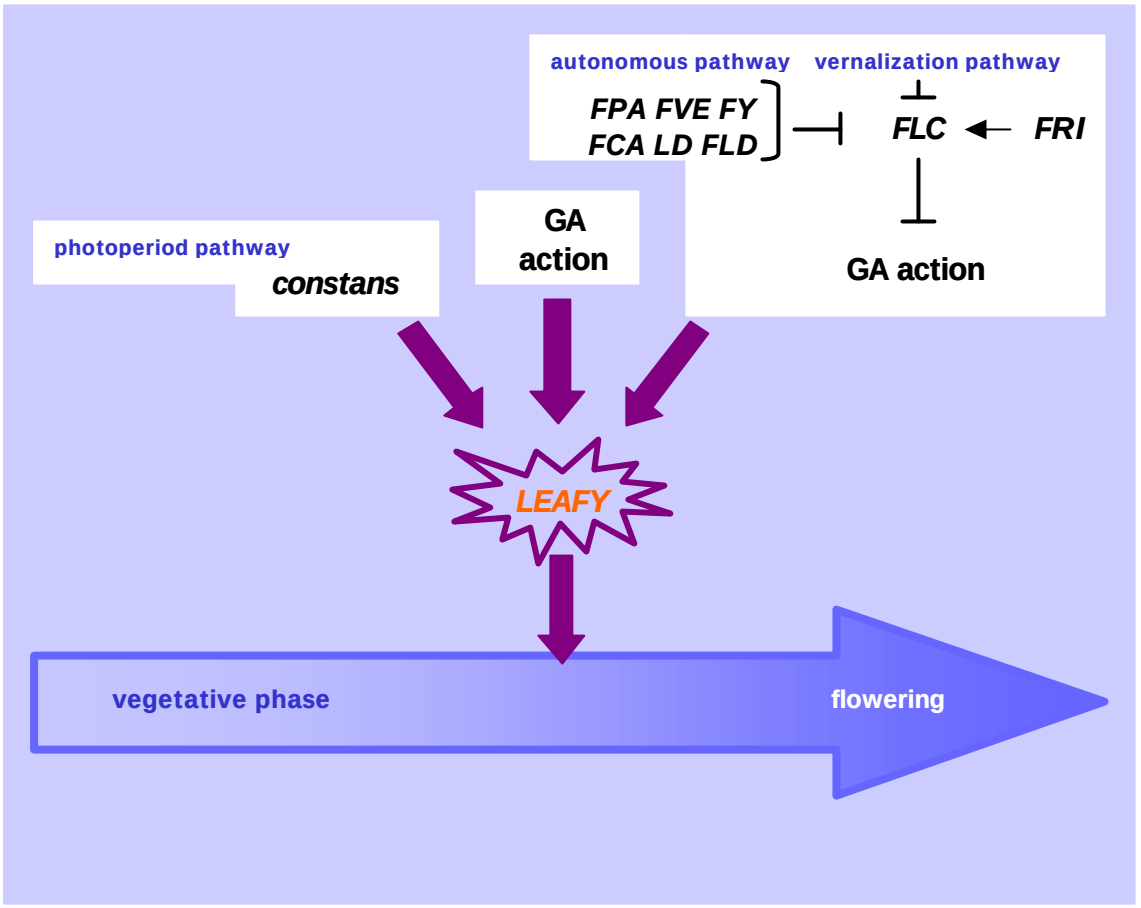


Figure 5: Pathways leading to the transition of flowering

infected with D188-5 or D188 (data not shown). When plants are grown under short day regime, D188 induces flowering slightly later than D188-5 for both ecotypes (data not shown).

8.3.3.2. Analysis of the flowering-inducing capacities of *R. fascians* by means of flowering and hormone mutants of *Arabidopsis thaliana*

Transition to the generative phase is considered as the most dramatic change in a plant's life cycle. A complex genetic network monitors both the developmental state of the plant and some environmental parameters such as light and temperature (reviewed recently by Araki, 2001). The characterization of so-called flowering-time mutants, mainly in *Arabidopsis*, made it possible to situate at present about 80 genes in the multiple pathways that control floral transition (figure 5). Signals from the environment are mediated by the photoperiod pathway and the vernalization pathway. It is assumed that the autonomous flowering pathway registers endogenous cues from the developmental state and genetic and physiological studies indicated that gibberellins (GA) are involved. For example, the GA deficient *ga1* and insensitive *gai* mutants are late flowering under short day conditions, while *spy*, in which the GA signaling is constitutively activated, flowers early (Jacobsen and Olszewski, 1993). However, it is still not

clear whether GAs exert their influence via or in parallel with the autonomous pathway, since it has been demonstrated by Koornneef and coworkers (1991) that *ga1* tends to flower earlier under long day conditions than mutants in some genes of the autonomous pathway, such as *fca* and *fve*. Therefore, it remains to be determined whether GA biosynthesis and signal transduction form a distinct promotion pathway. Ultimately, the multiple flower promotion pathways converge leading to the upregulation of the floral meristem identity genes, such as *LEAFY* and *APETALA-1*, upon which the vegetative meristems turn into inflorescence meristems and give raise to the formation of a inflorescence.

The public availability of many *Arabidopsis* mutants impaired in flowering allowed us to dissect in more detail which flowering pathway(s) might be influenced upon interaction with *R. fascians*. To reduce

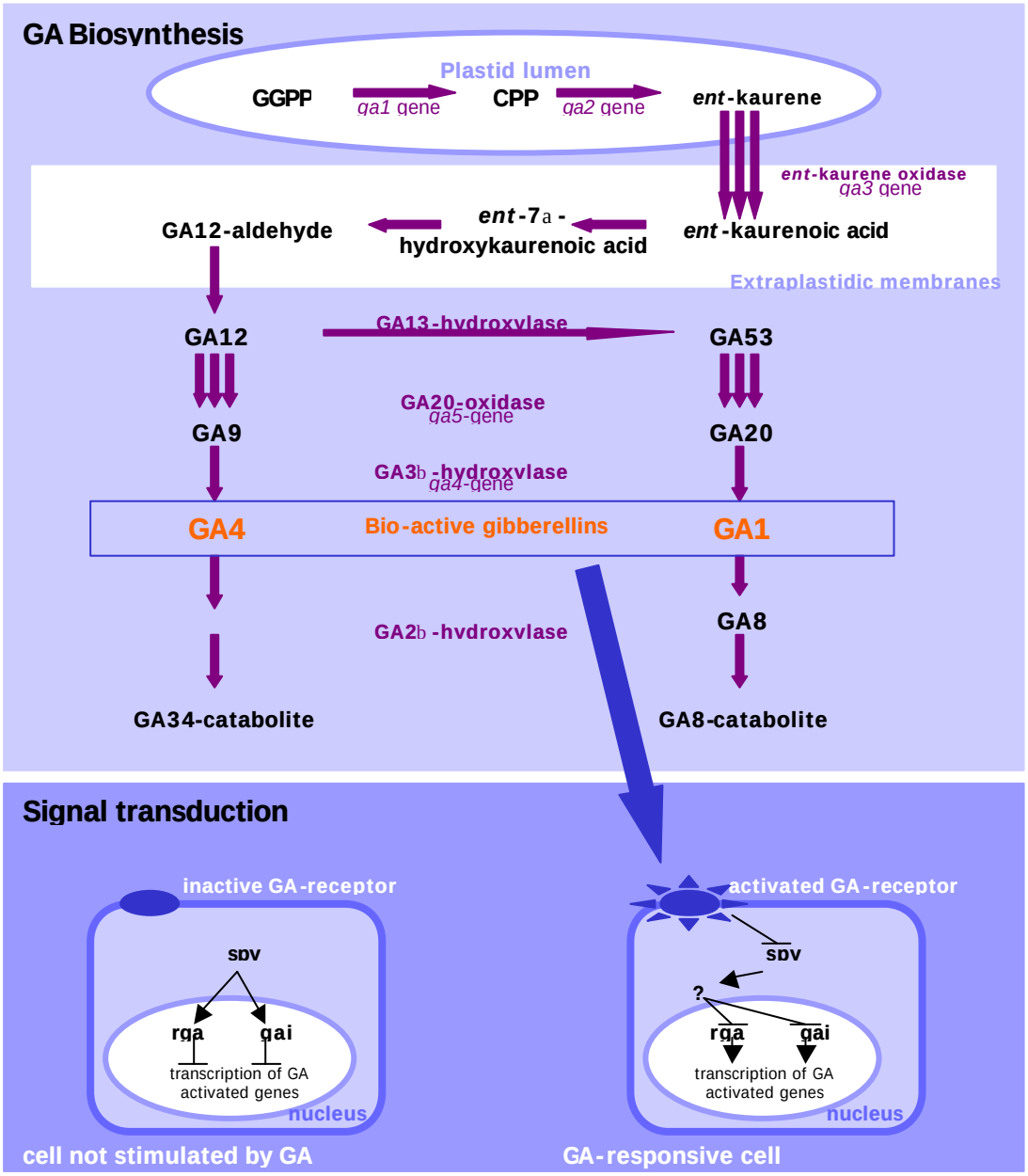


Figure 6: Biosynthesis and signal transduction pathways of GA

the complexity of the different pathways involved and to discriminate between endogenous and exogenous signals inducing flowering, one week old Landsberg *erecta* plants, grown under either short day (SD) or long day (LD) light conditions, were dipped in a suspension of the non-virulent strain D188-5 at 1 week after seed germination.

Short day conditions

Under short day conditions, GA synthesis and signal transduction play an essential role in the induction of floral transition: mutation of the strong allele *ga1-3*, which severely reduces endogenous levels of GAs, has been shown to abolish the upregulation of *LEAFY* and to prevent flowering under short days. The exogenous application of bioactive GA restores both (Wilson *et al*., 1992; Blazquez *et al*., 1998). In a first step towards the elucidation of the promotion of flowering by *R. fascians*, flowering times of gibberellin-biosynthesis or -response mutants of Landsberg *erecta* were compared after infection with *Rhodococcus* strain D188-5 or dipping in water as a control. Figure 6 gives an overview of the complex biosynthesis and signal transduction pathway of GA and the steps disrupted in the different mutants used. The biosynthesis mutants *ga5* and *ga4* are blocked in the genes encoding GA 20-oxidase and GA 3ß hydroxylase respectively (Talon *et al*., 1990; Xu *et al*., 1995; Hedden, 1998; Williams *et al*., 1998) and have a dwarfed phenotype, but produce normal flowers and siliques. The *gai* responsive mutant has a dwarfed phenotype, flowers later than wild type plants and is resistant to the external application of bioactive GAs (Koornneef *et al.,* 1985). The *gai* mutation has been shown to be of dominant-negative nature and the cloning of the corresponding gene revealed that it encodes a negative regulator of GA response (Peng *et al*., 1997). The *gar2* mutant was isolated as a suppressor of the *gai* phenotype (Wilson and Somerville, 1995). The mutant is dominant and affects the dose-response relationship of diverse gibberellin responses (including flowering; Peng *et al*., 1999). The gene disrupted in this mutant is thought to encode a negative regulator of GA response. Because of the dominant nature of this mutation, it is difficult to predict a function of GAR2 in GA signal transduction, as has been done for other isolated genes from resistant mutants (figure 5; for review see Sun, 2000).

Besides analyzing the effect of *R. fascians* infection on control medium, experiments were performed where wild type Landsberg *erecta* and the *gai* mutants after infection were placed on a medium containing paclobutrazol, a well-known inhibitor of the early steps of GA biosynthesis.

The experiments with the wild type Landsberg *erecta* plants showed that in a SD regime the difference in flowering between plants infected with D188-5 and the control batch is more pronounced than in LD conditions (3 days difference compared to 1 day, figure 7a1). When these plants are placed on a medium containing paclobutrazol, this effect is abolished (figure 7a2).

Although infection of the biosynthetic mutants *ga4* and *ga5* does lead to earlier flowering compared to uninfected plants, infected mutants still flower considerably later than wild-type plants. Moreover, as demonstrated in panel B of figure 6, infection by strain D188-5 leads to the formation of longer flower stalks compared to the uninfected control plant.

In the *gai*-repressor mutant *gar2*, the situation is reversed: infection with strain D188-5 leads to a delay of flowering compared to the uninfected control and the *ga5*-mutant. On the *gai* mutant D188-5 is able to induce flowering earlier than in the control batch. When the *gai* plants are placed on a paclobutrazol-containing medium, D188-5 infection does not lead to premature flowering anymore. This indicates that although it has been shown that exogenous GA application does not lead to stem elongation, the plants are able to respond to endogenous concentrations of GAs, hereby transducing a signal for flowering. The observation that paclobutrazol affects GA-dependent responses in this mutant has been previously reported (Silverstone *et al*., 1997).

In conclusion, the results obtained on plants grown under SD conditions suggest that infection with *R. fascians* is associated with an upregulation of GA production by altering of the biosynthesis pathway, resulting in the induction of flowering before time

Long day conditions

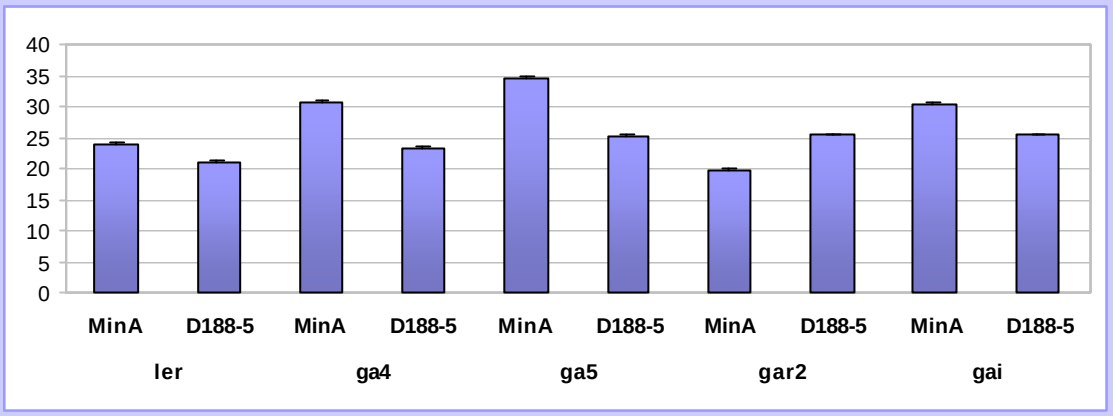
Under LD conditions, the situation becomes more complex than under SD light regime, since other pathways become implemented (Figure 5). The most important pathway promoting flowering under LD conditions is the photoperiod pathway. A key gene in this pathway is *constans*. A mutant in *constans* reduces greatly levels of *LEAFY* expression, whereas a rapid upregulation of *LEAFY* is observed upon activation of *constans* (Putterill *et al*., 1995; Nilsson *et al*., 1998). Nevertheless, GAs still play a significant role, since only in a *co*;*ga1* double mutant floral transition is abolished (Blazquez and Weigel, 2000).

The mutants used under LD conditions are the hormone mutants described above for the SD conditions and the late-flowering mutants *constans*, *fca* and *fve* (the latter two are part of the autonomous flowering pathway).

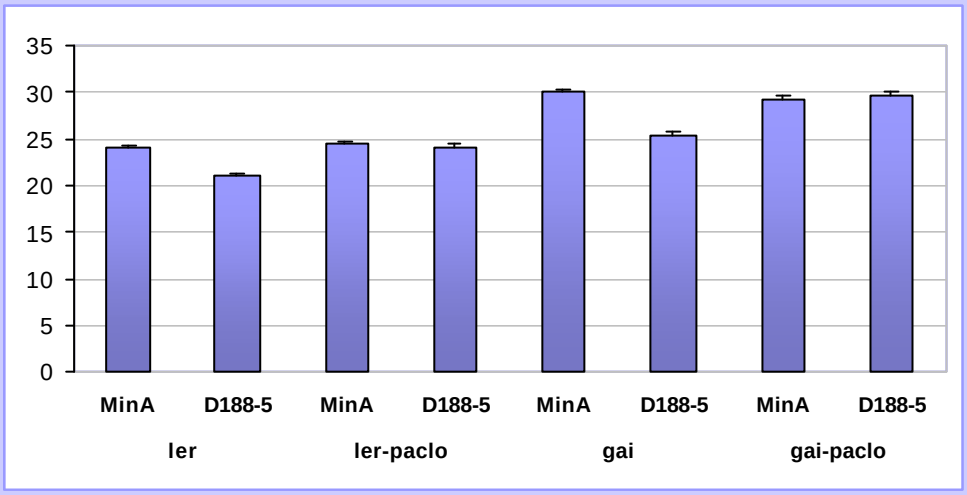
Figure 8a shows the results obtained with the mutants *constans*, *fca* and *fve* in comparison to the wild-type Landsberg *erecta* plants. In all mutants, D188-5 infection leads to an earlier flowering compared to the control infection (approximately 14 days for *constans*, 9 days for *fca* and 12 days for *fve*), suggesting that the flower-promoting effect of the bacterium is independent of these pathways. Moreover, since the difference in flowering time observed for the mutants (between 9 and 14 days) is much bigger than for the wild type plants (1 day), the pathway modulated by the bacterium must be operative in these mutants and has become more important, having a bigger impact.

From figure 8b, it is apparent that the results with the GA mutants under LD conditions are similar as seen under SD conditions. Early flowering upon infection with D188-5 is abolished when the wild-type plants are placed on paclobutrazol-containing medium, but this is not the case with the *ga4* mutants. Here, paclobutrazol shortens the difference between D188-5 infected plants and the controls with one day), but the difference in flowering-induction remains significant. In the *gar2* mutant, D188-5 and the control behave similarly.

To conclude, the experiments performed under LD conditions strongly suggest that an elevated GA production would be responsible for the earlier induction of the generative phase upon infection with *R. fascians*.



A1



A2

Figure 7:
A. Monitoring floral transition under short day conditions
The mutants *ga4, ga5, gai,* and *gar2* and wild type Landsberg *erecta* plants were tested. Infections were performed with the non-virulent strain D188-5 and compared with control dippings in water. Panel A1 shows the average age of flowering (days) for the plants tested under short day conditions. A2 shows the results for *gai* and *Ler* plants placed on medium with paclobutrazol (0.25mg/l)
B. Comparison of *ga4* plants infected with D188-5 (middle) and D188 (right) with non-infected plants (left). Infection with D188-5 leads to elongation of the flower stalks

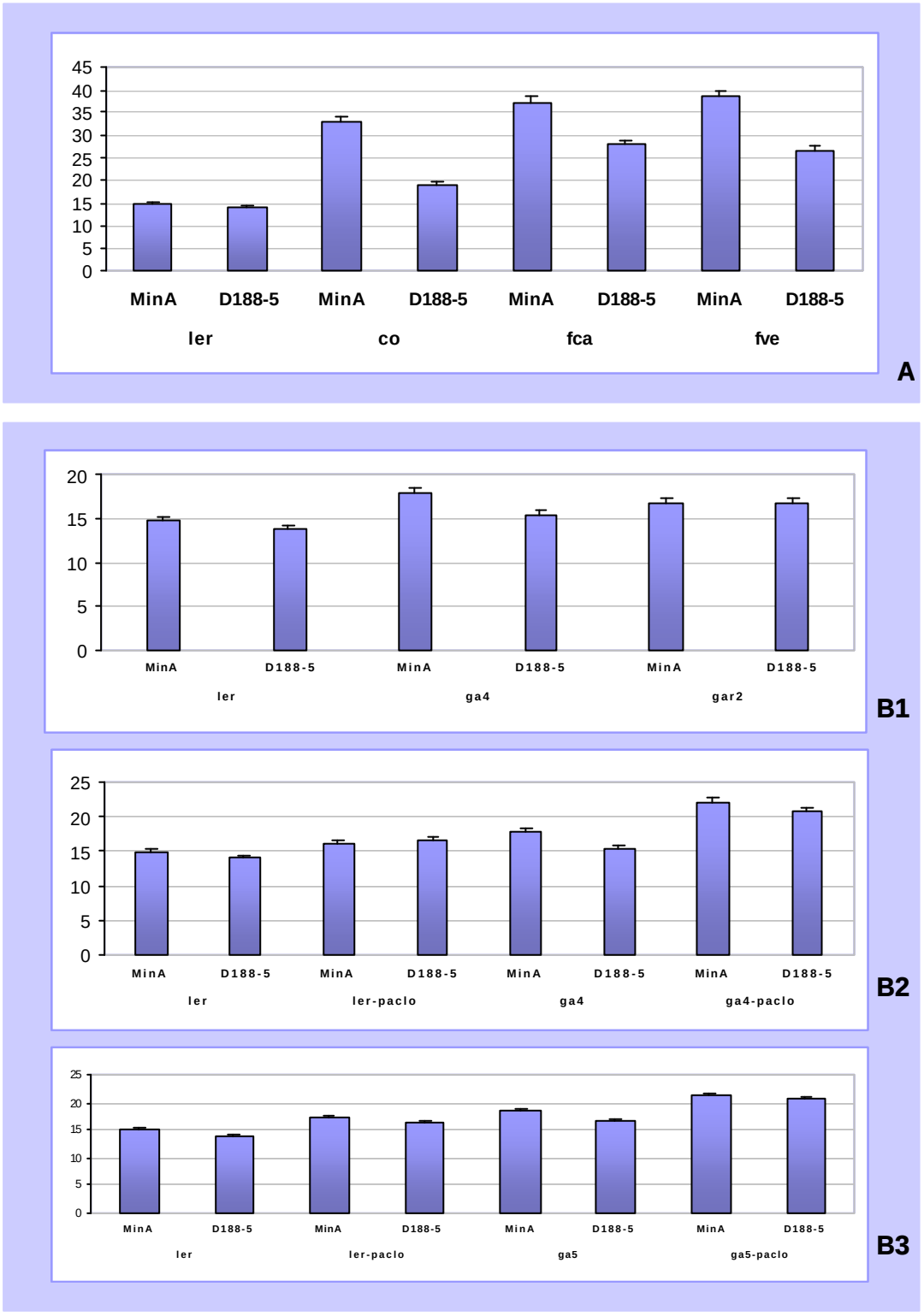


Figure 8: Monitoring floral transition under long day conditions by D188-5 (MinA dipping as a contro)l

- Comparison of wild type plants with the mutants *constans, fca, fve*
- Comparison of wild type plants with gibberellin biosynthesis and response mutants
  (B1) results obtained with *ga4* and *gar2*, compared with wild type plants
  (B2) results obtained when placing *ga4* mutants and the wild type plants on a paclobutrazol-containing medium (0.25mg/l)
  (B3) results obtained with *ga5*

8.4. Discussion and future perspectives

The results presented in this chapter indicate that the infection of *A. thaliana* by the phytopathogen *R. fascians* leads to symptoms that are comparable as those described on other plant species. Upon infection, there is a loss of apical dominance, an activation of axillary meristems in the rosette, and the formation of many shoots inhibited for further outgrowth at these sites. The comparison of infection phenotypes on different ecotypes of *Arabidopsis* made it to analyze in detail the effects of the bacterium on generative development. The two ecotypes used, C24 and Landsberg *erecta*, have a different timing of transition of the generative phase. Landsberg *erecta* is known to flower a few weeks earlier than C24. This is a consequence of the presence of an early allele of the *FRIGIDA* gene in Landsberg *erecta* plants, one of the two major loci (next to *FLC*) that control flowering time (Lee *et al.*, 1994). This implies that at the moment of infection, the two ecotypes are in a different developmental phase and thus that the meristems affected by *R. fascians* are in another developmental state. Besides shoot induction, severe aberrations in flower development were observed upon infection of both ecotypes. Flowers are formed, of which the gynoecium appears to be affected. As a result of uncontrolled proliferation of carpel and stigmatic tissue, the development of a normal silique with viable seeds is hampered. This phenotype is in some respects reminiscent of the gynoecia observed in malformed flowers formed on inflorescences of the mutants in the *PINOID, PIN-FORMED* and *ETTIN* genes in *A. thaliana*. It has been suggested that the first two genes might be involved in auxin-related processes, whereas the latter gene encodes a product related to auxin-responsive factors (Alvarez and Smyth, 1998; Nemhauser *et al.*, 2000). Indeed, *R. fascians* does produce auxins and induces in shoots typical auxin-related effects (Vereecke *et al.*, 2000; Mondher El Jaziri, unpublished results). However, in the above mutants all 4 the whorls of the flowers are affected, which is not the case for the flowers observed on plants infected with *R. fascians* strain D188.

The inflorescences, developing on infected plants are fleshy and slightly shorter. In some extreme cases, true fasciations can be observed. Most notable however is the extreme inhibition of the elongation of the co-inflorescences, which develop in the axils of the cauline leaves. In later stages of infection, a "flower gall", a generative analogue of the "leafy gall" of the vegetative phase can develop. Many features are comparable for the two types: a massive proliferation of meristematic tissue, covered with wildgrowth of organs originating from meristems, either shoots or sepals, petals and pappillae.

Another important observation, not related to bacterial virulence, is the premature floral transition upon interaction with the bacterium. The faster onset of flowering is most pronounced upon infection with the non-virulent strain D188-5, while the presence of the linear plasmid in strain D188 seems to temper this effect. The availability of several gibberellin and late-flowering mutants in *Arabidopsis* enabled us

to analyze this effect in more detail under both long and short day light regimes, where we compared the flower-inducing effect of the strain D188-5 with control infections with water. Under SD conditions, the effect exerted by D188-5 is abolished by paclobutrazol, which blocks the early steps of GA-biosynthesis indicating that the *R. fascians* effect would be mediated through GA biosynthesis. However, infection of *ga4* and *ga5* biosynthetic mutants with D188-5 still leads to earlier flowering although not as pronounced as on wild-type plants. Although these mutants have a dwarfed phenotype, bioactive GAs have been measured in these plants. Another indication for the leakiness of the mutation is that flowering - although delayed- is possible under a SD light regime (in contrast to *ga1-3* mutants, which don't flower at all under these circumstances). On the other hand, it became clear that in *Arabidopsis* and some other plant species several GA-20 oxidase genes are expressed. It is assumed that, even if each gene would encode for a GA 20-oxidase enzyme that specifically contributes to a certain developmental process, the loss of one enzyme could be partially compensated by the activity of others (Hedden and Kamiya, 1997; Hedden and Proebsting, 1999). Additionally, it seems likely that the GA 3ß hydroxylases also are encoded by multiple genes (Hedden and Proebsting, 1999). Whatever the explanation of the mutant phenotypes, infection with strain D188-5 enables the circumvention of the loss of this one enzyme and amplifies the GA-effect leading to flowering even in circumstances where GA-synthesis partially is blocked. Our hypothesis is further substantiated by the observations that in the *gar2*-mutant, infection no longer leads to earlier flowering, whereas in the *gai* mutant earlier flowering can still be induced upon infection. The finding that placing infected *gai* plants on paclobutrazol abolishes the effect, what can be explained by assuming that the *GAI* locus encodes a redundant component of the GA-signal transduction pathway, as suggested in literature (Peng and Harberd, 1993; Sun, 2000). Additionally, we observed that strain D188-5 is capable to fasten flower onset in the slender *spy1* mutant (data not shown), which fits with the assumption that GA production is elevated, since it has been reported that this mutant is not saturated in its GA response (Peng *et al.,* 1999). With the mutants *constans, fve* and *fca*, infection by D188-5 still leads to earlier flowering independent of the light conditions and the difference is much bigger than observed on wild type plants. This strongly suggests that in these flowering mutants, the pathway, which is modulated by the bacterium, is still functional and gained importance for flower induction, and that D188-5 does not act through *CONSTANS* or the autonomous pathway.

Altogether, the results presented here strongly suggest the capability of *R. fascians* to boost the production of bioactive gibberellins *in planta*, ultimately leading to an earlier induction of *LEAFY* expression. The observations of the inflorescence stalks in normal plants (figure 1) as well as in gibberellin mutants (figure 7b) infected by the non-virulent strain further solidify this vision, since their inflorescences are longer than the control batch of uninfected plants. How can this be achieved by the bacterium? A first explanation could be that *R. fascians* itself is producing bioactive gibberellins when in contact with its host plant. Some recent reports document gibberellin production by bacteria, such as certain *Bacillus, Acetobacter* and *Streptomyces* strains (El-Shirbiny and Hashem, 1997; Bastian-Fabiola *et al*., 1998; Gutierrez-Manero *et al*., 2001). Preliminary results suggest

that *R. fascians* would indeed be capable of gibberellin production, but this awaits further confirmation (Olivier Vandeputte and Mondher El Jaziri, personal communication). A second possibility could be that *R. fascians* modulates a specific step in gibberellin biosynthesis by the plant. Lately, evidence has been presented that IAA is responsible for the induction of expression of both GA 20-oxidase and GA 3ß hydroxylase in pea (Hedden and Philips, 2000). Moreover, Hanzawa *et al.* (2000) showed that the expression of *ACAULIS5,* a gene required for stem elongation and involved in GA signal transduction or biosynthesis, is upregulated by auxin. Recently, *R. fascians* strain D188 has been demonstrated to produce significant amounts of IAA. Preliminary results indicate that the same holds through for D188-5 (Olivier Vandeputte and Mondher El Jaziri, personal communication). Thus, IAA production by *R. fascians* could elevate the production of GAs and consequently lead to early flowering. The intermediate flowering effect of D188 (best distinguished in the C24 ecotype) could be explained by the presence of the linear plasmid pFiD188 on which the *fas* locus resides, responsible for the production of a component with cytokinin-like activity. This FAS molecule could be an important factor to counteract the induced gibberellin effects (see section 7.4.1.). Moreover, using the differential display technique a GA-2-oxidase from tobacco plants was isolated, which was specifically induced a few days post-infection with D188 (Filip Cnudde and Carmen Simon-Mateo, unpublished results). GA 2–oxidase is known to be a key enzyme involved in the degradation of bioactive gibberellins to less active forms (Thomas *et al.*, 1999). Further confirmation of this working hypothesis could be obtained by monitoring the expression levels of the genes encoding GA 20-oxidase and GA 3ß-hydroxylase following infection with *R. fascians*, as well as the measurement of the amounts of bioactive gibberellins present in infected plant tissue.

The results presented in this study are a further demonstration of the fact that following the infection with *R. fascians* the plant hormone balance is redefined, leading to a flower-promotive effect and other morphological and developmental changes. However, virulence of the bacterium is not dependent on the plant hormone homeostasis: when analyzing the shoot-inducing capacities of strain D188 on different plant hormone mutants, it is clear that *R. fascians* is able to establish it's characteristic phenotype on each mutant tested (Tita Ritsema, unpublished results). This is possibly due to the ability of this phytopathogen to produce hormones, which are the keys for symptom induction on the infected plant. As such, hormone levels and balances are affected and the bacterium actively reshapes the plant's hormone landscape.

Acknowledgments

Without the support of Dr. Tita Ritsema in both assisting in the practical work and giving useful comments for the interpretation of the results, this chapter would never have been like it is now. Olivier Vandeputte and Prof. Dr. Mondher El Jaziri from the ULB in Brussels are thanked for their generosity in sharing some preliminary results.

Part III

Nederlandse Samenvatting

Chapter Nine

Nederlandse Samenvatting

Deel I: Genoom-wijde expressie-analyse van het celdelingsproces in planten

Celdeling is een proces van levensbelang voor elk levend wezen, waarbij uit één cel twee identieke dochtercellen gevormd worden. In essentie voltrekt het celdelingsproces zich op cyclische wijze en verloopt het universeel voor elk species: In eerste instantie wordt de DNA-inhoud verdubbeld, waarna de identieke chromosomen gelijk verdeeld worden over de resulterende twee cellen, welke uiteindelijk fysisch van elkaar gescheiden worden. Dergelijke processen en de coördinatie van hun onderlinge opeenvolging verlopen in hoge mate gecontroleerd. Vanzelfsprekend vereist dit een optimaal functionerend regulatiesysteem. Het is reeds lang geweten dat de proteïnen vereist om dit controlesysteem op te bouwen, reeds vroeg in de evolutie verschenen zijn en daardoor sterk geconserveerd blijken bij hogere eukaryoten. In essentie ageren op welbepaalde controlemomenten proteïne kinase-complexen, bestaande uit cycline-afhankelijke kinases (CDK's, catalytisch gedeelte) en cyclines (activerend gedeelte). Enkel wanneer deze complexen geactiveerd worden, kan een volgende fase van de celcyclus beginnen, door middel van de kinatie van welbepaalde sleutelenzymen die hierin betrokken zijn. Verschillende controle-pathways zijn verantwoordelijk voor het aktiveren of inhiberen van de cycline/CDK complexen, waardoor de correcte opeenvolging en uitvoering van de verschillende processen tijdens de celdeling door verscheidene factoren gereguleerd kunnen worden. Bovendien is gebleken dat dergelijke regulatie niet alleen gebeurt op proteïneniveau, maar ook ter hoogte van de genexpressie of de afbraak van de betrokken eiwitten.

Op moleculair-genetisch vlak werd tijdens de laatste 25 jaren enorm veel vooruitgang geboekt in het bestuderen van de celdeling, vooral bij gist en menselijke cellen. Ook bij planten werden reeds belangrijke genen geïsoleerd en gekarakterizeerd. Hierbij wordt het steeds meer duidelijk dat verschillende mechanismen van de celdeling sterk geconserveerd zijn bij deze drie groepen. Het is evenwel zo dat voor bepaalde genklassen plantspecifieke leden geïsoleerd werden. Dit wijst er op dat bepaalde aspecten van de plantenceldeling afwijken. Het leidt immers geen twijfel dat de onbeweeglijke levenstijl van planten, alsmede het feit dat plantencellen omgeven worden door een rigide celwand, specifieke aanpassingen van het celdelingsmechanisme vereisen.

De recente opkomst van moleculaire technieken, waarbij op genomische schaal genexpressie onderzocht kan worden, maakt het mogelijk om op relatief snelle wijze een globaal overzicht te krijgen van het expressiegedrag van verschillende betrokken genen in de celcyclus. Dergelijke studies, waarin gebruik gemaakt werd van micro-arrays, werden reeds gepubliceerd voor gist en menselijke fibroblastcellen (Spellman *et al.*, 1998; Cho *et al.,* 2001) en stellen dat enkele honderden genen een celcyclus-afhankelijk expressiegedrag vertonen. Dit toont aan dat transcriptionele regulatie een belangrijk element van het celcyclusapparaat vomt. In deze doctoraatsverhandeling wordt een dergelijke studie voorgesteld in planten. Hierbij was het in

eerste instantie onze bedoeling om de transcriptionele regulatie van het celdelingsproces zo volledig mogelijk in kaart te brengen, door middel van een genoom-wijde expressieanalyse techniek in tabaks BY-2 celsuspensies. De bekomen resultaten zullen ongetwijfeld leiden tot een significante uitbreiding van de moleculair-genetische kennis van het celdelingsproces in planten. Bovendien kunnen vergelijkingen gemaakt worden met studies in gist en mens, wat ons in staat zal stellen om geconserveerde elementen terug te vinden naast meer plant-specifieke componenten.

In **Hoofdstuk 2** wordt de optimalisatie van de transcript profiling techniek om onze genoom-wijde expressieanalyse van de plantencelcyclus uit te voeren beschreven. Het feit dat voor dergelijke studies optimaal gesynchroniseerde celculturen onontbeerlijk zijn, deed ons immers besluiten om de tabakscellijn BY-2 te gebruiken. Het gevolg van deze keuze was dat het onmogelijk is om micro-arrays aan te wenden voor onze studie, aangezien sequentie-informatie van tabak nauwelijks voorhanden is. Het protocol van de cDNA-AFLP-techniek, welke reeds verschillende keren succesvol toegepast werd om differentiële genexpressie te bepalen, werd aangepast om gebruikt te worden voor genoom-wijde expressieanalyse. Hierdoor werd het mogelijk om zonder voorafgaande kennis van de genomische sequentie een groot deel van de transcriptionele veranderingen in tabaks BY-2 cellen te visualizeren. Daarnaast werd met behulp van de Quantar-Pro™ software (Keygene), een protocol opgesteld om de bekomen expressieprofielen kwantitatief te bepalen. Op basis van de clustering van de bekomen resultaten van de histongenfamilie kon aangetoond worden dat deze cDNA-AFLP-gebaseerde techniek een sensitieve en *bona fide* manier is om aan genoom-wijde expressieanalyse te doen. Bovendien heeft deze techniek als voordeel dat zelfs sterk homologe sequenties afzonderlijk geïdentificeerd kunnen worden.

Het experiment werd uitgevoerd op BY-2 cellen die gesynchroniseerd werden door behandeling met aphidicoline. Na het wegwassen van deze DNA-polymerase-inhiberende drug, werden om het uur stalen genomen, waardoor de celdeling gevolgd kan worden vanaf de vroege S-fase tot in het begin van de G1-fase van de celcyclus. De cDNA-AFLP-gebaseerde transcript profiling analyse werd op deze stalen uitgevoerd. Op deze wijze kon een collectie van genfragmenten geïsoleerd worden, welke ongeveer overeenkomt met 1500 genen, waarvan de expressie fluctueert tijdens het celdelingsproces. **Hoofdstuk 3** beschrijft de resultaten bekomen na karakterisering van de sequentie voor de meerderheid van deze verzameling van celcyclusgemoduleerde fragmenten. Er kan duidelijk gesteld worden dat een groot deel van de gevonden genen geen enkele homologie vertoont met de sequenties aanwezig in de databank. Zelfs indien de korte lengte van de AFLP-fragmenten in rekening gebracht wordt, alsmede de mogelijkheid dat deze gegenereerd werden uit het niet vertaald gebied van het mRNA, blijkt dat onze screening veel potentieel nieuwe genen opgeleverd heeft, welke gerelateerd kunnen zijn met ongekende, mogelijks plant-specifieke, processen. Het deel dat functioneel geannoteerd kon worden, bevat veel componenten waarvan de link met celdelings-gerelateerde processen reeds bewezen werd. Bovendien werden er homologen van celcyclusgenen gevonden, welke nog nooit

voordien in planten aangetoond werden. Vanzelfsprekend zijn deze interessant voor verdere karakterizering, wat ons in staat zal stellen om een verdere ontrafeling van de controlemechanismen en de regulatorische netwerken van het celdelingsproces in planten mogelijk te maken.

In **Hoofdstuk 4** wordt ingegaan op het expressieprofiel van de geïsoleerde genen. De gekwantificeerde expressieprofielen werden geclusterd zowel door middel van hiërarchische clustering, als door de adaptive quality-based clustering methode. Hierdoor kan duidelijk aangetoond worden dat de meerderheid van de celcyclus-gemoduleerde genen een piek van expressie vertonen tijdens de mitose. Voorts werden de genen geklasseerd in functionele groepen. Dit stelde ons in staat om eventuele over-representatie tijdens een bepaalde celcyclusfase na te gaan. De bekomen resultaten werden vergeleken met deze bekomen bij de studies in gist en menselijke fibroblastcellen. Vaak werden vergelijkbare resultaten gevonden, welke de hoge conservatie van de celcyclus in eukaryoten benadrukt. Eveneens werden er enkele verschillen vastgesteld, die mogelijks gerelateerd zijn aan verschillen in celcyclusprogressie tussen de verschillende species. Bovendien werd aangetoond dat veel ongekende genfragmenten significant geëxpresseerd worden tijdens de M-fase. Mogelijks duidt dit aan dat mitose en cytokinese in planten verloopt volgens tot nog toe ongekarakteriseerde processen.

Als besluit kan gesteld worden dat met succes een genoom-wijde studie van de celcyclus bij planten uitgevoerd werd met behulp van een cDNA-AFLP-gebaseerde techniek op tabakscellen als modelsysteem. Aan de hand van de bekomen resultaten kon een globaal beeld van de transcriptionele regulatie van celcyclus-gerelateerde genen geschetst worden. Hierbij werd duidelijk dat de globale mechanismen sterk aanleunen bij deze in andere hogere eukaryoten. Toch konden enkele wezenlijke verschillen aangetoond worden. Bovendien zal de grondige karakterizering van de resulterende genencollectie een sterke basis vormen voor de verdere ontrafeling van verschillende netwerken en dit zowel op algemeen als meer plant-specifiek niveau.

Deel II-ADDENDUM: Moleculaire en fysiologische aspecten van de interactie van de fytopathogeen *Rhodococcus fascians* met zijn gastplanten

Rhodococcus fascians is een Gram-positieve fytopathogene bacterie, welke in staat is om hyperplasia's te induceren bij diverse plantenspecies, behorend tot zowel monocotyle als dicotyle families. Dergelijke plantmisvormingen zijn waarschijnlijk het gevolg van het feit dat deze bacterie in staat is om het ontwikkelingsprogramma van de plant grondig te ontregelen. De manier waarop dat de bacterie dit bewerkstelligt, is nog steeds grotendeel ongekend, maar is hoogstwaarschijnlijk gerelateerd aan de aanpassing van de interne hormonenbalans van de geïnfecteerde gastplant. Moleculair-genetische karakterizering van deze fytpathogeen toonde aan dat de virulentie afhankelijk is van de aanwezigheid van een lineair plasmide, pFiD188. Hierop werden enkele loci (*fas*, *att* en *hyp*) gekarakterizeerd, welke verantwoordelijk zijn voor het virulente gedrag van deze bacterie. De sequentie van het *fas*-locus, essentieel voor virulentie, toonde aan dat het vermoedelijk instaat voor de aanmaak van een gemodificeerd cytokinine. In **Hoofdstuk 6** wordt aangetoond dat de expressie van de *fas*-genen enkel door extracten van geïnfecteerd plantweefsel geïnduceerd kan worden. Daarnaast werd een gen, *fasR* genaamd, geïsoleerd dat codeert voor een AraC-type regulator en noodzakelijk blijkt te zijn voor de pathogeniciteit en voor de expressie van de genen van het *fas*-operon. Experimenten, uitgevoerd in een gedefinieerd medium onder *in vitro* condities, tonen aan dat de expressie van dit operon strikt gereguleerd is. Het is gebleken dat inductie optreedt onder invloed van verscheidene milieufactoren zoals de pH, koolstof- en stikstofbronnen, fosfaat en zuurstofgehalte. Daarnaast werd bewezen dat regulatorische proteïnen betrokken zijn. Tenslotte werd aangetoond dat de expressie van de *fas*-genen zowel op het transcriptionele als het translationele niveau gereguleerd wordt. We vermoeden dat een dergelijk complex expressiepatroon gerelateerd is aan het feit dat verscheidene signalen geïntegreerd worden. Bovendien benadrukt dit het belang van het *fas*-operon voor de pathogeniciteit van *R. fascians.*

Naast de moleculaire karakterizering van de bacteriële pathogen, werd er ook onderzoek verricht naar het gewijzigde ontwikkelingspatroon van de gastplant tengevolge van infectie met *R. fascians.* In **Hoofdstuk 7** werd de interactie tussen *R. fascians* en *Arabidopsis thaliana* grondig bestudeerd. Hiervoor werden de fenotypes van enkele ecotypes veroorzaakt door infectie bestudeerd en vergeleken. Uit deze experimenten bleek ondubbelzinnig dat het infectiefenotype afhankelijk is van de ontwikkelingsfase waarin de plant zich bevindt op het moment van infectie. Wanneer de planten zich nog in de vegetatieve fase bevinden, heeft dit het verlies van de apikale dominantie en een massieve amplificatie van scheutgroei en vervormde bladeren in de rozet tot gevolg. Dergelijke scheuten zijn evenwel niet in staat om te elongeren, met een karakteristiek "bushy" fenotype tot gevolg. De infectie van planten, waarbij de overgang van de vegetatieve naar de generatieve fase zich reeds voltrokken heeft,

leidt tot sterke vervormingen ter hoogte van de bloeistengel en de bloemen die daarop gevormd worden. Daarnaast werd vastgesteld dat de infectie van zowel de virulente als de niet-virulente *R. fascians* stam leidt tot het vroegtijdige bloeien van de plant. Met behulp van bloei- en hormoonmutanten werd nagegaan waardoor dit fenomeen veroorzaakt wordt. Onze resultaten tonen aan dat het bloeibevorderende effect van *R. fascians* zich situeert ter hoogte van het van de gibberellinesynthese. Dit resultaat is een verdere bevestiging van de reeds bestaande vermoedens dat *R. fascians* in staat is om het hormonenlandschap van de plant te herdefiniëren, met een grote variatie aan morfologische en ontwikkelingsveranderingen tot gevolg.

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