The Effect of Stress Factors on Gene Expression in Higher Plants

PhD Thesis

of

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To my friends from abroad and from home

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ABBREVIATIONS

ABA	abscisic acid
ABRE	ABA-responsive element
BLAST	basic local alignment search tool
BRs	brassinosteroids
CDE	cell cycle dependent element
CDK	cyclin-dependent kinase
CDKA	A-type CDK
CDKB	B-type CDK
CHR	cell cycle gene homology region
cv.	cultivar
CYC	cyclin
DRE	dehydration responsive element
ERE	ethylene-responsive element
EREBP	ethylene-responsive element binding protein
EST	expressed sequence tag
G1, G2	gap phases of the cell cycle
GA	gibberillin
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTRR	gluthamyl – tRNA – reductase
GUS	β-glucuronidase
HU	hydroxyurea
JA	jasmonic acid
lt	low temperature and early drought induced protein
LTP	lipid transfer protein
luc	luciferase
M	mitosis phase of the cell cycle
MIF	mitosis-inducing factor
MSA	M-phase-specific activator *cis*-element
ORF	open reading frame
P-box	gibberellin-responsive element

PEG	polyethylene glycol
PPT	phosphinotrycin
Q-PCR	Quantitative Reverse-Transcriptase PCR
RB-B	Retinoblastoma – B domain
rcaA2	Rubisco activase A2
rcaB	Rubisco activase B
rbcL	Rubisco large subunit
rbcS	Rubisco small subunit
Rubisco	ribulose-1,5-biphosphate carboxylase
S	DNA synthesis phase of the cell cycle
SA	salicylic acid
TC	tentative contig
TCA	wound-responsive element
TCTP	translationally controlled tumor protein
35S	cauliflower mosaic virus 35S promoter
2,4-D	2,4-dichlorophenoxyacetic acid
TF	transcription factor
UCE	ubiquitine – conjugating enzyme
UP_79	unknown protein
UTR	untranslated region
WUN	wound-responsive element

OBJECTIVES OF THE PRESENT WORK

In the present study, we aimed to achieve a better understanding about the effect of stress factors on gene expression in higher plants. In the first part of our work, we investigated the expression of genes up-regulated in response to drought/osmotic stress. We chose as a source for gene isolation a drought tolerant wheat (*Triticum aestivum* L.) cultivar and we applied a subtraction molecular approach to select differentially expressed genes related to drought/osmotic stress. This project was done in collaboration with the Department of Plant Physiology of the University of Szeged with the purpose to collect information about the molecular mechanism of drought tolerance of wheat. These data are aimed to serve as molecular markers for fast identification of drought tolerant species, as well as for the establishment of drought tolerant transgenic lines at a later stage.

In the second part of our work, we aimed to reveal the complex integration of cell cycle phase-specific gene into the wound stress response. We focused on analyzing the regulation of B-type cyclin dependent kinase (B-type CDK, or CDKB) in alfalfa (*Medicago sativa* L.). The CDKBs are key regulators of the G2/M checkpoint of the cell division cycle progression in plants. These kinases are characteristically regulated at the transcriptional level and promoter studies are a useful approach to define different signal pathways that can affect the expression of these cell cycle kinases. There are two CDKB classes – CDKB1 and CDKB2 (the expression of CDKB1 preceeds the expression of CDKB2). In an earlier work, Magyar et al. (1997) demonstrated that in synchronized alfalfa cell cultures the proteins of two CDK genes, assigned as c*dc2MsD* and *cdc2MsF*, accumulate at the G2/M cell cycle-phase transition. According to the recent nomenclature these genes were re-named as *Medsa;CDKB1;1* and *Medsa;CDKB2;1*, respectively (Joubès et al., 2000). In the present work, we aimed at the cloning and characterization of the upstream region of the Medsa;CDKB2;1 kinase. Up to now no detailed investigations have been performed on promoters of B2-type CDKs, which highlights the requirement and the interest to improve our understanding about their regulation.

The aims of the present work are:

- Identification of genes expressed differentially in wheat in response to drought/osmotic stress

- Cloning of the promoter region of alfalfa mitotic kinase (*CDKB2;1*) and revealing the possible regulation mechanisms

1
INTRODUCTION

1.1. Survival of an organism is based on the regulated gene expression

Survival of the living organisms is based on their ability to adapt to the continuously changing environments. The processes that ensure the proper reactions of the organism in response to external signals, as well as the processes responsible for its growth and development, are all carried out by different functional classes of proteins. The information about how, when and where to produce each kind of protein, comes from the genetic material, the deoxyribonucleic acid (DNA) molecule. The genes are the functional units of the DNA where the genetic information is stored. The gene's promoter enables a gene to be transcribed. Promoters are a means to demarcate which genes should be used for messenger RNA creation - and, by extension, control which proteins the cell manufactures. Each cell of the organism contains the full set of genes, but only some of these genes are active and used for synthesizing proteins. Multiple developmental and environmental signals, including injury and infections, influence which genes a cell uses at a given time during its life cycle. Transcription of the eukaryotic genome is a dynamic process in which genes are being switched on and off. Genomic DNA exists in a chromatin structure, which is subjected to the action of sequence-specific transcription factors (TFs) and their associated coactivator complexes that regulate the transcription of target genes (Shikama et al., 2000).

1.2. Levels for the control of gene expression

Theoretically, regulation at any one of the various steps in gene expression could lead to differential production of proteins in different cell types or developmental stages or in response to external conditions (Figure 1-1).

1.2.1. Chromatin organization

DNA is packed into chromatin composed by nucleosome units where DNA is wrapped tightly around a core of histone proteins. For initiation of transcription, the enzyme RNA polymerase must bind to the promoter region of the genes, however, the DNA within the nucleosome is hardly accessible, which prevents the action of the general TFs necessary to position the RNA polymerase at the correct start site. The interaction between the DNA molecule and the histones can be influenced by modifications of the histone tails that are amino acids extending from the surface of nucleosomes. The histone tails may undergo modifications that neutralize their positive charge and

decrease the interaction with the negatively charged DNA molecule, which results in chromatin decondensation.

1.2.2. Synthesis of mRNA and protein

After DNA becomes available for expression, general TFs help RNA polymerase to initiate transcription. The protein-coding genes are transcribed by RNA polymerase II which characteristically terminates the primary RNA transcript only after transcribing a sequence that directs the further cleavage and polyadenylation at the 3'-end. In eukaryotic genes, the coding sequences, exons, are separated by non-coding segments, introns. Splicing of the primary RNA transcripts results in the removal of introns and joining of the exons. The functional eukaryotic mRNAs produced by RNA processing retain non-coding regions (untranslated regions: UTRs), at both the 5'- and 3'-ends. The mRNA exits the nucleus through the pores and initiates translation on ribosomes in the cytosol. Synthesis of all polypeptide chains begins with the amino-terminal amino acid methionine specified by the AUG start codon, and terminates with the stop codons UAA, UGA, or UAG that do not specify amino acids. The sequence of codons between a specific start codon and a stop codon is known as a reading frame. Further posttranslational modifications define the activity of the protein.

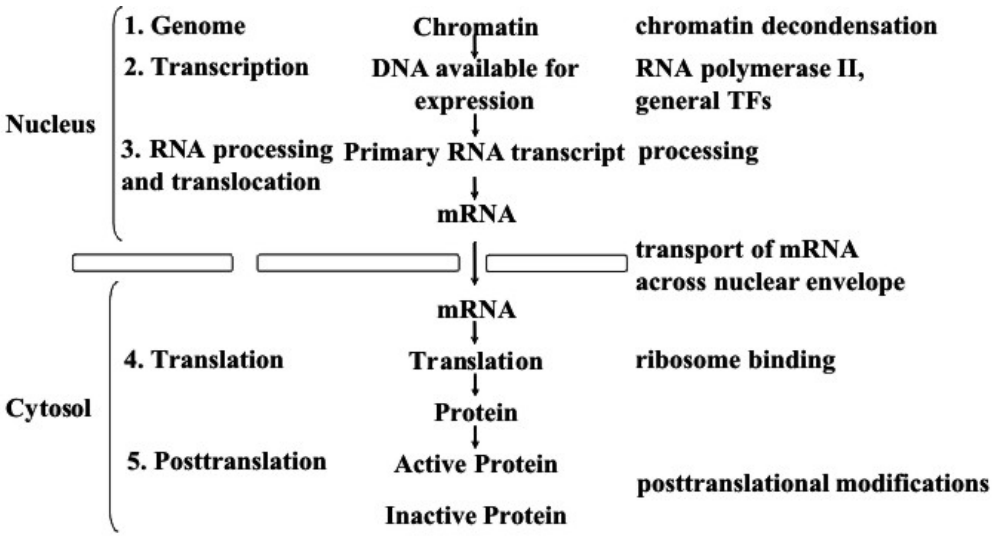

Figure 1-1 Levels of gene expression
The gene expression can be controlled inside the nucleus at chromatin and RNA level. The generated mRNA is transported through the nuclear pores into the cytosol where protein synthesis occurs.

1.3. Control of transcription initiation and promoter structure

Although regulation at each step in gene expression can occur, in most cases the first step – control of transcription initiation, is the most important in determining at what level genes are expressed. Transcriptional regulation relies on multiple regulatory sequences within the promoter regions of the genes. These control elements are termed *cis*-acting sequences, since they are adjacent to the transcription units they regulate. The *cis*-regulatory sequences in the promoter are recognized by TFs that typically contain DNA-binding domains.

The promoter structure characterizes with a 'core' or 'minimum' promoter that is essential for gene expression (Figure 1-2). The minimum promoter of genes transcribed by RNA polymerase II usually extends about 100 bp upstream of the transcription initiation site. It contains a short sequence called TATA-box that serves as the site of assembly for the transcription initiation complex. The minimum promoters may contain several additional regulatory sequences that are referred to as proximal control elements.

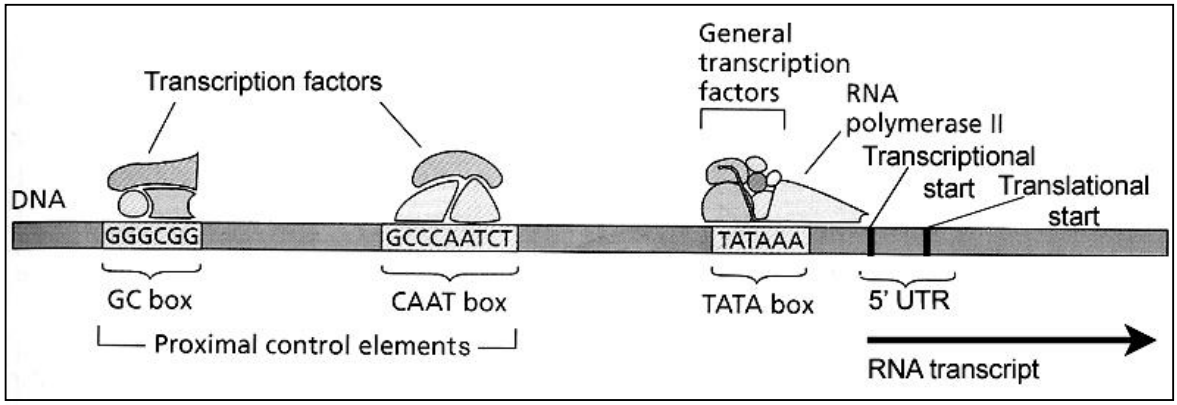

Figure 1-2 Features of a typical eukaryotic minimum promoter and proteins that regulate gene expression.
The minimum promoter contains TATA-box, where the transcription initiation complex assembles, and proximal control elements (CAAT box, GC box) that are bound by specific TFs. The positions of the non-coding 5' UTR, as well as the transcriptional and translational starts are shown.

Distal regulatory sequences located up- or downstream of the proximal promoter sequences can exert either positive or negative control over eukaryotic promoters. TFs that influence transcription positively (activators) or negatively (repressors) may bind to these sites. Gene expressions can also be regulated by enhancers that are distantly positioned positive regulatory sequences, either up- or downstream of the promoter. The large number of control elements that can modify the activity of a single promoter gives immense versatility for differential gene regulation in eukaryotes.

1.4. Signal transduction pathways

Coordination of the organism's responses to different stimuli requires an array of signalling mechanisms. The perception of the stimulus by cell receptors triggers one or more sequential biochemical reactions that connect the stimulus to a cellular response. Such a sequence of reactions is known as signal transduction pathway. These pathways often involve the generation of second messengers, transient secondary signals inside the cell that amplify the original signal. The signal cascade leads to changes in protein phosphorylation through the activation of protein kinases or protein phosphatases. The effects of these enzymes modify the activity of a target protein in different ways, activating or inhibiting its function.

A cell typically receives more than one signal at a time. The response to each signal, or condition, is often influenced by another one. The signalling molecules function primarily by regulating the activity of transcription factors that in turn regulate gene expression. One or more extracellular signals may activate one transcription factor. Moreover, multiple transcription factors regulate the transcription of a single gene. This integration of signals can prevent inappropriate responses and permit more nuanced reactions to multiple signals.

1.5. Plants and stress

Owing to their sessile lifestyle, plants have to respond to local environmental conditions by changing their physiology and redirecting their growth. Signals from the environment include light, temperature, water, nutrients, gravity, pathogen attack, wounding. The perceived stimuli are communicated across the plant body through chemical messengers, hormones, which affect diverse aspects of plant growth and development. Since the rigid cell wall does not allow mobilizing of specialized cells for stress response, plants developed the capacity of making cells competent for the activation of defense responses. The mechanisms for increased tolerance to environmental stress involve physiological changes, or expression of genes that results in modification of molecular and cellular processes (Reymond and Farmer, 1998; Aarts and Fiers, 2003). The information about the perceived stress is transmitted through a signal transduction pathway. In some cases, the pathways triggered by different stress factors are common to more than one stress type, which can be due the action of similar protective mechanisms (Knight and Knight, 2001). Indeed, there are common stress genes responding to various abiotic and biotic stress factors. These genes have specific sites in their promoter regions that are recognized by TFs regulating transcription initiation. Identifying the DNA *cis*-elements in the gene promoters as well as the corresponding TFs may improve the understanding about the regulatory networks acting during stress adaptation.

1.5.1. Drought stress

Environmental factors that impose water-deficit stress, such as drought, salinity and extreme temperatures, place major limits on plant productivity (Tanford, 1978). Drought stress is one of the major factors causing yield loss in different crops and in particular cereals that are the main foodstuff sources for men and animals (Cushman and Bohnert, 2000; Mundree et al., 2002). To overcome these limitations and improve production efficiency in the face of a fast-growing world population, more stress tolerant crops must be developed (Oliver and Bewley, 1996). Traditional approaches are limited by the complexity of stress tolerance traits, low genetic variance of yield components under stress conditions, and the lack of efficient selection techniques (Cushman and Bohnert, 2000). A more complete understanding of the complexity and interplay of osmotic, desiccation and temperature tolerance mechanisms, and their corresponding signalling pathways, is needed and will come from integrative whole genome studies. The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation will provide the basis of effective engineering strategies leading to greater stress tolerance.

In order to understand the plant defense strategies in response to stress conditions, the identification of differentially expressed genes is needed. General gene expression shows a big variation in the levels of different mRNAs, which makes difficult the capture of rare mRNAs from cDNA libraries (Bonaldo et al., 1996). Therefore, additional approaches have been developed to enable researchers to compare two populations of mRNA and obtain clones of genes that are differently expressed (Reddy et al., 2002).

The putative products of the isolated genes could be easily identified by applying *in silico* search. Vast amounts of DNA sequence, including the entire genomic sequence of key experimental organisms, are already available in online data banks. A computer program known as BLAST (*b*asic *l*ocal *a*lignment *s*earch *t*ool) is used for comparing amino acid sequences of protein encoded by a newly cloned gene and proteins of known function providing clue about the role of the protein of interest (Altschul et al., 1990). Putative functions can subsequently be tested in plants. The functional determination of genes that participate in stress adaptation or tolerance reactions are expected to provide an integrated understanding of the biochemical and physiological basis of stress responses in plants.

In addition, it is of great importance to investigate the gene regulation because tolerance to one type of stress may be co-ordinated with tolerance to another (Mundree et al., 2002). This phenomenon relies on the similar *cis*-acting elements in the promoters of different stress genes. Many stress-related genes are induced by abscisic acid (ABA) and contain potential ABA-

responsive elements (ABRE) with the sequence (PyACGTGGC) in their promoter regions. ABRE-DNA-binding proteins were also identified. The G-box (CACGTG) resembles the ABRE motif and functions in the regulation of plant genes in a variety of environmental conditions. Myb binding sites (T/C)AAC(T/G)G could be also identified in ABA-responsive genes, as well as in defense genes (Cheong et al., 2002; Euglem, 2005). Another sequence, TACCGACAT, found in stress-induced genes is termed dehydration responsive element (DRE) and is involved in ABA-independent gene expression. DRE is essential for the regulation of dehydration-responsive gene expression and it was found to function as a *cis*-acting element involved in the gene induction by low temperature. DRE-binding proteins participate in transcription activation of genes containing DRE sequence. Interestingly, the ethylene-responsive element binding protein (EREBP) that regulates ethylene-responsive gene expression can bind DRE, too. The co-operative action of *cis*-elements is crucial for the adequate gene expression in response to more than one signal (Mundree et al., 2002).

1.5.2. Mechanical wounding

Wounding is a common damage that occurs to plants caused by factors such as wind, rain, and biotic factors, especially insect feeding. An open wound caused by mechanical injury is a potential infection site for pathogens; thus, expression of defense genes at the wound site is necessary for the plants to build a barrier against opportunistic microorganisms. In addition to pathogen resistance, wounding pathways may also interact with other signalling processes involving abiotic stress responses (Cheong et al., 2002). Dissecting the cross talk between these pathways is critical to the understanding of the plant response to environmental cues in general and to wounding in particular.

1.5.2.1. Short and long term wound response

According to the response speed of the tissues to mechanical injury the wound response can be classified as short and long term. Short term response starts minutes after wounding, whereas long term response occurs 6 hours or more after the mechanical injury (Reymond et al., 2000). Curiously, it has been shown that the short term wound response occurs in the non-wounded parts of the plant, involves early-induced genes encoding predominantly signal transduction and transcription regulation factors (Delessert et al., 2004). In contrast, the long term wound response is characteristic for the wounded region and the expressed genes encode mainly proteins with metabolic functions related to photosynthesis, sugar, lipid, and carbon metabolism. The long term reaction involves the possibility of cell proliferation as a defense mechanism during vascular regeneration in stem and roots (Nishitani et al., 2002). These evidence suggest that the immediate

reaction of a plant to wounding is to signal the threat to the whole plant and produce defense mechanism against further attack by invaders, and that the healing process in the wound area is established later.

Wound-inducible genes were found to contain regulatory sequences, AATTT, recognized by WUN (wound-inducible) TFs (Siebertz et al., 1989; Lescot et al., 2002). However, the presence of additional *cis*-elements facilitates the cooperative interactions of TFs enabling the fine-tuned expression of defense transcriptome (Rushton et al., 2002; Eulgem, 2005).

1.5.2.2. Wounding and defense

Wounding and pathogen responses involve a number of plant hormones including jasmonic acid (JA), salycilic acid (SA) and ethylene (Reymond and Farmer, 1998). Cross-talks between the signal pathways involving these hormones appear to be very common and important in the regulation of gene expression in response to wounding, pathogens and other stress factors (Raymond and Farmer, 1998; Wang et al., 2002). It has been shown that many defense-related genes share similar regulatory mechanisms (Goldsbrough et al., 1993; Rushton et al., 2002). In plants, members of several TF families, bZIP, EREBPs, Myb, WUN, TCA-binding and WRKY have been implicated in the transcriptional control of wound- and pathogen-related genes (Rushton and Somssich, 1998; Eulgem, 2005).

JA. JA can modulate different aspects of plant growth and development. This compound and its derivatives activate genes involved in wounding, pathogen and insect resistance implying the role for JA in plant defense (Creelman and Mullet, 1997). Recent studies have demonstrated the involvement of JA in the early wound response locally, at the place of infection, and systemically, throughout the plant (Delessert et al., 2004). Evidence have revealed that wounding activates JA biosynthetic pathway for production of a long-distance signal to the distal plant organs suggesting that JA, or its derivatives, may act as a transmissible wound signal. JA accumulates in wounded plants and activates expression of various defense genes (Creelman and Mullet, 1997). Several classes of transcription factors are known to function in the jasmonate pathway and, in some cases, these proteins provide integrating points between important defensive and developmental pathways (Creelman and Mullet, 1997; Wang et al., 2002). G-boxes are found in JA-inducible promoters and in genes regulated by ABA, light, UV-radiation and wounding, as well as pathogen signals. Often they function in concert with other *cis*-acting elements. G-box elements are bound by bZIP proteins, a well characterized class of transcription factors also found in animals where they regulate, both positively and negatively, various cellular processes.

SA. The synthesis and accumulation of SA appear to be necessary for the activation of defense responses, both locally and systemically (Durner et al., 1997). It has been suggested that this compound cannot serve as a messenger in a long-distance signaling, but still its mobility remains unclear. SA has been shown to be a signaling molecule involved in the induction of systemic acquired resistance that defines an enhanced ability of plants to defend themselves against pathogens (Ryals et al., 1996). The promoters of genes involved in desease resistance mediated by SA, and genes induced by multiple stress factors as pathogen attack, wounding and abiotic stress, have the TCA-binding sequence, TCAT(T/C)(T/C), recognized by a 40 kDa nuclear protein in a SA-dependent manner (Goldsbrough et al., 1993). Also, W-box sequence, (T)GACC/T, recognized by WRKY TFs presents in the promoters of both SA- and defense-related genes (Durner et al., 1997; Euglem et al., 2000; Cheong et al., 2002).

Ethylene. Ethylene has a wide spectrum to regulate growth and developmental processes in plants (Abeles et al., 1992; Zhong and Burns, 2003). It was regarded as a stress hormone because its synthesis is induced by a variety of stress signals, such as mechanical wounding, chemicals and metals, drought, extreme temperatures, and pathogen infection (Kende, 1993; Johnson and Ecker, 1998). Many of the ethylene response TFs such as EREBPs are rapidly induced by wounding. These TFs may directly participate in the activation of ethylene-responsive genes. Ethylene is involved in the early stress response, and its transient induction is followed by a controlled quenching, which might be a way for plants to limit the propagation of cell death (Wang et al., 2002). A GCC-box (AGCCGCC) is found in the promoter regions of many pathogen-responsive genes. This *cis*-acting element has been shown to function as an ethylene response element in some ethylene-regulated genes (Rushton and Somssich, 1998). EREBPs bind to GCC-boxes. Ethylene-responsive element (ERE), A(T)TT(C/G)AAA, is also often found in genes involved in the ethylene signal pathways (Lescot et al., 2002).

1.5.2.3. Wounding and abiotic stress

Wounding activates a number of genes regulated by abiotic stress response pathways triggered by drought, cold, high salt, heat shock, and others (Cheong et al., 2002). DREB TFs containing EREBP domain bind the DRE *cis*-element in the promoter region of these genes.

1.6. Cell division cycle progression

Cells have the extraordinary ability to make nearly identical copies of themselves by the process of cell division. The controlled progression through the cell division cycle is of great importance for the correct copy, or replication, and subsequent distribution of the genetic information, as well as partitioning of the cell content into the daughter cells.

Nowadays, investigating the mechanisms of the cell cycle is mostly focused on the questions how in eukaryotic organisms the cell division is integrated into processes like growth, development and cell death, and how stress can influence the cell division cycle progression (Figure 1-3). Plants offer exceptional opportunities to significantly contribute to this study (Inzé, 2005).

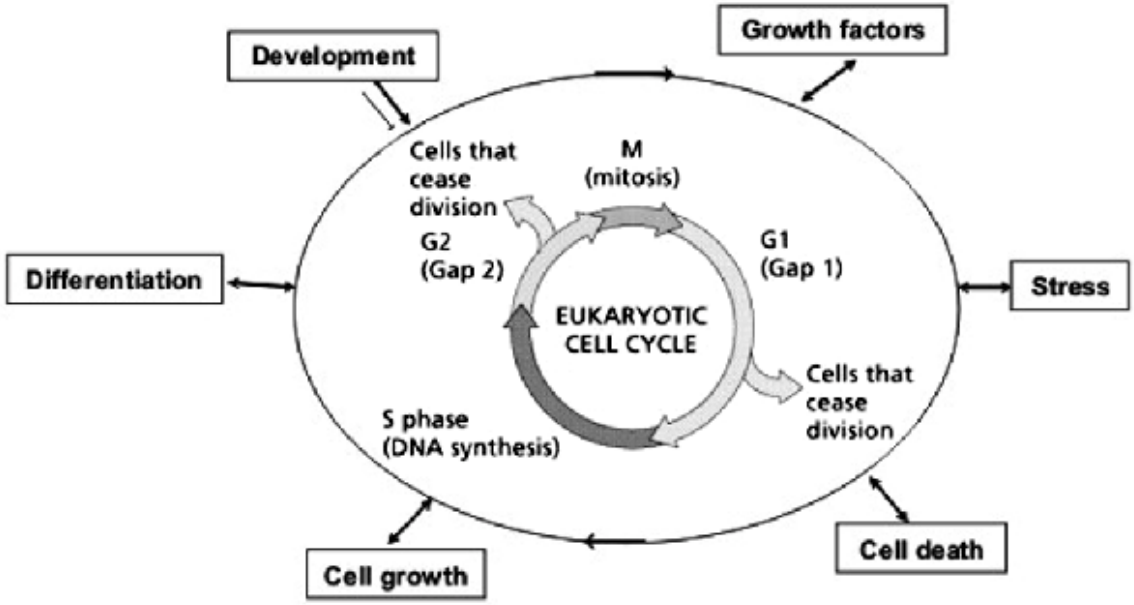

Figure 1-3 Cell cycle progression.
Understanding how the cell-cycle machinery integrates into different processes and how stress can influence the cell division cycle progression is a major challenge for the future (Stals and Inzé, 2001). Bars indicate inhibition; single-headed arrows indicate activation; double-headed arrows indicate unknown regulatory mechanism. The inner circle represents the cell cycle progression including the two active phases – DNA synthesis (S phase) and mitosis (M phase), preceeded by the regulatory G1 (Gap 1) and G2 (Gap 2) phases. The outer circle is a schematic view of the cell cycle progression and shown interacting factors.

1.6.1. The cell division cycle in plants

The overall control of the cell cycle is broadly similar between plants and other eukaryotic organisms. In recent years, the understanding of the cell proliferation and cell cycle control has increased considerably (Inzé, 2005). The most common form of cell division cycle, or cell cycle, includes two active phases – the S-phase when DNA synthesis occurs, and the M-phase (or mitosis) during which the chromatin is folded and compacted into chromosomes that are equally distributed to the daughter cells (Figure 1-3). Regulatory gap phases, G1 and G2, precede the S- and M-phases, respectively. Cells decide whether or not to proceed through the cell cycle during the G1- and G2-phases (Dewitte and Murray, 2003). After the cell has completed mitosis, it may initiate another complete cycle (G1 through mitosis) or it may leave the cell cycle. This choice is made at the critical G1 point, before the cell begins to replicate its DNA (Stals and Inzé, 2001). Characteristically, plant cells can leave the cell division cycle either before or after replicating their DNA, i.e., during G1 or G2 (Figure 1-3; Bergounioux et al., 1992; Devitt and Stafstorm, 1995).

1.6.2. Cell division cycle and cylin-dependent kinases (CDKs)

The G1/S- and G2/M-phase transitions are considered to be the major control points in the cell cycle at which decisions are taken with respect to division, differentiation, programmed cell death or adoption of quiescent state (Meijer and Murray, 2001). Both transitions are controlled by serine/threonine kinases that become functional by binding to a regulatory protein known as cyclin and are therefore termed as cyclin-dependent kinases (CDKs) (Morgan, 1997). The CDKs are key regulators of the cell cycle (De Veylder et al., 2003). They phosphorylate substrate proteins at serine or threonine residues, thereby modifying the substrate's properties. Known substrates include transcriptional regulators and components of the cytoskeleton, nuclear matrix, nuclear membrane, as well as cell cycle proteins (Dewitte and Murray, 2003).

1.6.3. Plant-specific B-type CDKs

Usually, the expression pattern of CDKs is constitutive throughout the cell cycle. Meanwhile the periodic expression of cyclins provides the characteristic cell cycle phase-specific timing of CDK activities (Morgan, 1997). However, plants contain a unique class of CDKs (B-type CDKs; CDKBs) with periodic gene expression and elevated protein accumulation at G2/M phases (Fobert et al., 1996; Segers et al., 1996; Magyar et al., 1997; Mészáros et al., 2000; Joubès et al., 2000; Porceddu et al., 2001; Sorrell et al., 2001; Menges and Murray, 2002; Vandepoele et al., 2002; Kono et al., 2003). Comparison with the expression pattern of marker genes with known cell cycle-phase dependency revealed the presence of two CDKB classes – CDKB1 and CDKB2 (Joubès et

al., 2000; Vandepoele et al., 2002). The *CDKB1* expression starts during S-phase and continues during the G2/M transition, overlapping the *CDKB2* expression that is specific to the G2- and M-phases (Fobert et al., 1996; Segers et al., 1996; Magyar et al., 1997; Porceddu et al., 2001; Sorrell et al., 2001; Menges and Murray, 2002).

1.6.4. Significance of the control of the G2/M transition

The regulation of G2-to-M-phase transition has special significance considering plant development because it is linked to the synthesis of the new cell wall and the possibility of endoreduplication. Endoreduplication is a modified cell cycle that involves repetitive chromosomal DNA replications without intervening mitosis or cytokinesis. As a result the genome is represented by several copies, i.e. higher ploidy (Larkins et al., 2001; Boudolf et al., 2004b). This leads to increased cell size and it is also characteristic for developmental processes like endosperm formation (Grafi and Larkins, 1995). Thus, controlling the switch between mitotic chromosome segregation and endoreduplication programs represents an important agronomical challenge for increasing yield. The understanding of the mechanisms governing S-G2-M-phase progression in plants is in the focus of cell cycle research.

1.6.5. Transcriptional regulation of the G2/M transition

The transcriptional control at the G2/M checkpoint is still to be revealed. The activity of B-type CDKs is characteristically regulated at the transcriptional level. Understanding of this control may help unravelling the regulation of G2/M transition. Additional knowledge comes from studies on the control of other G2/M-specific genes in plants and animals.

1.6.5.1. MSA *cis*-element recognized by Myb factors

It is well known that cyclin B acts as a regulatory subunit of a CDK and has a critical role in G2/M progression of the cell cycle (Pines and Hunter, 1990). *Cyclin B* genes have been identified in many different plant species and their expression was shown to be specific for the G2 and M phases (Fobert et al., 1994; Kouchi et al., 1995; Setiady et al., 1995; Ito et al., 1997; Menges and Murray, 2002). It has been demonstrated that the phase-dependent mRNA accumulation of *cyclin B* gene is, at least partly, directed by the promoter activity that is regulated during the cell cycle (Shaul et al., 1996; Ito et al., 1997; Tréhin et al., 1997). Detailed study by deletion or point mutation of *Catharantus roseus cyclin B* promoter, *CYM*, in heterologous system of tobacco (*Nicotiana tabacum*) cells resulted in identification of the MSA (M-phase-specific activator) *cis*-element (Ito et al., 1998). The MSA, with the core sequence of AACGG, is responsible for cell cycle phase-

dependent expression of the reporter gene since its absence from the promoter abolishes G2/M-phase-specific expression, and also this element alone is sufficient to direct G2/M-phase-dependent promoter activity of the basal part of cauliflower mosaic virus 35S promoter (*35S*).

The presence of MSA elements is not restricted to promoters of *cyclin B* genes, but they are also present in the promoters of genes expressed with similar kinetics. Mutational analysis has shown that the G2/M-periodic activity of tobacco *NACK1* promoter is dependent on the presence of the MSA sequence (Ito et al., 2001). NACK1 is an M-phase-specific kinesin-like motor protein that has been identified as an activator of NPK1, a mitogen-activated protein kinase kinase kinase that is required for the formation of the cell plate during cytokinesis in tobacco (Nishihama et al., 2002). In addition, a report suggests that another gene essential for cytokinesis in *Arabidopsis*, *KNOLLE*, may also be regulated by the MSA elements, and that such regulation is important for its cytokinetic function (Volker et al., 2001).

Thus, it seems likely that the wide range of G2/M-phase-specific genes are co-regulated by a common mechanism based on the presence of the MSA element. Supportingly, a genome-wide analysis of the *Arabidopsis* transcripts from synchronized cell cultures, applying the microarray approach, has revealed 93 genes with periodic expression similar in kinetics to B-type cyclin genes (Menges et al., 2002). Among them, approximately 20 % of the genes have MSA-like motifs in their promoter regions, as defined by a 1 kb region upstream of the translation initiation sites.

It has been found that tobacco Myb factors are able to bind *in vitro* to the MSA DNA sequence (Ito et al., 2001). These Myb factors belong to the evolutionarily most conserved group of Myb TFs in plants and contain three conserved motifs within the DNA-binding domain (three-repeat Myb, or 3R Myb). However, in contrast to vertebrates where they regulate the cell cycle specifically at the G1/S transition, in plants the 3R Myb proteins have a role in the entry and progression through the M-phase (Ito, 2005). The MSA-binding 3R Myb factors in tobacco, NtmybA1 and NtmybA2, were transiently expressed in tobacco Bright-Yellow-2 protoplasts, and they could activate promoters from *cyclin B* and *NACK1* genes. By contrast, expression of NtmybB caused decreased activity of these promoters. In addition, transcription of the NtmybA1 and NtmybA2 genes themselves is also G2/M cell cycle-phase regulated but preceding their target genes, while the NtmybB is constitutively expressed. Based on these data, it was assumed that a balance between activators and repressors is may be required to ensure stringent cell cycle-phase-specific expression (Ito, 2005). Since the tobacco genes coding for 3R Myb factors are also transcribed at the G2/M transition, analysis of their transcriptional control is required for understanding the primary control of G2/M-phase-specific transcription in plants.

It is necessary to note that in the promoter of *Nicotiana sylvestris cyclin B* (*Nicsy;CYCB1;1*) the core MSA sequence is present, enssuring cell cycle activity without cell cycle-phase-dependence (Tréhin et al., 1999). Therefore, it was suggested that related but different proteins bind the MSA-like element. The evidence supporting the mitotic function of plant 3R Myb factors has been obtained using cell culture systems. Functional analysis of 3R Myb genes *in planta* is necessary in order to confirm their role.

1.6.5.2. Other cis-elements and TFs (CDE/CHR elements, CCAAT-box, E2F factors)

In mammalian cells, the G2/M-phase transcription of *cyclin A, cdc2* (equivalent to CDK), *cdc25C* (encoding a CDK-activating phosphatase) and *cyclin B2* genes has been shown to be controlled by tandemly organized repressor promoter elements: CDE (cell cycle dependent element) and CHR (cell cycle gene homology region) with the GGCGGNNNNTTTGAA sequence (Lucibello et al., 1995; Zwicker et al., 1995a; Dohna et al., 2000). Subsequent experiments have revealed that CDE/CHR-mediated repression occurred only in the presence of a functional upstream activating sequence that includes CCAAT element (Zwicker et al., 1995b). In the promoter of the human *cyclin B2* gene, a sole CHR element (TTTGAA) in the proximity of the CCAAT-box provides the cell cycle regulation (Wasner et al., 2003). It is believed that a repressor protein binds to these elements during the G1-phase, and that subsequent dissociation of the repressor in late S- and G2-phases activates transcription.

E2F transcription factors regulate S phase entry (Dyson, 1998; Harbour and Dean, 2000), but recent studies have revealed their involvement in the regulation of the G2/M transition, too (Ishida et al., 2001; Ren et al., 2002). Zhu et al. (2004) demonstrated the direct role for E2F factors in both the repression and activation of human *cdc2* and *cyclin B1* promoter activity. Mutation analysis of E2F-binding sites revealed the presence of distinct *cis*-elements recognized by activator and repressor E2F factors, respectively, at G2/M. In addition, the role of neighboring CCAAT and CHR *cis*-elements was also demonstrated. Mutation of the CCAAT-element recognizable by positive-acting TFs (Maity and de Crombrugghe, 1998) abolished *cdc2* promoter activity. In contrast, mutation of the CHR element (the identity of the CHR-binding TFs has not been established yet) led to an increase of *cdc2* promoter activity. In addition, the significance of Myb factor for G2/M regulation was also shown. Taken together, these results suggest a role for multiple elements: E2F-binding sites, CCAAT, CHR, and Myb-binding sites to control the activity of the *cdc2* promoter in both positive and negative manner. It was suggested that the combination of these *cis*-elements recognizable by corresponding TFs ensures cooperative interactions between the TFs, which is required for specific gene regulation (Zhu et al., 2004).

Wheather a similar mechanism for cell cycle regulation exists in plants, it is still unknown. In *Arabidopsis* the E2F factors can be functionally subdivided into activators (E2Fa and E2Fb) and a repressor (E2Fc) (DeVeylder et al., 2003). Since the E2F factors act as heterodimers with DP factors required for high-affinity sequence-specific DNA binding, transgenic *Arabidopsis* plants overproducing the E2Fa-DPa heterodimer were generated and studied (Boudolf et al., 2004b). It was observed that the expression of the B-type CDK, *CDKB1;1*, was upregulated in these plants. Also *CDKB1;1* promoter activity was stronger than in the control plants, as shown by promoter: *GUS* (*β-glucuronidase*) reporter fusion. These data suggest a direct stimulation of *CDKB1;1* promoter activity by E2Fa-DPa factor. Consistently, an E2F-like binding site can be detected in the *CDKB1;1* promoter (Boudolf et al., 2004b). More recent research highlighted the role of the other activating factor, E2Fb, in the regulation of *CDKB1;1* (Magyar et al., 2005). It was emphasized that in E2Fa-DPa overproducing *Arabidopsis* plants, the *E2Fb* expression was also upregulated, and the *E2Fb* promoter contains E2F-binding site. Together with studies on tobacco cell cultures overexpressing the *Arabidopsis* E2Fa or E2Fb factors, the data suggested that E2Fa indirectly increases *CDKB1;1* expression, perhaps through E2Fb. Supporting evidence is that in hormone-free conditions no CDK expression and activity can be observed in cultured tobacco cells, however, the expression of E2Fb but not E2Fa promotes cell division, including increased CDK expression and activity (Magyar et al., 2005). In addition, mutation in the *CDKB1;1* promoter impairing the binding of E2F-DP resulted in substantial decrease of the promoter activity in tobacco cells (Boudolf et al., 2004b). Deletion of the E2F-binding site did not result in increased transcriptional activity in maturing leaves supporting the regulation by activating E2F factors. The combinatorial nature of E2F factors demonstrated in mammals (Zhu et al., 2004) is still to be investigated in plants. Characterizing the promoter structure of cell cycle phase-specific genes in plants is expected to elucidate the involvement of TFs regulating the gene expression.

The knowledge about the regulation of G2/M-specific genes in plants increases. In particular, the regulation of B-type CDKs, is on the focus of current studies. Investigating the transcription control of both B1- and B2-type CDKs is necessary to clarify the mechanism of the switch between mitosis and endoreduplication.

1.6.6. The role of B-type CDKs

Currently, the knowledge about the function of the B-type CDKs comes from studies on B1-type CDKs. The gene expression and activity data suggest a role of this B-type kinase in the switch between mitosis and endoreduplication (Porceddu et al., 2001; Boudolf et al., 2004a,b). The *CDKB1;1* expression is characteristic for cell cycle activity-requiring stages of plant growth and

development, including seed germination and organ formation, as well as pathogen invasion (de Almeida Engler et al., 1999; Beeckman et al., 2001; Himanen et al., 2002; Boudolf et al., 2004b; Barrôco et al., 2005). In addition, it was demonstrated the role of this cell cycle phase-specific kinase in seedling development in relation to cell elongation, light response and plastid differentiation, which could be uncoupled from the cell cycle activity (Yoshizumi et al., 1999). Further experiments, as well as additional studies on B2-type CDKs, are necessary to clarify the function of B-type CDKs during the plant life cycle.

1.6.7. Effects of hormones and wounding on the regulation of cell cycle progression

Different hormones, as well as wounding, and possibly other stress factors, seems to affect the regulation of cell cycle genes. The data suggest a complex transcriptional control that modulate gene expression according to the requirements of the plant, and address the question how a cell cycle phase-dependent genes, such as *CDKB*s, is affected by environmental stimuli.

1.6.7.1. Hormonal regulation of CDK expression

Auxin. Since long, auxins have been put forward as potent stimulators of cell division (Gautheret, 1939). It has been shown that the initiation of lateral roots is an ideal model system for studying how auxin signalling activates cell cycle progression (Himanen et al., 2002; Casimiro et al., 2001, 2003; Vanneste et al., 2005). Deprivation of auxin keeps pericycle cells (having principal role during the first stages of lateral root initiation) in G1-phase, whereas re-addition of auxin promotes the G1/S transition (Casimiro et al., 2003). Recent studies focused on the auxin role at the G2/M checkpoint and highlighted auxin induction of B-type CDK genes that may be mediated by E2Fb pathway (Magyar et al., 2005; Vanneste et al., 2005).

Cytokinin. Cytokinins are necessary, in concert with auxin, for cell division at the G1/S transition (Riou-Khamlichi et al., 1999; Meijer and Murray, 2000). Studies on the G2/M progression in tobacco cell cultures suggested that cytokinin controls the activity of a phosphatase whose substrate is CDK with an inhibitory phosphate group. The subsequent dephosphorylation increases CDK activity (Zhang et al., 2005).

Brassinosteroids (BRs). BRs act at the G1/S checkpoint (Hu et al., 2000). *In planta* investigations suggested that the BRs are part of the regulatory system controlling *CDKB1;1* expression (Yoshizumi et al., 1999; Stals and Inzé, 2001).

Gibberellin (GA). Upon submergence in water, cell division and cell elongation are accelerated in the intercalary meristems of deep-water rice internodes by GA. This mitogenic

hormone initially induces the G1/S transition (Sauter, 1997). At the G2/M transition, *CDKB1;1* is induced and following increase in mitotic cyclin gene expression occurs (Fabian et al., 2000).

ABA. The stress-responsive hormone ABA inhibits cell division in response to adverse environmental cues. ABA prevents tobacco cells from entering S-phase, but does not affect the progression through the other cell-cycle phases (Swiatek et al., 2002). *In planta* experiments supported the role of this hormone in the G1/S transition (Wang et al., 1998). An indirect effect of ABA at the G2/M boundary might be exerted by counteracting the GA response (Sauter et al., 1995).

1.6.7.2. Effect of defense-related hormones and wounding on CDK regulation.

JA, SA and ethylene mediate in particular the defense response caused by pathogen attack or wounding (Ryals et al., 1996; Creelman and Mullet, 1997; Durner et al., 1997; Reymond and Farmer, 1998; Wang et al., 2002). Their effects depend on cellular context defined by various receptors and signaling pathways that are active under different conditions (Vanacker et al., 2001; Wang et al., 2002; Euglem, 2005). In addition to the defense mechanism these molecules affect cell division, which suggests the regulation of cell cycle genes in response to stress.

JA. The application of JA on synchronized tobacco cells blocked both G1/S and G2/M transitions (Swiatek et al., 2002). It was suggested that JA prevents entering into mitosis by arrest in early G2, before the induction of expression of the G2/M genes, mitotic cyclin (*CYCB1;1*) and *CDKB1;1*, take place (Swiatek et al., 2004).

SA. SA regulates cell growth by specifically affecting cell enlargement, endoreduplication, as well as cell division (Vanacker et al., 2001). Interestingly, *Pseudomonas syringae*, a pathogen that induces cell death and SA in *Arabidopsis*, also stimulates the expression of *CDKB1;1* (Vanacker et al., 2001). The *CDKB1;1* induction was evidenced by the induction of GUS activity in transgenic plants bearing 5 kb upstream promoter region and first codon of *CDKB1;1* fused to the *GUS* reporter.

Ethylene. Ethylene may induce expression of mitotic cyclins in rice stem cuttings, cause cell death at the G2/M boundary in tobacco cells, inhibit the progression through the G2/M transition and induce endoreduplication in cucumber (*Cucumis sativus*) hypocotyl epidermis (Lobriecke and Sauter, 1999; Herbert et al., 2001; Dan et al., 2003; Kazama et al., 2004).

Wounding. Wound-responsive genes are not only induced under conditions of stress, but are also expressed in specific cells during plant development (Siebertz et al., 1989; Guiderdoni et al., 2002; Preston et al., 2004). Interestingly, the *Arabidopsis* cell cycle-related A-type CDK, expressed constitutively during the cycle, could also be induced by wounding in the absence of cell division

(Hemerly et al., 1993). This wound-induced expression was correlated to an increased competence for cell proliferation. Wound-activated expression of a mitotic cyclin (*CYCB1;1*) was also reported in *Arabidopsis*, and function in structural reinforcement was suggested (Colón-Carmona et al., 1999). It was also shown that cell proliferation could act as a defense mechanism during vascular regeneration in the stem and the roots (Nishitani et al., 2002).

2
MATERIALS AND METHODS

2.1. Plant material manipulations

2.1.1. Wheat (*Triticum aestivum* L.)

2.1.1.1. Material for construction of subtraction libraries

Hydroponic cultures of the drought tolerant wheat cv. Kobomugi (Erdei et al., 1990; Nagy and Galiba, 1995) and the mild drought tolerant cv. Öthalom (Szgeletes et al., 2000) were used for construction of subtraction libraries. Growing in the absence or in the presence of polyethylene glycol 6000 (PEG) were performed, according to Erdei et al. (2002), in order to generate control and drought/osmotic-stressed plantlets. Caryopses were imbibed in distilled water and after 24 hours the viable germs were placed into complete 0.5x diluted Hoagland solution (Kerepesi and Galiba, 2000; complemented with 200 mOsm (14.3 % PEG). The nutrient solution without addition served as control. Plants were grown under controlled conditions in phytotron (Conviron, type EF7) in 14 h day/ 10 h night period and 24/18°C day/night temperature. The containers were aerated and the nutrient solution was exchanged weakly.

2.1.1.2. Material for expression analysis

Both, cv. Kobomugi and cv. Öthalom seedlings were grown in hydroponic conditions in the green house under natural light (13 h day/ 11 h night). On the 10^{th} day, treatment with increasing concentrations of PEG was applied, as follows: 100 mOsm (after the 10^{th} day), 200 mOsm (after the 12^{th} day), and 400 mOsm (after the 14^{th} day).

2.1.2. Alfalfa (*Medicago sativa* L.)

2.1.2.1. Plant transformation (alfalfa, tobacco, *Arabidopsis*)

Agrobacterium tumefaciens –mediated transformation of leaf discs from tetraploid alfalfa (*Medicago sativa* L., RegenS line, tetraploid) was performed according to Barbulova et al. (2002b). Transformed regenerants were generated via indirect somatic embryogenesis on selective - 4 mg/l phosphinotrycin

(PPT), B5II medium (Gamborg et al., 1968, with modifications: B5 micro- and macroelements, 500 mg/l Tripton, 500 mg/l Myo-Inositol, 30g/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg/l kinetin, 6g/l agar). Embryogenic calli were further grown and regenerated into plants on MS basic medium (Murashige and Skoog, 1962) in the presence of PPT (Barbulova et al., 2002a).

A. tumefaciens transformed *Arabidopsis* (*Arabidopsis thaliana*, ecotype Columbia-0) lines were generated by in planta transformation using vacuum infiltration (Bechtold and Pelletier, 1998). Seeds from infiltrated plants were germinated on selective (4 mg/l PPT) 0.5xMS basic medium.

The transgenic plants were grown *in vitro* in a phytostat at 23°C and under 16 h light/ 8 h dark. After adaptation to green house conditions, the plants were grown in the green house under natural light (13 h day/ 11 h night).

2.1.2.2. Synchronized alfalfa cell cultures

Two-week-old callus tissue from transformed plants - *Medicago sativa* (RegenS line) were used for generation of cell suspensions. A relatively homogeneous and fast-growing cell suspension culture was established and maintained in the presence of 1 mg/l 2,4-D and 0.5 mg/l kinetin in B5II liquid medium (Bögre et al., 1988 with modifications) containing the selective agent PPT (4 mg/l). The cell suspensions were subcultured once a week and cultivated at 150 rpm, at 23°C.

Synchronization of the cell division in exponentially growing (three-day-old) cell suspension cultures by 10 mM hydroxyurea (Sigma) has been done according to Ayaydin et al. (2000). After 36 h incubation, the inhibitor was released by three times washing with fresh B5II medium, containing PPT. Finally, the cells were resuspended in the original volume of medium from 4-day-old culture.

2.1.2.3. Wounding of alfalfa seedlings and leaf explants; hormone treatments

The leaves of *in vitro* transgenic alfalfa seedlings were wounded by strong cutting with a blade (1-3 random cuttings across the adaxial surface of each leaflet of the leaf). Mature leaves from alfalfa green house plants were detached, sterilized (70 % EtOH for 30 s, 70 % bleach for 15 min, water rinse), wounded, and cultivated on solid B5II medium without hormones. In a parallel experiment, wounded leaf explants were cultivated on medium supplemented with hormones – 2,4-D (1 mg/l or 4,5 μM) and ethephon /2-chlorethanephosphoric acid/ (10 mg/l or 0.7 μM). Treatments were also performed with GA (50 μM); kinetin (0.5 mg/l or 2 μM); ABA (50 μM); SA (1 to 100 μM); JA (0.1 to 500 μM).

2.2. DNA and mRNA analysis

2.2.1. Total RNA isolation

The photosynthesizing part (leaves and stems) of non-treated and PEG-treated wheat plantlets was used for RNA isolation. The guanidinium thiocyanate approach was applied for purifying RNA (Chomczynski and Sacchi, 1987). Briefly, the plant material is homogenized in a denaturing solution (4 M guanidinium thiocyanate, 25 mM Na-citrate pH 7, 0.1 M 2-mercaptoethanol, 0.5 % N-lauroylsarcosine). The homogenate is mixed sequentially with 2 M Na acetate pH 4, phenol, and chlorophorm/isoamyl alcohol. The resulting mixture is centrifuged yielding an upper aqueous phase containing total RNA. In this single step extraction the total RNA is separated from proteins and DNA that remain in the interphase and in the organic phase. Following isopropanol precipitation, the RNA pellet is redissolved in the denaturing solution, reprecipitated with isopropanol, and washed with 75 % ethanol. Total RNA from alfalfa cell cultures was isolated by using TRIzol reagent according to the manufacturer's instructions (Invitrogen).

2.2.2. Construction of a subtracted cDNA library

Poly A (+) RNA was purified from total RNA using oligo(dT)-cellulose (Sigma) in high salt buffer, washed extensively and eluted in salt free buffer (Sambrook et al., 1989). First and second cDNA strands were synthesized with the PCR-Select cDNA Subtraction kit and PCR-Select cDNA subtraction was performed according to manifacturer's instructions (Figure 2-1; Clontech). The subtracted PCR-ed fragments were directly inserted into a T/A cloning vector (pGemT Easy vector, Promega, or derived from pBlueScript/SK+ vector, Stratagene). The constructed cDNA libraries were transformed in *Escherichia coli* DH5α competent cells. The insert content was tested on randomly picked colonies by colony PCR using the nested primers, or the T3/T7 primers (in the case of pBlueScript/SK+).

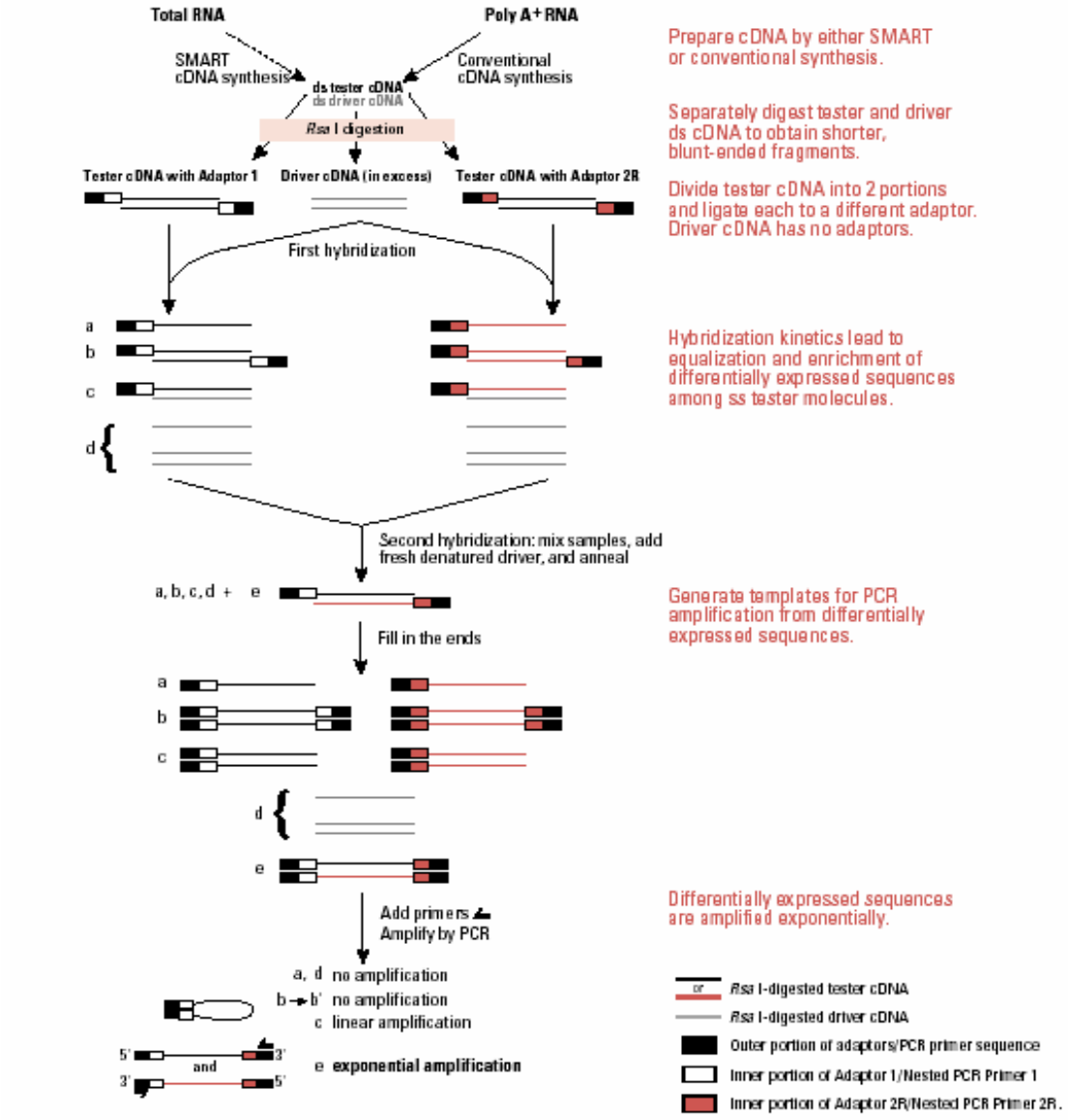

Figure 2-1 Clontech PCR-Select cDNA Subtraction procedure
Double stranded (ds) cDNA was digested with a four-base-cutting restriction enzyme *Rsa*I (in order one gene to be represented several times by several short fragments). The dscDNA population supposed to contain specific (differentially expressed) transcripts is defined as a "tester", and the control dscDNA as a "driver". The tester cDNA is subdivided into two portions and each is ligated with a different cDNA adaptor (1 or 2R) provided with the kit. The ends of the adaptor do not have a 5'-phosphate group, so only one strand of each adaptor attaches to the 5'-ends of the digested cDNA. The two adaptors have stretches of identical sequence to allow annealing of the polymerase chain reaction (PCR) primer after the recessed ends have been filled in. Subsequently, two hybridizations were performed. In the first, an excess of driver was added to each sample of tester. The samples were heat denatured and allowed to anneal, generating the type ***a***, ***b***, ***c*** and ***d*** molecules in each sample. The concentration of high- and low-abundance sequences is equalized among the type ***a*** molecules because re-annealing is faster for the more abundant molecules due to the second-order kinetics of hybridization. At the same time, the single stranded (ss) type ***a*** molecules are significantly enriched for differentially expressed sequences, as cDNAs that are not differentially expressed form type ***c*** molecules with the driver. During the second hybridization, the two primary hybridization samples were mixed together without denaturing. In this step, only the remaining equalized and subtracted ss tester cDNAs can re-associate and form (see next page)

Figure 2-1 (continued) new type ***e*** hybrids. These new hybrids are ds tester molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA was added (again, without denaturing the subtraction mix) to further enrich fraction ***e*** for differentially expressed sequences. After filling the ends by DNA polymerase, the type ***e*** molecules – the differentially expressed tester sequences – have different annealing sites for the nested primers on their 5'- and 3'-ends. The entire population of molecules was then subjected to PCR to amplify the desired differentially expressed sequences. The primers are supplied with the kit. The first used primer is universal for both the adaptors. Next, a secondary PCR amplification was performed using nested primers (1 and 2R) to further reduce any background PCR products and to enrich for differentially expressed sequences. Only type ***e*** molecules, which have two different adaptors, were amplified exponentially. Theoretically, these are equalized, differentially expressed sequences.

2.2.3. Sequencing and analysis of subtracted EST clones

Randomly selected clones (amplified by PCR) from the subtracted libraries were sequenced using the Nested primer 1, or T3 primer (in the case of pBlueScript/SK+) and Applied Biosystems Sequencer (Forster City, CA). The ESTs were analyzed using the BLAST program (Altschul et al., 1990). In order to increase clone specifisity, the selected clones were extended using GenBank (www.ncbi.nlm.nih.gov) and TIGR database (www.tigr.org) together with contig construction software (Contig Express, Vector NTI Suite 6). The results are shown in details in the Appendix. Primer Premier 5 (Premier Biosoft International) was applied for designing of primers for the extended clones. The sequences of these primers are shown in Table 2-1.

Table 2-1 Oligonucleotides used for PCR amplification of wheat genes corresponding to subtracted EST clones

Gene name*	Sequence	Purpose
lt	5' gagaattCATCACTCTACTCCACCTACC	Northern blot, forward/ mz5**
	5'gaggatccGCTAATCATCAAGCACGAA	Northern blot, reverse/ mz6
rd22	5'GTAACGAGGGTCAGAGAGG	Northern blot, forward/ mz15
	5'CGTAGCCACACGCAGG	Northern blot/ RT-PCR, reverse / mz16
rd22	5' GCTCACGCTGCTGCTTA	RT-PCR, forward/ mz47
rca2	5'gagaattCAAGGCCAACAGGTTCACA	Northern blot, forward/ mz3
	5'gaggatcCGTCACTCCTCGCCGTT	Northern blot, reverse/ mz4
rcbS	5' CGTGATGGCTTCGTCGGCTAC 3'	Northern blot, forward/ las70
	5' CCTCGCAACCCGGTGGCCTG 3'	Northern blot, reverse/ las71
TCTP	5'CGCTACATCAAGAACCTC	Northern blot, forward/ mz13
	5'AATACTGACGACCGCATA	Northern blot, reverse/ mz14
TCTP	5' GGACCTTCAGTTCTTTGTTGGC	RT-PCR, forward/ mz21
	5' GACGACCGCATAGATTTTAGCAT	RT-PCR, reverse/ mz22
UCE	5'CGCATAAGATCGCAACATTT	RT-PCR, forward/ mz52
	5'CAGGCGTACCCAGGAGAT	RT-PCR, reverse/ mz34
UP_79	5'gagaatTCAACAAGGCTGTGGACG	Northern blot, forward/ mz7
	5'gaggatccATAAAGGCTCGCCAGAAG	Northern blot, reverse/ mz8
UP_79	5'CTCGTGCTGCGTAGATT	RT-PCR, forward/ mz27
	5'CGAAGCCCTTGAACCA	RT-PCR, reverse/ mz28

* see Appendix for full names
** working name of the primers

First strand cDNA was used as template for PCR amplification with primers specific for cDNAs corresponding to extended differentially expressed EST clones. The PCR products were subsequently cloned into T/A cloning vector derived from pBlueScript/SK+ vector, and their identity and orientation were confirmed by sequencing.

2.2.4. Cloning of the promoter of *Medsa;CDKB2;1*

Cloning of genomic DNA fragments from tetraploid alfalfa (*Medicago sativa* L., RegenS line) upstream of the *Medsa;CDKB2;1* coding sequence was carried out with the method of PCR-based genome-walking (Siebert et al., 1995). An *Eco*RI-adapter was ligated to *Eco*RI-digested genomic DNA fragments and served as a 5'-primer sequence for PCR amplification together with the *Hind*III-site carrying 3' primer complement to the mRNA of alfalfa CDKB2;1. The generated genomic DNA fragments were contiguous from the start codon up to the endogenous *Eco*RI site including the 5'-UTR. The PCR products were digested with *Eco*RI and *Hind*III restriction enzymes and subcloned upstream from the *β-glucuronidase* (*GUS*) gene in the pCambia3301 vector (Bayer Crop Sciences). *E. coli* transformation was performed and several resistant colonies were used for sequencing the *Eco*RI-*Hind*III inserted clones. The vector pCambia3301 was used for generation of constructs where the longest DNA fragment, named *fpr15*, was fused to reporter genes coding for GUS, (*fpr15:GUS*) and luciferase (*fpr15:luc*, or, *fpr15:luc_35S:GUS*). The resulting plasmids were introduced into *A. tumefaciens* (LBA4404).

2.2.5. Northern blot assay

Twenty micrograms of total RNA were loaded, following Qiagen BenchGuide instructions, on formaldehyde (FA)-containing 1.5 % agarose gel, then subjected to electrophoresis in FA-running buffer (6 % FA, 20mM 4-morpholinepropanesulfonic acid/MOPS, 5mM sodium acetate, 1mM ethylene diamine tetra-acetic acid, pH 7.0). Following separation, RNA was capilliarity-blotted onto Hybond N membrane (Amersham) (Sambrook et al., 1989). The used oligonucleotide sequences for probe generation are shown in Table 2-1 and 2-2. Radiolabeling with [α-^{32}P]dCTP was performed by PCR or random-priming (Megaprime DNA labeling systems, Amersham). The membrane containing the transferred RNA was first pre-hybridized for 1 hour in Church-Gilbert solution (1 mM ethylene diamine tetra-acetic acid, 0.25 M $NaHPO_4$, 7 % sodium dodecyl sulphate/SDS) at 65°C. Then the labeled DNA probe was added to the pre-hybridization buffer and hybridized with the membrane-bound RNA overnight at 65°C. The filter was washed with SSC solution (SSC: 0.15 M NaCl, 0.015 M

Na-citrate, pH 7.0) supplemented with 0.1 % SDS, at 65°C, as follows: twice with 2x SSC for 10 min; once with 0.5x SSC for 10 min; and once with 0.1x SSC for 10 min. Hybridization signals were detected by Phosphor Imager 445 SI (Molecular Dynamics) and analyzed by the Image Quant NT 5.0 software.

2.2.6. Quantitative Reverse-Transcriptase PCR (Q-PCR)

cDNA templates for Q-PCR were synthesized according to the manifacturer's instructions (First-strand cDNA synthesis kit, Fermentas) from 2 µg total RNA. PCR reactions were made in 25 µl volume by using SYBR green PCR Master Mix (Applied Biosystems), 2 µl 100x diluted cDNA template and 0.3 µM gene specific primers (Table 2-1). The PCR assays were performed and analyzed using the ABI Prism 7000 Sequence Detection System instrument and 1.1 software (Applied Biosystems).

Table 2-2 Oligonucleotides used for PCR amplification of alfalfa genes

Gene name	Sequence	Purpose
Medsa;CDKB2;1 (Cdc2MsF*)	5'-CTTCATGAAGATGATGAA-3'	Northern blot, forward
	5'-CTAAAGATGGGTCTTGTCCA-3'	Northern blot, reverse
Medsa;CDKA;1 (Cdc2MsA)	5'-CGGGTACACCGAATGAGGAA-3'	Northern blot, forward
	5'-GGCTGATTTAAAATCAGGCAATG-3'	Northern blot, reverse
Firefly *luciferase* gene in pGL3-Enhancer vector	5'-AACGGATTACCAGGGATTTCAG-3'	Northern blot, forward
	5'-AGACTTCAGGCGGTCAACGA-3'	Northern blot

* In parenthesis is given the original name of the gene.

2.2.7. Sequence data

The wheat sequences are shown in details in the Appendix. The TCTP-coding gene is a wheat homologue of the alfalfa *Msc27* gene (Gyorgyey et al., 1991). The rest of the sequence data have been deposited with the GenBank data libraries under accession numbers: DQ136188, promoter region of *Medsa;CDKB2;1* (*fpr15*); X97317, *Medsa;CDKB2;1* (Magyar et al., 1997); M58365, *Medsa;CDKA;1* (Hirt et al., 1993); AC144481 BAC clone containing the 5' promoter region of *Medicago truncatula CDKB2;1*; D10851 (At3g54180), *Arath;CDKB1;1*; AJ297936 (At1g76540), *Arath;CDKB2;1*; U47297, Firefly luciferase gene in pGL3-Enhancer vector; AF234314, *GUS* gene in binary vector pCAMBIA-3301.

2.3. Flow cytometric analysis and mitosis index determination

Nuclei for flow cytometric analysis were isolated after protoplastation (1 h, 80 rpm, RT, with 2 % cellulase YC and 0.2 % pectolyase in enzyme buffer: 0.2 M manitol, 0.08 M $CaCl_2$, 0.1 % (or

5 mM) 2-[N-morpholino] ethansulfonic acid, pH 6.0) and lysis in Galbraith buffer (45 mM $MgCl_2.6H_2O$, 30 mM $Na_3Citrate.2H_2O$, 20 mM MOPS, pH 7.0, 0.3 % Triton-X-100) (Galbraith et al., 1983). After staining with 1 ng/ml propidium iodide, the nuclei were sorted by Becton&Dickinson FACS Calibur and the data were analyzed by ModFit software. For checking the mitotic index, cells were fixed with 6 % formaldehyde (Sigma) in 1xPBS (Sambrook et al.,1989), stained with 4,6-diamidino-2-phenylindole (1 ng/ml) and watched under fluorescent microscope.

2.4. Protein analysis

Protein extraction and immunoblotting were performed according to Magyar et al. (1997). Total protein from alfalfa leaf explants were extracted by grinding cells with quartz sand in homogenization buffer (25 mM Tris-HCl pH 7.5, 15 mM $MgCl_2$, 75 mM NaCl, 15 mM ethylene glycol tetra acetic acid, 60 mM β-glycerophosphate, 1 mM dithiothreitol, 0.1 % Nonidet P-40, 0.1 mM Na_3VO_4, 0.5 mM NaF, 1 mM phenylmehylsulfonyl, 10 mg/l aprotinin, antipain and leupeptin, 5 mg/l chymostatin and pepstatin), and subsequent centrifugation (45,000 g, 30 min, at 4°C). For immunoblotting, SDS-polyacrylamide gel was transferred onto polyvinylidene difluoride (Millipore) membranes in 50 mM Tris-base-50 mM boric acid buffer at a constant 10 V overnight. The filters were blocked in 5 % milk powder-0.05 % Tween 20 in TBS (25 mM Tris-CI, pH 8.0, 150 mM NaCI) buffer for 2 hours at room temperature. Following reaction was performed with the first antibody (rabbit Medsa;CDKB2;1; 1:500 dilution) in blocking buffer for 2 hours at room temperature. The filter was washed three times with TBST (0.2 % Tween 20 in TBS, pH 7.5), and incubated for 1 hour in blocking solution with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:10000 dilution; Sigma). The reaction was visualized using Super Signal West Pico Chemiluminescent Substrate (PIERCE).

2.5. Histochemical assays

2.5.1. GUS assay – plant staining, sections

GUS staining was carried out by the method described by Jefferson et al. (1987) with some modifications. Briefly, the plant tissues were incubated for 12 h at 37°C in a 50 mM sodium phosphate buffer (pH 7.0) containing 1 mM ethylene diamine tetra-acetic acid, 0.1 %SDS, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide. Before use, 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronide substrate was added. The stained tissues were bleached by several replacements with 70 %

ethanol. Images of GUS stained plants were taken with Leica MZFL III microscope and Leica DC 300F camera (Leica Microsystems), and analyzed by Leica IM 50 1.20 software.

For sectioning, GUS stained plants were fixed in 4 % formaldehyde or a fixative mixture (1.85 % formaldehyde, 5 % acetic acid and 63 % ethanol), at 4ºC, overnight, and embedded in glycol methacrylate resin according to the manufacturer's instructions (Technovit 7100). 5 μm sections were generated using glass knife and mounted in a water drop on a slide for microscopy. Nomarski phase-contrast images were taken with Axioscop2 MOT microscope and camera AxioCam HR (Zeiss) and analyzed by AxioVision 3.1. software.

2.5.2. Immunohistochemistry

Root tips were fixed as it was mentioned above. The immunohisochemical work was done in BayBioMed, Szeged according to Krenacs et al. (1999). Briefly, glycol methacrylate sections (5 μm) were subjected to wet heat-induced antigen retrieval in citrate buffer (pH 2.5) using the facilities of BayBioMed. The sections were blocked in 5 % milk powder in TBS and washed 5 min in TBS. Rabbit primary antibodies anti-luciferase (1:500 dilution; Sigma) and anti-Medsa;CDKB2;1 (1:1000 dilution; Magyar et al., 1997), were applied on separate sections (1 hour, room temperature). For visualisation of immunoreactions, goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase and diaminobenzidine substrate were used according to the manifacturer's instructions (EnVision).

2.5.3. Luciferase activity

The activity of the luciferase enzyme was monitored 10 min after addition of 1 or 2 mM luciferin substrate (Biosynth). The substrate stability persisted until the 4^{th} day of measurement and light excitation at different time points was detected with a CCD camera system (Visilux Imager, Visitron Systems GmbH). Bioluminescence images (after 10 to 25 min exposure time) were processed using the Metaview 4.5r6 software (Universal Imaging Corporation).

3
RESULTS

3.1. Identification of genes expressed differentially in wheat in response to drought/osmotic stress

3.1.1. Construction of subtracted cDNA libraries

In order to select genes that are expressed differentially during drought/osmotic stress, the subtractive hybridization technique was applied. As a starting material two wheat (*Triticum aestivum* L.) cultivars: cv. Kobomugi, known as drought tolerant, and cv. Öthalom, resistant only to a mild drought stress, were chosen. PEG treatment was applied in order to simulate osmotic stress that is coupled to the drought stress. Non-treated and PEG-treated seedlings from both cultivars were generated. Samples for RNA isolation were collected from the photosynthesizing part (leaves and stems) of two-week-old seedlings. Two subtracted cDNA libraries, assigned as dKobomugi and dPEG, were constructed.

3.1.1.1. dKobomugi subtraction - where driver cDNAs generated from non-treated seedlings were "subtracted" from tester cDNAs generated from PEG-treated seedlings of the drought tolerant wheat cv. Kobomugi (Figure 3-1):

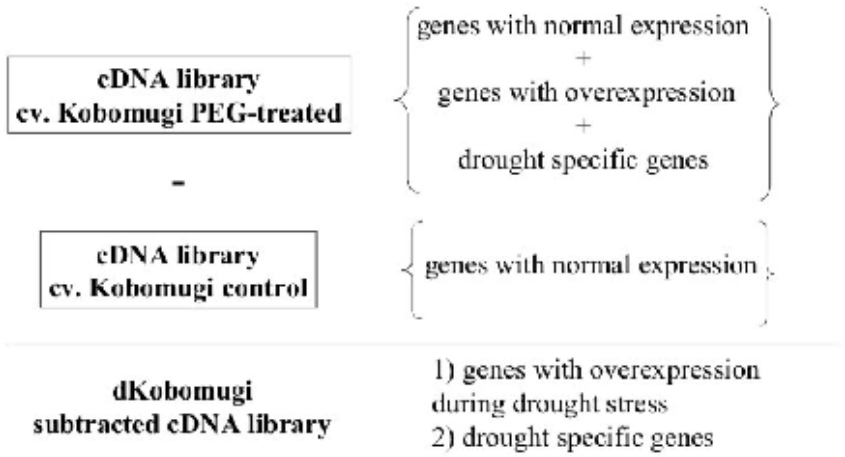

Figure 3-1 dKobomugi subtraction
In order to obtain differentially expressed genes, two cDNA populations differing from each other are used. The tester cDNA library generated from PEG-treated cv. Kobomugi seedlings contains several sets of genes: genes with normal expression, overexpressed genes and drought-specific genes. In the driver cDNA library from non-treated cv. Kobomugi seedlings, there are genes with normal expression. Scheme of the subtraction of these cDNA populations is shown. This approach results in a new, subtracted cDNA library, enriched in genes up-regulated in response to stress (overexpressed and drought-specific).

The resulting cDNA population covered a wide range in length between 150 bp and 1.5 kb. The PCR products were inserted into T/A cloning vector. Nearly 1000 independent transformant bacterial colonies were obtained. 78 randomly picked clones were PCR-amplified using the nested primer pair of the subtraction. The purified PCR products were subjected to sequence determination. 11 of the clones did not give readable sequences. 3 clones with no homology in the database (mainly because of their relatively short size: <60 bp) were defined as "no hits". The rest of the clones generated ESTs that were screened against the current GenBank database using the BLAST algorithm. On the basis of the search results, we classified the subtracted EST clones according to the characteristics of the proteins coded by homologous genes (Table 3-1):

Table 3-1 Putative functions assigned to ESTs from subtracted library prepared from non-treated and PEG-treated seedlings of the drought tolerant wheat cv. Kobomugi

Functional categories	Number of clones	Description of the homologues	Organism
Osmoprotection	1	CAA64683 ABA-inducible; homologue of Osr40-gene family	*Oryza sativa*
	1	Q09134 abscisic acid and environmental stress inducible protein	*Medicago sativa*
	1	dehydratation induced AY072821 rd22-like protein	*Gossypium hirsutum*
	1	AAM46894 low temperature and early drought induced protein	*Oryza sativa*
	1	NM_115116 putative E2, ubiquitin-conjugating enzyme	*Arabidopsis thaliana*
	1	Q40070 photosystemII 10 kDa polypeptyde, chloroplast precursor	*Hordeum vulgare*
Metabolism	13	BAB 19812 Rubisco small subunit (rbcS)	*Triticum aestivum*
	12	Rubisco large subunit (rbcL)	*T. aestivum(chl. genome)*
	2	Q40073 Rubisco activase (rcaA2)	*Hordeum vulgare*
	1	T02955 probable cytochrome P450 monooxygenase (fragment)	*Zea mays*
	1	Q96563 glutamil-tRNA reductase 2 (GTTR)	*Hordeum vulgare*
	1	spQ41048 chloroplast precursor, 16 kDa subunit of oxygen-evolving system of Photosystem II	*Zea mays*
	1	P20143 chloroplast precursor, PSI-H subunit of Photosystem I reaction center	*Hordeum vulgare*
	1	AP000615 glyceraldehyde-3-phosphate dehydrogenase	*Oryza sativa*
Protein synthesis	1	AJ307662 putative plastid ribosomal protein L19 precursor	*Oryza sativa*
	1	NC_003075 60S ribosomal protein L15 homologue	*Arabidopsis thaliana*
	1	NC_003070 putative translation initiation factor IF2	*Arabidopsis thaliana*
	1	weakly similar to sp:P02996 elongation factor G	*Escherihia coli*
Membrane-associated	4	Q42842 non-specific lipid transfer protein	*Hordeum vulgare*
	1	AF130975 plasma membrane intrinsic protein (aquaporin; water channel protein)	*Zea mays*
	1	AB032839 pyrophosphate-energized vacuolar membrane proton pump	*Hordeum vulgare*
Cell wall-associated	1	U34333 proline-rich 14 kDa protein	*Phaseolus vulgaris*
Cytoplasm-associated	1	AAL87157 putative kinesin light chain gene	*Oryza sativa*
	1	P49969 signal recognition particle 54 kDa (SRP54)	*Hordeum vulgare*
Unclassified proteins	9	AC092750 hypothetical protein	*Oryza sativa*
	1	T50804 hypothetical protein	*Arabidopsis thaliana*
	1	BAA84613 and AAD48936 putative proteins	*Oryza sativa* *Arabidopsis thaliana*
	1	unknown protein (UP_79)	
	1	AC025098 unknown protein	*Oryza sativa*
"No hit"	3		
Non-readable	11		

A high percentage of the clones that were picked up from the subtracted library show similarity to genes coding for ribulose-1,5-biphosphate carboxylase (Rubisco) small subunit (rbcS). We checked the expression pattern of *rbcS* by Nothern blot assay hybridizing the same total RNA used for construction of the subtracted libraries (Figure 3-2). It was observed that in the mild drought tolerant cv. Öthalom the *rbcS* expression decreases under stress conditions and the drought tolerant cv. Kobomugi shows a moderately increased *rbcS* level. Also, we were interested in the expression of a gene coding for a protein with no assigned function – unknown protein (designated as *UP_79*) (Figure 3-2). Its amount in cv. Kobomugi seems to be much higher than in the cv. Öthalom. Although there is no sharp difference between the *UP_79* expression levels in non-treated and PEG-treated cv. Kobomugi seedlings, measurement of the radiolabeled signal revealed increased level in stress conditions (data not shown).

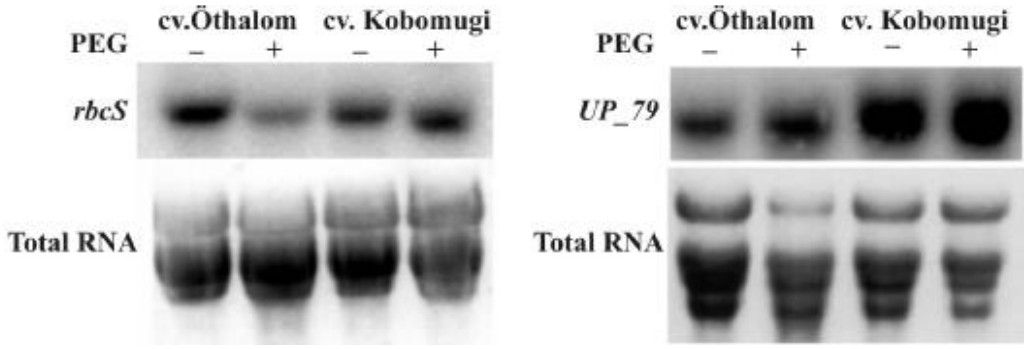

Figure 3-2 Change in the mRNA level of Rubisco small subunit (rbcS) and unknown protein (UP_79) under drought/osmotic stress simulated by PEG treatment.
Total RNA (20µg) from non-treated (indicated with "-") and PEG-treated (indicated with "+") cv. Öthalom and cv. Kobomugi seedlings (two-week-old) was hybridized with PCR-radiolabeled probe specific for the gene of interest.

Altogether, the obtained data demonstrated that our small scale monitoring for drought/osmotic stress-induced genes in the drought-tolerant wheat cv. Kobomugi resulted in the identification of some interesting genes whose role in drought tolerance should be further evaluated *in planta.*

3.1.1.2. dPEG subtraction – where driver cDNAs generated from PEG-treated seedlings of the mild drought tolerant wheat cv. Öthalom were subtracted from tester cDNAs generated from PEG-treated seedlings of the drought tolerant wheat cv. Kobomugi (Figure 3-3):

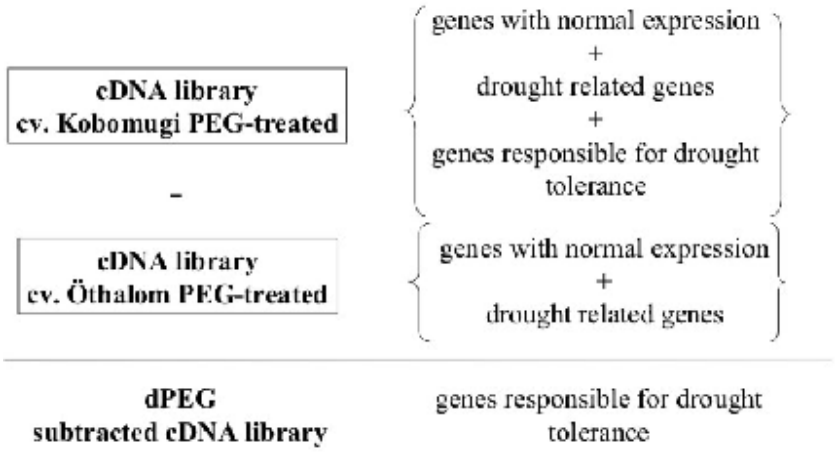

Figure 3-3 dPEG subtraction
In order to obtain differentially expressed genes, two cDNA populations differing from each other are used. The tester cDNA library generated from PEG-treated cv. Kobomugi seedlings contains several sets of genes: genes with normal expression, drought-related genes and genes responsible for drought tolerance. In the driver cDNA library from PEG-treated cv. Öthalom seedlings, there are genes with normal expression and drought-related genes. Scheme of the subtraction of these cDNA populations is shown. This approach results in a new, subtracted cDNA library, enriched in genes responsible for drought tolerance.

In this case, during the subtractive hybridization, we used 'driver' cDNAs from the PEG-treated cv. Öthalom seedlings where *rbcS* expression is strongly suppressed (Figure 3-2). On the other hand, in the 'tester' cDNAs from PEG-treated cv. Kobomugi, the *rbcS* transcription level is increased (Figure 3-2). That means that the newly constructed dPEG subtracted library will be enriched in *rbcS* clones. To resolve this problem, we amplified the *rbcS* gene, the obtained PCR product was *Rsa*I digested and added to the driver. In such a way, we expected to eliminate the abundance of *rbcS*-transcripts.

The resulting cDNA population covered a range between 150 bp and 2 kb. The PCR products were purified and inserted into T/A cloning vector derived from pBlueScript/SK+. After transformation, 120 independent colonies were obtained. The presence of insert was checked by colony PCR using T3 and T7 primers for pBlueScript/SK+. Among the checked colonies, 57 gave PCR product bigger in size than the control 160 bp PCR product (i.e. the T3/T7 amplified region of pBlueScript/SK+). Sequences of 44 of these clones were analyzed. From these 44 clones, 8 contained only primer, 4 did not give readable sequences, and 6 were cloning artefacts. The rest 26 contained 33 inserts (there were up to 3 inserts in some of the cloning vectors) correspondable to ESTs (Table 3-2). The latter were screened against the current GenBank database using the BLAST algorithm. On the basis of the search results, we classified the subtracted EST clones according to the characteristics of the proteins coded by the homologous genes.

Table 3-2 Putative functions assigned to ESTs from subtracted library prepared from PEG-treated seedlings of the drought tolerant wheat cv. Kobomugi and the mild drought tolerant wheat cv. Öthalom

Functional categories	Number of clones	Description of the homologues	Organism
Osmoprotection	8	AAM46894 low temperature and early drought induced protein	*Oryza sativa*
Metabolism	2	BAB19812 Rubisco small subunit (rbcS)	*T. aestivum*
	7	Q40073 Rubisco activase (rcaA2)	*Hordeum vulgare*
Cell wall-associated	12	U34333 proline-rich 14k Da protein	*Phaseolus vulgaris*
Unclassified proteins	2	AC092750 hypothetical protein	*Oryza sativa*
Artefacts	7		
Non-readable	4		
No insert	8		

The results obtained from the dPEG subtraction confirmed the previous observation in the dKobomugi subtraction that during drought/osmotic stress there is increased expression of transcripts coding for: low temperature and early drought induced protein (lt); Rubisco small subunit (rbcS); Rubisco activase (rcaA2); proline-rich protein; and a protein similar to *Oryza sativa* AC092750 hypothetical protein (Table 3-2).

3.1.2. Extension of selected clones by *in silico* search

On the base of the subtraction results and literature data, we focused our attention on some of the genes with putative role in the response to drought/osmotic stress. In order to ensure higher specificity, some of the subtracted EST clones were extended by finding the corresponding mRNAs in the database. If no mRNA was available, overlapping GenBank ESTs were used to construct the longer tentative contig (TC). These TCs were compared with the available TCs from the TIGR wheat database. Detailed description of the obtained longer sequences is shown in the Appendix, and in most cases it includes the sequence of the expressed gene with highlighted open reading frame and deduced amino acid sequence.

In addition to the chosen subtracted genes, we searched also for the expressed sequences of genes coding for: Rubisco activase B (*rcaB*) that is the other subunit of Rubisco activase whose *rcaA2* subunit was present in the obtained subtracted libraries; Retinoblastoma-B domain (*RB-B*) that is a functional domain of a cell division cycle repressor and putatively can be used as a marker for cell division supression under stress conditions; and Translationally controlled tumor protein (*TCTP*) used as a constitutive control for Q-PCR (Appendix).

3.1.3. Expression analysis

We started the characterization of chosen subtracted clones by applying Northern blot assay for expression studies. In some cases, we use the more sensitive Q-PCR technique. A new experiment simulating drought/osmotic stress similar to this in field conditions was set up. Briefly, 10 days after germination seedlings were treated with increasing concentration of PEG (from 100 mOsM to 400m OsM), and samples were collected from both non-treated and PEG-treated plantlets on the 10^{th}, 12^{th}, 14^{th}, 17^{th}, 19^{th}, 21^{th} and 23^{th} days of cultivation. The expression patterns of the investigated genes are shown in Figures 3-4 and 3-5.

The expression data showed a striking 35-fold up-regulation of the *lt* gene during PEG-treatment of cv. Kobomugi plantlets (Figure 3-4). In cv. Öthalom the *lt* transcript also increased in stress conditions – up to 3-fold. In the case of *rcaA2*, no significant difference between treatments and cultivars were observed (Figure 3-4). The *rbcS* transcript level was considerably higher in the cv. Kobomugi, and PEG treatment caused a gradual increase, up to 5 times (Figure 3-4). In cv. Öthalom the *rbcS* expression level decreased in PEG conditions. The mRNA pattern of genes coding for rd22, ubiquitine-conjugating enzyme and unknown protein (*UP_79*) was followed during development of cv. Kobomugi plantlets in the absence or presence of PEG (Figure 3-5). The rd22 and ubiquitine-conjugating enzyme transcript level did not change during stress. The *UP_79* mRNA increased up to 2-fold after addition of PEG, in contrast to the control where a clear decrease was observed. Further repetition of these experiments are necessary to confirm the obtained results.

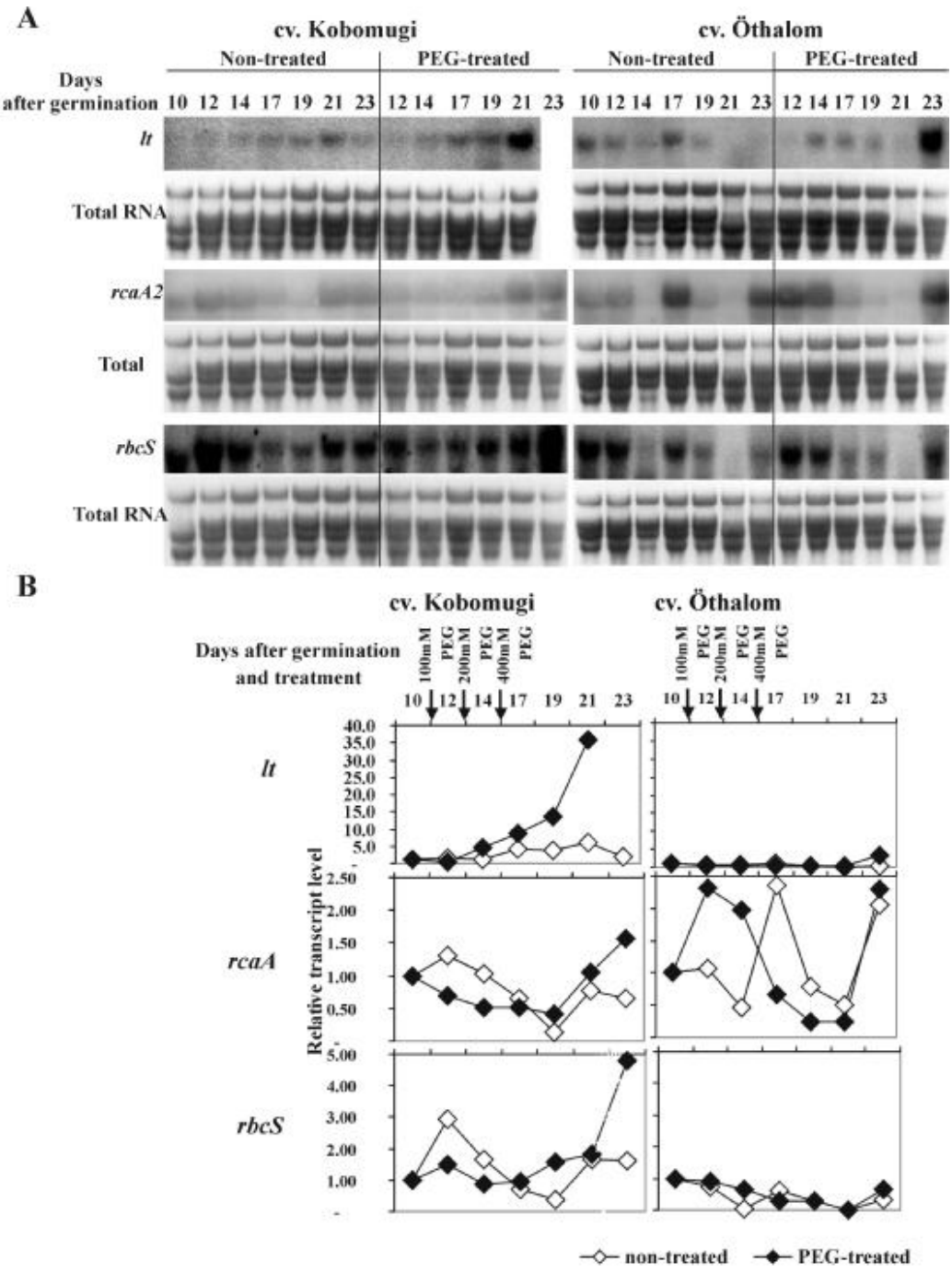

Figure 3-4. Changes in the mRNA levels of selected genes are monitored by Northern blot assay.
Samples were collected during growth in absence or presence of PEG. **A**, Northern blot assay demonstrates the oscillation of low temperature and early drought induced protein (*lt*), Rubisco activase A2 (*rcaA2*), and Rubisco small subunit (*rbcS*) mRNAs. Total RNA (20µg) from non-treated and PEG-treated cv. Öthalom and cv. Kobomugi plantlets was isolated at the shown time points during growth and hybridized with PCR-radiolabeled probe specific for the gene of interest. For estimating the accuracy of loading, picture of the total RNA run on a gel before hybridization is shown. **B**, The relative transcript level of the genes in (A) is shown in respect to the absolute control – the sample from 10-day-old non-treated plantlets. The radiolabeled signal was quantified by PhosphoImager and the related software.

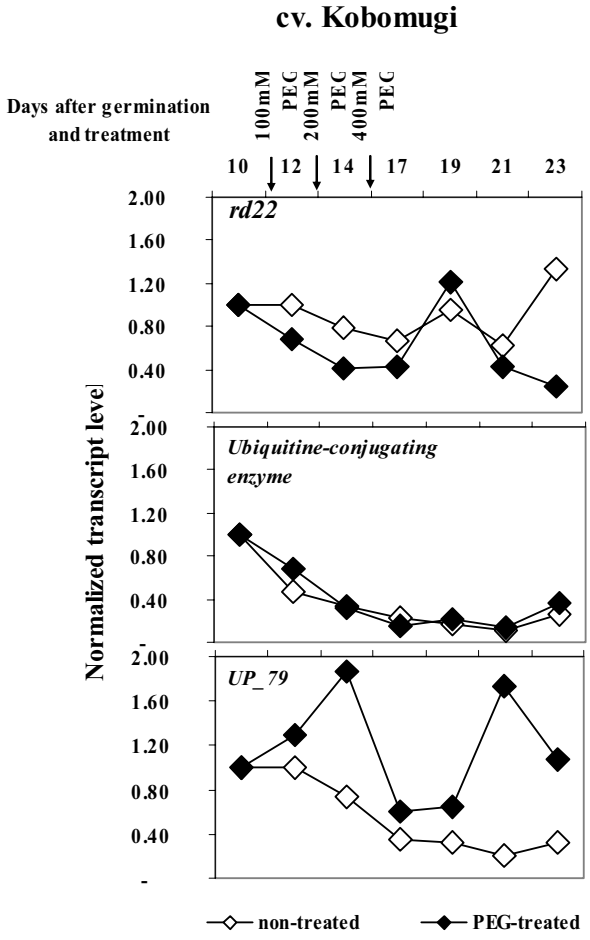

Figure 3-5. Changes in the mRNA levels of selected genes are monitored by Q-PCR.
Samples were collected during growth in absence or presence of PEG. Q-PCR was applied to follow the oscillation of rd22, ubiquitine-conjugating enzyme and unknown protein (*UP_79*) mRNAs. The same samples were used as in Figure 3-4. The transcript levels were normalized to the constitutively expressed *TCTP* gene.

3.2. Isolation and characterization of the promoter region of alfalfa *CDKB2;1*

3.2.1. Cloning of a 360 bp length upstream region of alfalfa *CDKB2;1* (*Medsa;CDKB2;1*)

Cloning of genomic DNA fragments from tetraploid alfalfa (*Medicago sativa* L., cv.Regen S) upstream of the *Medsa;CDKB2;1* coding sequence was performed. The generated genomic DNA fragments were contiguous from the start codon including the 5' UTR and around 300 bp upstream region. Several randomly selected transformed *E. coli* colonies were picked up, and the cloned upstream regions what they contained were analyzed. Since the clones contained a couple of deletions or insertions that could not be generated by PCR, therefore, they were divided into 3 different groups (*fpr10*, *fpr13*, *fpr15*) according to their sequence similarity. The alignment representing these 3 groups and the recently published sequence of a corresponding genomic region of *Medicago truncatula* (AC144481) is shown in Figure 3-6. The longest (360 bp) cloned fragment, assigned as *fpr15*, was used for further investigation (GenBank accession number: DQ136188).

3.2.2. *In silico* analysis of the cloned upstream region of *Medsa;CDKB2;1*

The *cis*-elements of the *Medsa;CDKB2;1* promoter region were analyzed by two different programs available online: the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/) (Higo et al., 1999) and PlantCARE (http://intra.psb.ugent.be:8080/PlantCARE/) (Lescot et al., 2002). The putative *cis*-elements found are indicated in Figure 3-6. The central element of the core-promoter with the putative TATA-box is located approximately –50 bp from the transcriptional start. The CAAT box enhancer element is represented by five potential copies in the *fpr15* sequence. In the *fpr15* sequence, there is a palindromic-like MSA motif $_{-89}$TCCGTTGCACAACGAT, in an almost perfect orientation, upstream a CCAAT-box was located, interestingly, a putative E2Fb binding site ($_{-165}$TTTGCAGC) was also recognized in *fpr15* (Figure 3-6). Comparison of the cloned upstream regions of *Medsa;CDKB2;1* and the orthologous *M. truncatula CDKB2;1* genes confirmed that most probably all of the *cis*-elements necessary to regulate the genes in a cell cycle-dependent manner are present on the relatively short *fpr15* fragment (360 bp). The longer *M. truncatula* genomic sequence does not include other known essential *cis*-elements in addition to those present in the *fpr15* fragment.

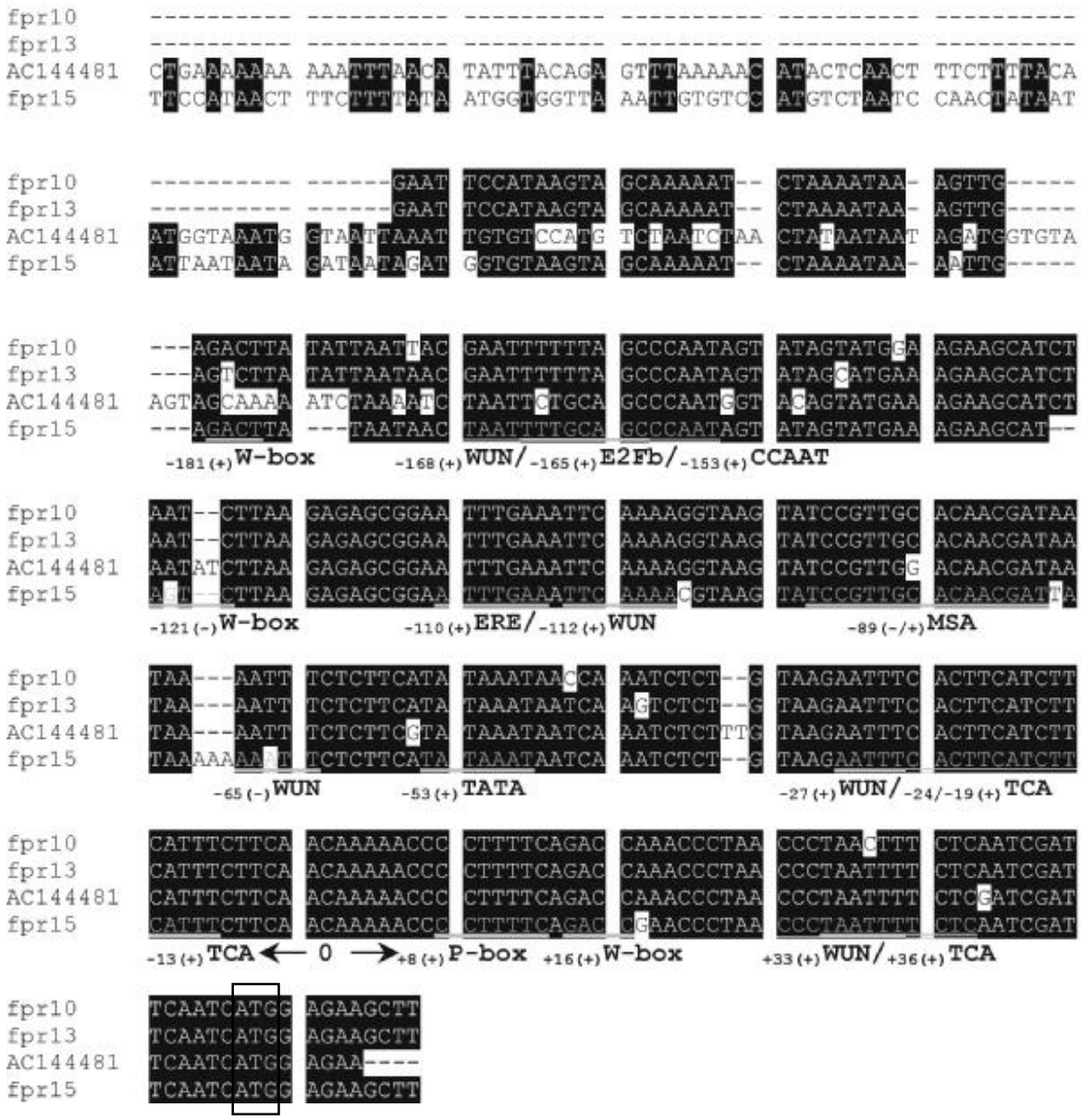

Figure 3-6 Alignment of cloned 5' upstream regions of *Medsa;CDKB2;1* gene.
Corresponding alfalfa genomic DNA fragments were classified (*fpr10*, *fpr13*, *fpr15*), and aligned with the homologous *Medicago truncatula* (AC144481) genomic sequence. Putative promoter motifs are underlined and indicated: W-box (WRKY binding site), WUN (wound-responsive element), E2Fb (E2Fb transcription factor recognizable element), CCAAT-box, ERE (ethylene-responsive element), MSA (M phase-specific activator sequence), TATA-box, P-box (gibberellin-responsive element), TCA (wound-responsive element). Numbers indicate the distance (base pairs) from the putative transcription start (0), and the sense or antisense orientation is shown: (+) or (-), respectively. The translational start is highlighted within a box.

The *in silico* promoter analysis revealed several other potential regulatory elements in the *fpr15* sequence which are unrelated to cell cycle regulation. Among these, light-responsive elements seem to dominate the promoter region with more than 10 different transcription factor binding sites for light (data not shown). In addition, *fpr15* contains wound-related elements like: ethylene-responsive element (ERE: $_{-110}$ATTTGAAA), TCA element (wound-responsive element: $_{-24/-19/-13/+36}$TCATTT/C), WRKY binding site (W-box: $_{-181/-121/+16}$GATC/T) and five WUN motifs ($_{-168/-112/-65/-27/+33}$AATTT). Interestingly, a gibberellin-responsive element (P-box: $_{+8}$CCTTTTC) was found in the 5' UTR.

3.2.3. The promoters of B-type CDKs have a defined order of *cis*-elements

The structures of three *CDKB* promoters were analyzed (Table 3-3):

Table 3-3 Locations of the putative regulatory motifs in the promoter regions of alfalfa and *Arabidopsis CDKB* genes.

	ABRE	E2Fb	WUN	ERE	MSA	TATA	P-box	TCA
Medsa;CDKB2;1	n/a	-216	-170	-165	-138	-106	-47	-23
Arath;CDKB2;1	-228	-225	-170	-168	-145	-104	-213	-24
Arath;CDKB1;1	-135	-151*	-434	-452	-170	-320	-157	-387

* The sequence of *Arath;CDKB1;1* contains E2Fa motif instead of E2Fb. The distance of the elements starts from the ATG codon.
The promoter regions of *Medsa;CDKB2;1*, *Arath;CDKB2;1* and *Arath;CDKB1;1* were analyzed for: abscisic acid-responsive element (ABRE); E2Fb transcription factor recognizable element (E2Fb); wound-responsive element (WUN); ethylene-responsive element (ERE); M-phase-specific activator sequence (MSA); core promoter element (TATA-box); gibberellin-responsive element (P-box); wound-responsive element (TCA).

The distances of the elements were calculated from the ATG start codon. The absolute positions of some *cis*-elements in the promoters of *CDKB2;1* genes of alfalfa and *Arabidopsis* are almost identical, so the arrangement of the E2Fb, WUN, ERE, MSA, TATA, TCA motifs seems to be fixed. The location of the putative hormonal response elements like the one for GA (P-box) can vary within the entire promoter sequence, but they are definitely part of it. The promoters of the orthologous *CDKB2;1* genes share the same basic structure, while the *Arath;CDKB1;1* gene promoter possesses similar elements, but they are found in a different order. In addition, a putative ABRE *cis*-element is present in the *Arabidopsis CDKB* promoters, but it was not recognized in alfalfa or *Medicago truncatula*.

The indicated motifs in the analyzed upstream regions do not share high sequence similarity. Nevertheless, a highly conserved region was identified and highlighted on Figure 3-7. It has palindrome-like structure and includes the ERE motif, as well as a WUN motif (Table 3-3; Figure 3-7).

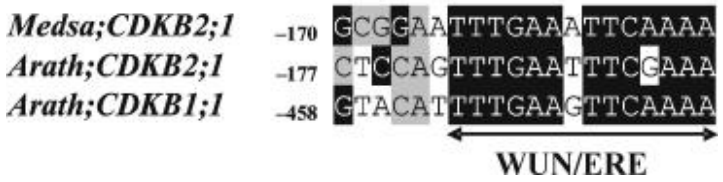

Figure 3-7 Highly conserved region in promoters of alfalfa and *Arabidopsis* CDKB kinases.
The sequence highlighted in black boxes is highly conserved between the analyzed alfalfa and *Arabidopsis* upstream regions. In it, the WUN and ERE *cis*-elements are recognizable. The 5' neighbouring region without significant sequence homology is also shown.

3.2.4. Generation of transgenic alfalfa and *Arabidopsis* plants in order to investigate the promoter activity of the cloned 360 bp alfalfa CDKB2;1 upstream region (*fpr15*)

Transgenic approach was chosen for characterization the *in planta* promoter activity of the *fpr15* cloned fragment. Therefore, alfalfa and *Arabidopsis* transgenic plants were generated with the plasmid constructs where the *fpr15* promoter fragment is fused to the *GUS* or *luciferase* reporter genes, as shown in Figure 3-8. pCambia3301 vector was used for generation of constructs, as follows: fpr15pC3301 construct, where the *35S* promoter regulating the *GUS* reporter gene was substituted by the *fpr15* promoter region (*fpr15:GUS*); fpr15pC3305 construct, where the whole "*35S – GUS*" cassette was replaced by "*fpr15 – luciferase*" (*fpr15:luc*); fpr15pC3315 construct, where the "*fpr15 – luciferase*" was inserted in front of the "*35S – GUS*" gene (*fpr15:luc_35S:GUS*). The resulting plasmids were introduced into *A. tumefaciens* (LBA4404) in order to perform stable plant transformation. Transgenic lines were selected as PCR as well as GUS and luciferase assay positive plants as follows: in alfalfa, *fpr15:GUS* (4), *fpr15:luc* (1) and *fpr15:luc_35S:GUS* (4); and in *Arabidopsis*, *fpr15:GUS* (10), *fpr15:luc_35S:GUS* (2).

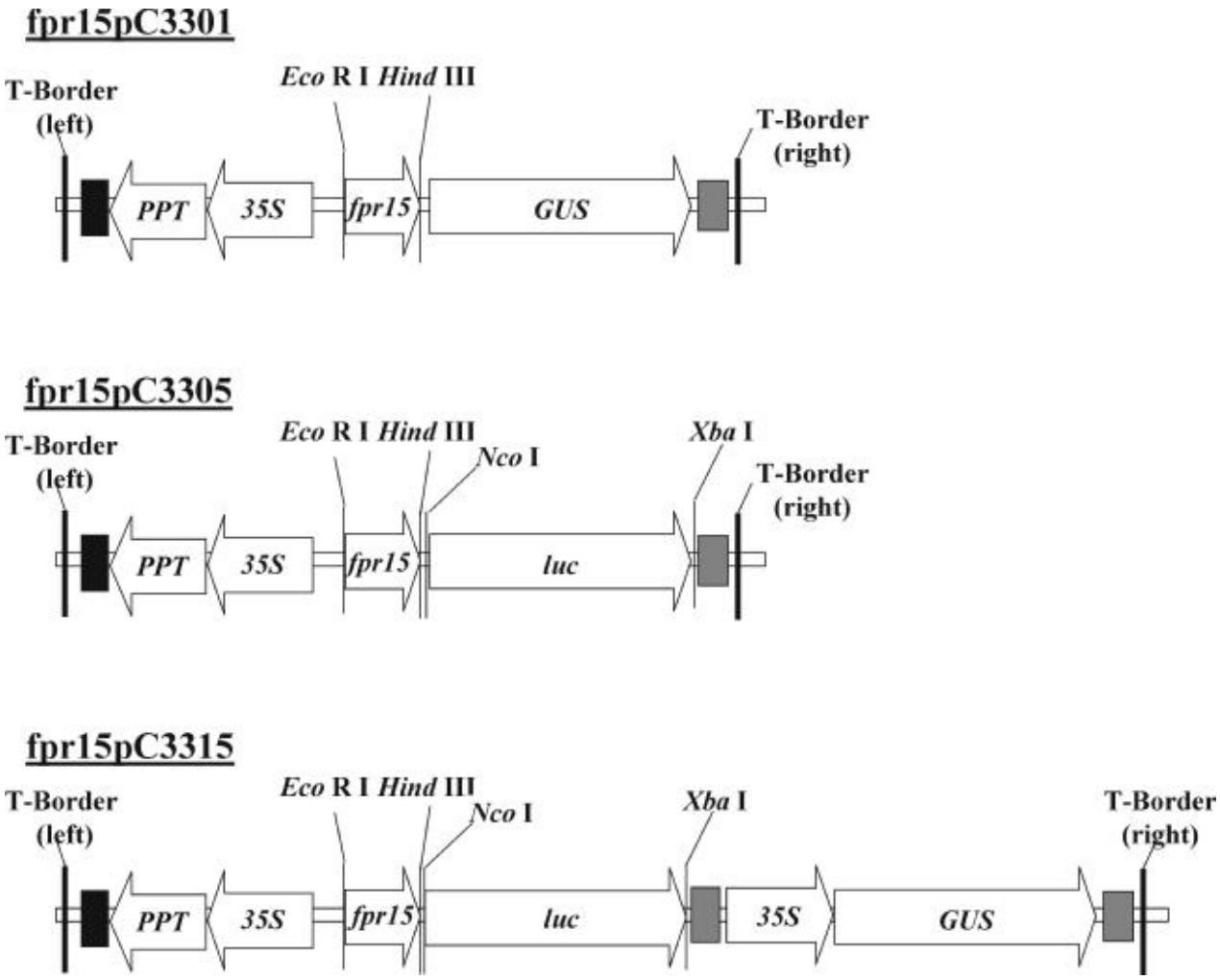

Figure 3-8 Schematic map of the constructs used for the generation of transgenic plants.

The constructs were developed from pCambia3301 vector. fpr15pCambia3301 contains *fpr15* fused to the β-glucuronidase reporter gene (*fpr15:GUS*), and fpr15pCambia3305 contains *fpr15* joined to luciferase reporter gene (*fpr15:luc*). The structure of fpr15pCambia3315 is *fpr15:luc_35S:GUS*. Black boxes represent CaMV35S polyA terminator sequence, and grey boxes - Nos poly-A terminator sequence.

3.2.5. Histochemical analysis of CDKB2;1 promoter fragment-driven reporter gene expression in alfalfa

In planta characterization of the activity of the *fpr15 CDKB2;1* promoter fragment was performed using transgenic lines that carry *luciferase* (4 lines) or *GUS* (4 lines) reporters (Figures 3-9 and 3-10). Transgenic *fpr15:luc* calli with high cell division activity exhibited strong signal after addition of luciferin substrate (Figure 3-9A). Different organs of *fpr15:GUS* transformed alfalfa plants were stained for GUS activity to monitor promoter function (Figure 3-9B to I):

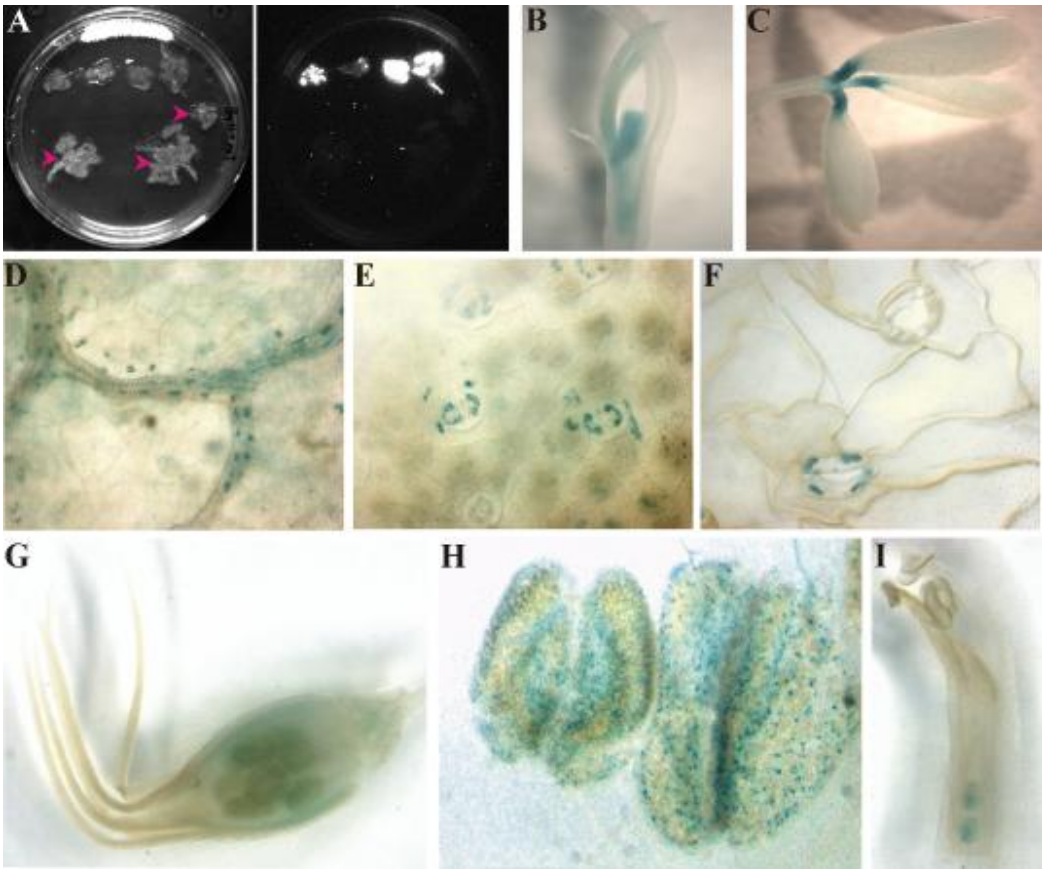

Figure 3-9 *In planta* characterization of the activity of *fpr15* promoter fragment.
A, *In vivo* luciferase assay was carried out on transgenic alfalfa (*fpr15:luc*) calli. Normal (on the left) and luminescent light images produced after spraying with luciferin (on the right), are shown. Red arrowheads point the not transformed control calli sprayed with luciferin. **B** to **I**, Histochemical localization of GUS activity (blue) is shown in *fpr15:GUS* alfalfa plants: shoot apex (**B**); young leaf (**C**); developing vascular tissue (**D**); developing stoma cells in 5-day-old cotyledons (**E**); stoma cells in 15-day-old cotyledons (**F**); flower buds (**G**); young anthers (**H**); seed primordia (**I**).

GUS expression was preferentially localized to the shoot apex (Figure 3-9B). In young leaves (Figure 3-9C), strong *GUS* expression was characteristic for the junction between the leaf and petiole, and moderate staining was detected in the region of developing vascular tissues, as well (Figure 3-9D). Interestingly, in 5-day-old cotyledons (Figure 3-9E), considerable GUS activity was observed in the epidermis during stomaformation, and, to a lesser extent, in 15-day-old cotyledons (Figure 3-9F). No expression was detected in mature leaves. The *fpr15* activity was also observed in flowers. Weak blue staining was characteristic for flower buds (Figure 3-9G), but not for open flowers. A patchy distribution of *fpr15* activity in anther tapetum was observed during microsporogenesis (Figure 3-9H). GUS activity was also detected in seed primordia (Figure 3-9I). The above results showed that the *fpr15* activity is characteristic for plant tissues and developmental stages where active cell division occurs.

More detailed investigation of the localization of *CDKB2;1* promoter fragment activity as well as the endogenous CDKB2;1 protein was performed in roots from transformed alfalfa plants (Figure 3-10). The *fpr15*-regulated *GUS* expression at different stages of lateral root development is shown on Figure 3-10A. The staining was strongest in the central cylinder (stele) of the main root, as well as in the pericycle-derived lateral root meristem. In ultrathin cross sections of the root, *GUS* expression was visible only in the stele and in emerging lateral root meristem but not in the epidermal and cortical layers (Figure 3-10B). In order to compare the accumulation of *fpr15*-controlled GUS reporter in roots with this of the endogenous CDKB2;1 protein, ultrathin cross sections were generated through GUS stained roots of *fpr15:GUS* transgenic alfalfa plants. The GUS staining and the immunohistochemical detection of the endogenous CDKB2;1 protein showed correlation co-expressing in the same cells of the lateral root meristem (Figure 3-10C).

The localization of the endogenous CDKB2;1 protein was compared in root cross sections above the elongation zone - closer and farther from the root tip (Figure 3-10D and E). In addition, cross sections were made from young roots of *fpr15:luc* transgenic alfalfa plants distantly from the root tip - in the maturation zone. Single-labeling immunohistochemistry was performed on consecutive sections with antibodies against alfalfa CDKB2;1 and luciferase, respectively. In both cases, immunopositivity showed similar patterns inside the stele (Figure 3-10F and G).

The histochemical assays in roots demonstrated correlation between the expression of the *fpr15*-regulated reporter genes and the endogenous alfalfa *CDKB2;1* gene.

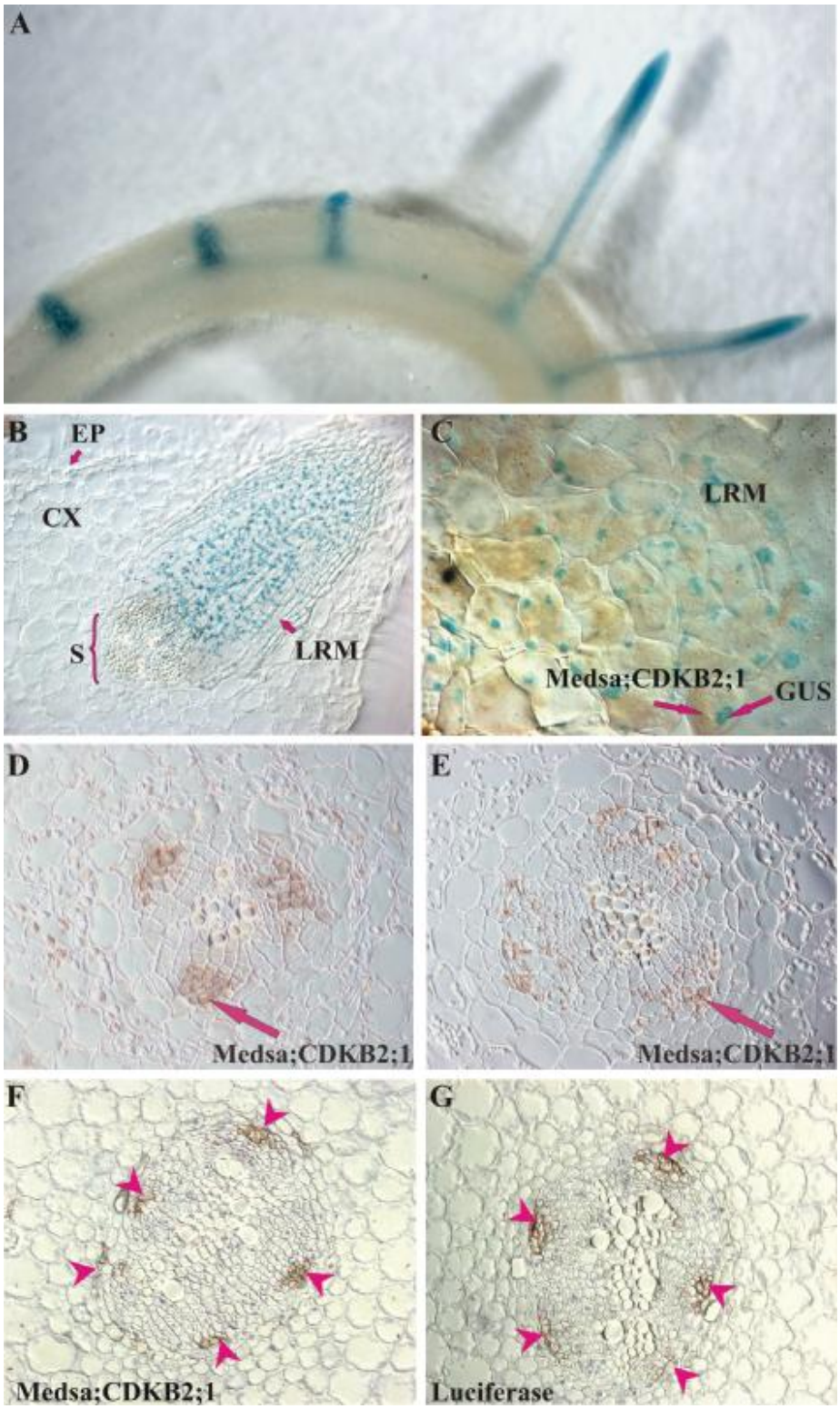

Figure 3-10 Localization of the activity of alfalfa *CDKB2;1* promoter fragment and endogenous protein in roots (see next page)**.**

Figure 3-10 (continued) Histochemical localization of GUS activity (blue) is shown in *fpr15:GUS* alfalfa lateral roots of different developmental stages (**A**) and in root cross section with lateral root meristem (**B**). **C,** Red arrows show the correlation between GUS activity staining and alfalfa CDKB2;1 immunolocalization (brown) in root cross section. Co-expression is visible in the same cells of the lateral root meristem. **D** and **E**, Red arrows show alfalfa CDKB2;1 immunolocalization in root cross sections made above the elongation zone – closer and further from the root tip, respectively. **F** and **G**, Immunohistochemical staining on consecutive sections (made in the root maturation zone) with alfalfa CDKB2;1 and luciferase antibodies, respectively. Similar pattern of brown spots (indicated by red arrowheads) are shown. **LRM** - lateral root meristem; **EP** – epidermis; **CX** – cortex; **S** - stele.

3.2.6. The 360 bp promoter of *CDKB2;1* is sufficient to restrict the expression of the reporters to G2/M cell cycle phase

The histochemical analysis showed that the cloned promoter fragment could provide cell division-dependent expression for the reporter genes, but the correct timing of the promoter activity (i.e. the cycle specificity) remained to be elucidated. The regulation of gene expression was characterized by comparisons between the expression pattern of the endogenous alfalfa *CDKB2;1* and *fpr15*-driven reporter genes in synchronized cell suspension cultures generated from the proper transgenic plants (2 lines) (Figure 3-11). Cell cycle progression was synchronized by the S-phase inhibitor hydroxyurea. After releasing the block, samples were taken at 3-hours intervals for flow cytometric analysis and mitotic index determination (Figure 3-11A), as well as for RNA isolation. The transcript levels of the genes of interest were compared by Northern blot assay (Figure 3-11B). Transcript levels of *CDKA;1*, a cell cycle-related kinase with constitutive expression during the cell cycle, were used as an indicator for the amounts of loaded RNAs. Accumulation of *CDKB2;1* transcripts was maximal in samples from 12 to 21 hours which correspond to the G2/M-phases (Figure 3-11A and B). The level of the *fpr15*-driven *luciferase* gene also exhibited a G2/M-phase-specific expression pattern in good agreement with that of *CDKB2;1*. The experiments with synchronized cell cultures clearly confirmed that the studied 360 bp DNA sequence is sufficient for the cell cycle-regulated, G2/M-specific promoter function.

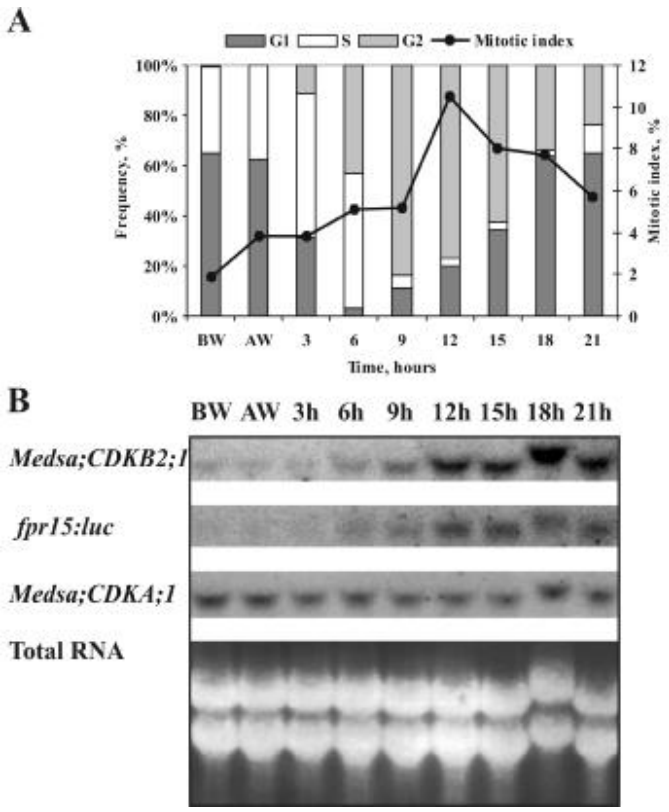

Figure 3-11 Expression analysis of reporter genes regulated by the 360 bp *CDKB2;1* promoter fragment, and endogenous cell cycle genes, in synchronously dividing alfalfa cell cultures.
Transcript level of genes of interest was monitored in hydroxyurea (HU) synchronized cell cultures initiated from transgenic alfalfa plants. Samples were taken every 3 hours after the release from HU block (**BW**– before removing HU; **AW**– after removing HU). In *fpr15:luc* synchronized cell culture, the frequency of cells in various cell cycle phases (G1, S, G2, mitosis) was determined by flow cytometry and by counting the mitotic index (**A**). Total RNA was hybridized with the radiolabeled specific DNA probes for *CDKB2;1*, *luciferase* and *CDKA;1* genes (**B**). The *CDKB2;1* promoter-regulated genes had maximal accumulation in the interval of 12-21 hours, and the *CDKA;1* expression was constitutive throughout the entire cell cycle.

3.2.7. Behaviour of the *fpr15* promoter in heterologous systems

The question was addressed whether the cloned *fpr15* promoter fragment can regulate gene expression in a cell cycle-dependent manner in plant species other than *Leguminosae*. For this reason, the constructs, *fpr15:GUS, fpr15:luc* and *fpr15:luc_35S:GUS* were introduced into *Arabidopsis* (12 lines). *GUS* expression of *fpr15:GUS* carrying transgenic *Arabidopsis* plants is shown in Figure 3-12. It was observed that, similarly to alfalfa, actively proliferating regions of different organs of the plants - shoot and root apical meristems, as well as expanding leaves - showed GUS signals (Figure 3-12A, B). In contrast, the constitutive *35S* promoter-controlled *GUS* gene showed expression in non-proliferating regions as well (Figure 3-12A, B insets).

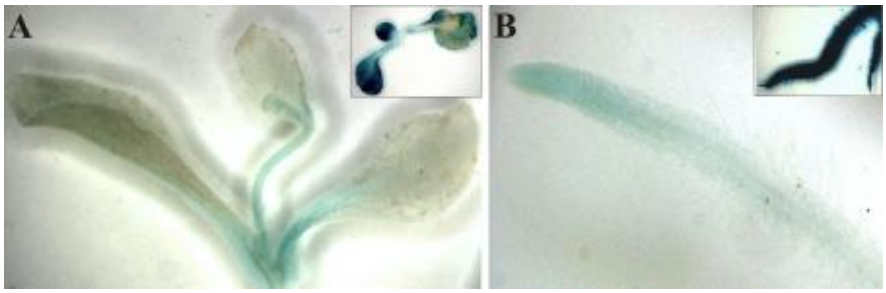

Figure 3-12 Activity of the alfalfa *CDKB2;1* promoter fragment in *Arabidopsis*.
The *fpr15:GUS* construct used for transformation of alfalfa was introduced into *Arabidopsis* plants. The *fpr15* promoter activity was found to be restricted to dividing regions in the shoot apex and basal part of expanding leaves (**A**), as well as in the root apical meristem (**B**). Insets show *35S*-regulated *GUS* expression in the same organs.

3.2.8. Activation of alfalfa CDKB2;1 by wounding and hormones

3.2.8.1. Activation of alfalfa CDKB2;1 by wounding in young seedlings

The wound response of *CDKB2;1* promoter, assumed on the basis of the *in silico* promoter analysis (see above) was tested *in vivo* on leaves of two-week-old seedlings grown in sterile conditions. The leaves were wounded and subsequently detached from the plants at 6, 12, 24 and 48 hours. Analysis of transgenic *fpr15:GUS* plants demonstrated increased GUS activity at the wounded site of leaves 6 and 12 hours after the injury (Figure 3-13A), and with the time the activation decreased. The level of CDKB2;1 was examined by Western blot assay on samples from leaves of non-transgenic seedlings and showed an expression peak at the 12^{th} hour sample (Figure 3-13B).

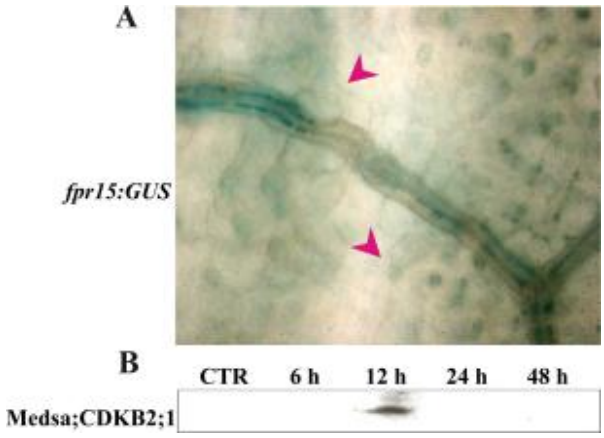

Figure 3-13 Activation of alfalfa CDKB2;1 by wounding in young seedlings.
Leaves of two-week-old alfalfa seedlings grown *in vitro* were wounded by cutting and detached from the plants at 6, 12, 24 and 48 hours after the injury. **A**, A representing picture of increasing GUS activity at the wounded site (indicated by arrowheads) in leaf from *fpr15:GUS* transgenic plants between 6 and 12 hours after the injury. **B**, Western blot assay using for samples leaves from non-transgenic seedlings revealed a peak of CDKB2;1 expression at 12th hour after injury.

3.2.8.2. Activation of alfalfa CDKB2;1 by wounding and hormones in leaf explants

In order to investigate in more detail the wound response of CDKB2;1, we chose as an experimental system leaf explants which limits the movement of endogenous hormones presenting throughout the plant body. Mature leaves from greenhouse-grown alfalfa plants were used to investigate the direct effect of different stimuli on CDKB2;1 activation.

The wound response of CDKB2;1 was studied in detached leaves wounded by cuttings, and then immediately placed onto B5II solid medium. Similarly, hormonal response was analyzed by exposing non-wounded leaves to salicylic acid, jasmonic acid, or ethephon (an ethylene precursor), and 2,4-D known to induce cell proliferation (Hemerly et al., 1993). Samples were taken at different time points: 0.5, 1, 3 and 4 days, and the expressions of the *CDKB2;1* promoter-regulated reporter genes were followed in transgenic plants, and the endogenous CDKB2;1 kinase was monitored in non-transgenic plants by Western blot analysis (Figure 3-14A, C, D). In the case of wounding, increasing GUS activity was observed from day 1 onwards (Figure 3-14A), and the accumulation of the endogenous CDKB2;1 protein changed synchronously with the *GUS* expression pattern (Figure 3-14A). The GUS activity spread symmetrically in the equatorial mesophyll cells of the leaves from the site of the injury (Figure 3-14B). *GUS* expression was hardly seen in the epidermis.

Most inducible defense genes are regulated via jasmonic acid-, salicylic acid-, and ethylene-related signal pathways (Reymond and Farmer, 1998; Wang et al., 2002). Therefore, we tested if these possible activating hormones could turn the *CDKB2;1* promoter on in detached leaves. Jasmonic acid

had no effect at all, and salicylic acid could barely activate the promoter (data not shown). However, the effect of ethylene, which was anticipated on the basis of *in silico* analysis, was more pronounced (Figure 3-14C). Luciferase activity was quantified in detached leaves treated with ethephon. The emitted light was well detectable from the 1st day, and became stronger in time. The level of the endogenous CDKB2;1 protein in ethephon-treated leaf explants was detectable one day after the treatment, and showed similar accumulation pattern as in the case of wounding (Figure 3-14C).

These experiments were performed in parallel with 2,4-D-treated leaf explants (Figure 3-14D). GUS activity staining of the auxin-treated leaves showed that *CDKB2;1* promoter is activated after 3 days (Figure 3-14D). Western blot assay confirmed this result, the CDKB2;1 kinase appeared on the 3rd day, and its level became highly elevated at the 4th day (Figure 3-14D).

For the analysis of the effects of wounding and hormone treatments on the activation of the cell cycle, nuclei were isolated for flow cytometry. Figure 3-14E shows that there was no change in the DNA content of the wounded or ethephon-treated leaf cells during the examined period similarly to the negative control (non-wounded leaves cultivated on hormone-free medium). However, in the 2,4-D-treated leaf explants, the increased frequency of S- and G2-phase cells indicated that the cell cycle was activated 3 days after the treatment.

Taken together, these data suggest, that the expression of CDKB2;1 can be controlled, not only in relation to the cell cycle but independently of it, by wounding. To pursue this phenomenon, wounded or 2,4-D-treated leaves of *fpr15:luc* transgenic plants were placed onto hydroxyurea-containing or control media for three days. Hydroxyurea, an inhibitor of S-phase progression, was expected to block only the 2,4-D-induced (i.e. cell cycle-dependent), but not the wound-induced expression of the *fpr15*-driven reporter. Figure 3-14F shows the luciferin-sprayed leaves after the three days treatment. The *luciferase* gene was switched on in wounded, as well as in 2,4-D-treated leaves, incubated in the absence of hydroxyurea. The presence of hydroxyurea, however, selectively blocked *luciferase* expression in 2,4-D-treated leaves but did not affect the wound-induced expression of the reporter. This indicates that the wound induction of *fpr15* is indeed independent of cell division. Other hormones as kinetin, ABA and GA did not show any significant influence on the activity of *CDKB2;1* promoter in leaf explants (data not shown).

Because of the lack of any indication of cell proliferation in the wounded and ethephon-treated leaves, we could conclude that there must be an independent regulation of the *CDKB2;1* gene for wounding and cell division, and the mitosis-inhibitor ethylene could be a putative mediator of the wound response.

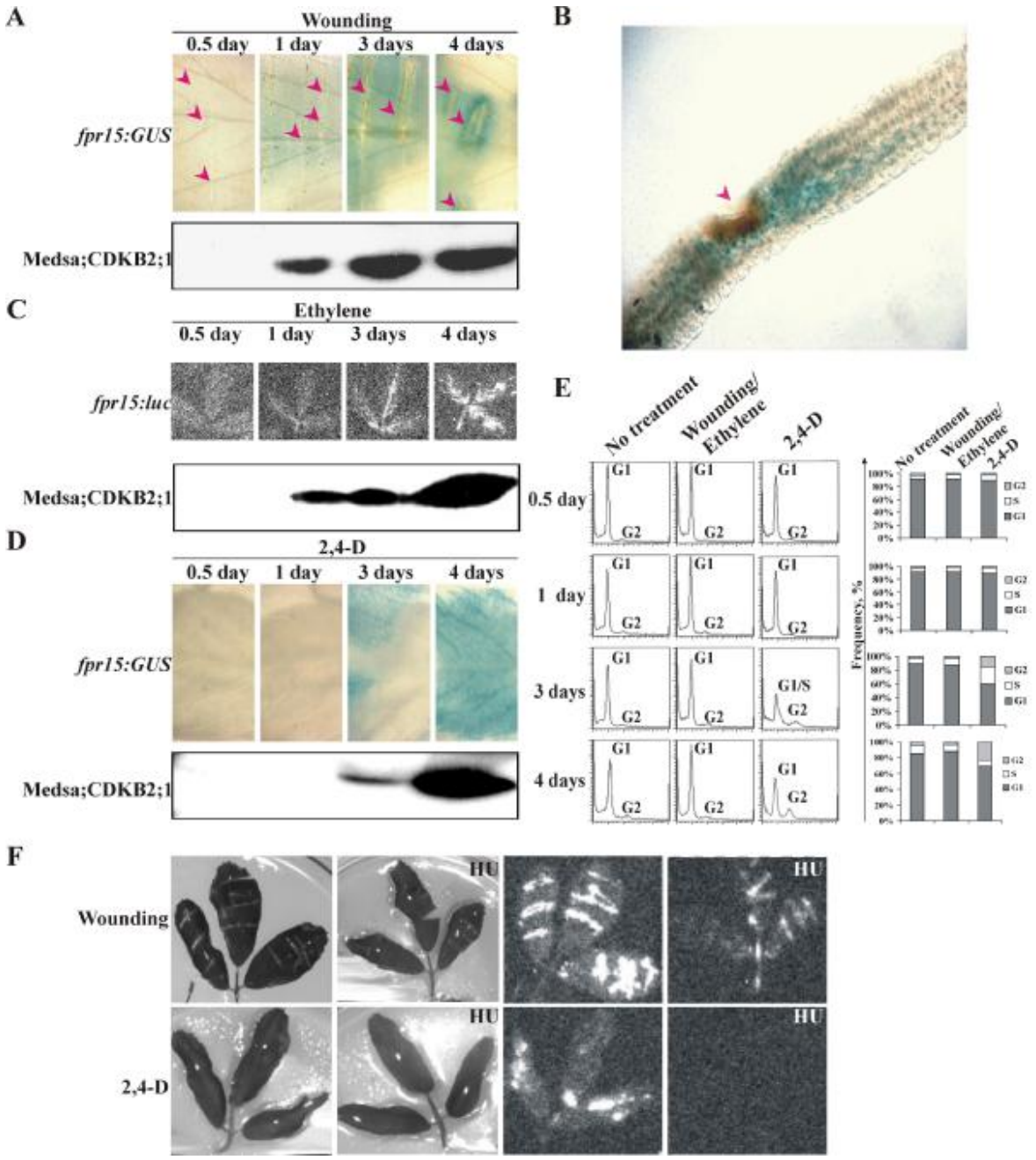

Figure 3-14 Induction of the *CDKB2;1* promoter and endogenous CDKB2;1 by wounding (A,B), ethephon (C), and 2,4-D (D).

Detached leaves were cultivated on B5II solid medium for 0.5, 1, 3, 4 days following the indicated treatments. **A**, *GUS* expression appeared (blue) adjacent to the injury (marked with red arrows) in *fpr15:GUS* leaves. Western blot revealed the endogenous CDKB2;1 kinase accumulation in non-transgenic leaves after wounding. **B**, Cross-section of a wounded leaf after 4 days of cultivation demonstrates that *GUS* expression spread farther from the edge of the wound in the equatorial mesophyl cells of the leaf (See next page). **C**, Arrowhead shows the wounding site. *fpr15:luc* leaves were placed on solid B5II medium supplemented with 10 mg/l ethephon, and the luciferase assay generated luminescent light was detected at given time points. In parallel experiments, Western blot assay was executed with antibody against CDKB2;1 on protein isolated from (see next page)

Figure 3-14 (continued)ethephon-treated, non-transgenic alfalfa leaves, which revealed an increasing amount of CDKB2;1 kinase with elapsed time. **D**, *fpr15:GUS* leaves were incubated on B5II medium, in the presence of 1 mg/l 2,4-D. The accumulation of the endogenous CDKB2;1 protein from the 3rd day in non-transgenic leaves is shown by Western blot. **E**, Cell cycle activity in wounded or hormone-treated leaves of alfalfa was characterized by flow cytometric analysis. The wounded or ethephon-treated samples had no change in relative DNA contents of cells, while 2,4-D-treated leaves exhibited an increased frequency of cells in the S and G2 cell cycle phases. Histograms represent average of three independent measurements. **F**, Wounded, or 2,4-D-treated *fpr15:luc* leaves were incubated on solid BII5 medium in the presence or absence of hydroxyurea (HU). On the left side the leaves are shown in normal light, and on the right side the emitted luminescent light was captured upon luciferase assay carried out on the 3rd day. In the absence of HU, the isolated promoter is active in the wounded, or in the 2,4-D-treated leaves. In the presence of HU, the *fpr15* remained active only in the case of wounding but was silent in the 2,4-D-treated leaves. This indicates that the wound-inducibility of the promoter is independent from the cell cycle.

4
DISCUSSION

4.1. Differential gene expression as a strategy to prevent the water defficit consequences

Life first arose in a watery environment, and the properties of this ubiquitous substance have a profound influence on the chemistry of life. Water is the most abundant molecule in biological systems constituting 70–80 percent by weight of most cells. This molecule is a fundamentally important component of the metabolism of all living organisms, facilitating many vital biological reactions by being a solvent, transport medium, and evaporative coolant (Bohnert et al., 1995). In plants and other photoautotrophs, water provides also energy necessary to drive the photosynthesis. Loss of protoplasmic water leads to concentration of ions (Cl^-, NO_3^-) causing osmotic stress that inhibits metabolic functions (Mundree et al., 2002). Additionally, the decrease of water molecules increases the chances of molecular interactions that can result in protein denaturation and membrane fusion (Hartung et al., 1998; Hoekstra et al., 2001). The survival strategy of plants "drying without dying" involves the co-ordinated expression of a large number of genes (Walters et al., 2002). In order to study the differentially expressed genes in response to osmotic stress that is coupled to the response to drought stress, we constructed subtracted libraries. We used as a stusing material non-treated and PEG-treated seedlings from two wheat cultivars, the mild drought tolerant cv. Öthalom and the drought tolerant cv. Kobomugi. Clones from the resulting cDNA populations were sequenced and subjected to database screen. According to the characteristics of the proteins coded by the homologous genes classification into several categories was performed.

4.1.1. Genes involved in osmoprotection

Following cellular perception of water loss, signalling mechanisms must be activated to induce specific genes (Bray, 1997). One of the major signals operating during water stress is the plant hormone abscisic acid (ABA) (Mundree et al., 2002). Stress conditions trigger its synthesis and accumulation, facilitating the adaptation processes in plants. The effect of ABA induces the closing of stomata, which decreases the transpiration, and on the other hand the hormone increases the water permeability of roots (Kimenov, 1993). The expression patterns of drought-induced genes are complex. Some genes respond to stress very rapidly, whereas others are induced slowly after the accumulation of ABA (Mundree et al., 2002). We found clones similar to ABA-inducible genes, including *rd22* that is responsive to dehydratation and salt stress, too (Yamaguchi-Shinozaki and Shinozaki., 1993). A clone in our library coding for a homologue of an early drought induced protein, assigned also as "low temperature and salt responsive", is another putative ABA-regulated candidate. More detailed expression analysis of this gene confirmed its up-

regulation during PEG treatment in the stress tolerant cv. Kobomugi – up to 35-fold in comparision with the control, and in the less stress resistant cv. Öthalom – up to 3-fold. By contrast, investigation of the *rd22* level during growth did not reveal significant induction by PEG.

We classified in this category also a clone coding for ubiquitin-conjugating enzyme that is involved in the ubiquitin pathway for degradation of target proteins (www.nottingham.ac.uk/biochemcourses/students/ub). It would be interesting to identify the function of this gene, however, our expression analysis did not confirm its up-regulation.

The successful working of the cell surviving strategies in leaves can be monitored by a marker gene for which we have a clone. This gene codes photosystem II 10kDa polypeptide that is related to the leaf senescence – terminal development phase in the life of a plant that leads to the death of the leaves (John et al., 1997). The gene is used as a marker for studying the onset of the senescence in tomato where it is down-regulated. We can conclude that its up-regulation in the PEG-treated wheat cv. Kobomugi demonstrates successful adaptation under drought conditions.

4.1.2. Genes playing role in the metabolism

Biochemical modifications are necessary to protect against metabolic imbalances caused by cellular water loss (Mundree et al., 2002). The genes related to the metabolism may be also up-regulated during stress, which ensures the normal development of the plant. We observed increased expression of the genes coding ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The synthesis of this enzyme in higher plants requires the coordinate expression of the nuclear genes for its small subunit (*rbcS*) and the plastid gene for its large subunit (*rbcL*) (Sugita and Gruissem, 1987). The rbcS is encoded by a multigene family of several members, whereas the rbcL is encoded by a single gene in the chloroplast genome that is present from a few to several hundred copies in the cell. The accumulation of *rbcS* mRNAs and/or proteins is modulated by light in several plant species, which is mainly the result of transcriptional activation of the *rbcS* genes (Sugita and Gruissem, 1987).

Rubisco is a complex with dual enzymatic function – its active center interacts with either CO_2 or O_2 molecules (Kimenov, 1993). In the first case CO_2 is transformed into carbohydrates. In the second case the process is called photorespiration and it has been shown that it can maintain the electron flux especially under stress conditions that lead to reduced rate of photosynthetic CO_2 assimilation. A high percentage of the clones that we selected from the subtracted library shows similarity to genes coding for rbcS. The expression analysis revealed that in the cv. Öthalom *rbcS* expression decreases under stress conditions, and cv. Kobomugi shows moderate increase of the *rbcS* transcript level. These data correspond to the results obtained by Erdei et al. (2002) that show higher accumulation of soluble carbohydrates in the drought tolerant cv. Kobomugi than the more sensitive cv. Öthalom. One of the

mechanisms that plants use to combat the detrimental effects of water loss is to synthesise compatible solutes, including sugars, that facilitate the maintenance of favourable turgor pressure during water stress (Mundree et al., 2002). In addition, these compounds may substitute water molecules during dehydration and prevent the interaction between a protein and destabilising molecules.

The presence in our library of clones homologous to *rbcL* may be an artefact since the mRNA transcripts in chloroplasts do not possess polyA(+) tail. We found an example in the database for existence of AAM08604 putative *rbcL* in chromosome 10 of *Oryza sativa*. It can be speculated that this could be a kind of conservative sequence that might be involved in Rubisco regulation but further investigation should be performed.

An important enzyme (for which we have clones) is Rubisco activase (*rca*)– a nuclear-encoded enzyme that regulates the activity of Rubisco in the chloroplasts, thereby limiting the photosynthetic process (Portis, 1990). Three Rubisco activase polypeptides are encoded by two closely linked, tandemly oriented nuclear genes (*rcaA* and *rcaB*) (Rundle and Zielinski, 1991). The rcaA subunit of the activase consists of two isoforms, rcaA1 and rcaA2, that arise from alternative splicing of a common transcript. In all higher plants examined, transcription of *rca* mRNA seems to be organ specific (photosynthesizing organs), developmentally regulated according to leaf age and light inducible (To et al., 1999). This multiple regulation can explain the observed oscillating expression pattern of *rcaA2* what we observed. Increased synthesis of activase may play a role in the moderation of the decline in the photosynthetic capacity in wheat plants exposed at high temperature. The Rubisco activase is a phosphoprotein that proved to be a GA-binding protein in rice (Komatsu et al., 1996). This suggests the involvement of the enzyme in signal transduction pathways resulting in the activation of specific genes.

Our results show the presence of the transcript for glyceraldehydes-3-phosphate dehydrogenase in drought/osmotic stress conditions. The enzyme is involved in the photosynthetic CO_2 assimilation (Loggini et al., 1999). In addition, one of the clones found is a homologue to a gene coding for cytochrome P450 monooxygenase. This enzyme play an important role in the synthesis of several regulatory substances (e.g. JA, auxins, GA), and various secondary metabolites (Chapple, 1998).

Increased expression of genes responsible for the photosynthetic apparatus may serve as a compensatory mechanism for successful maintaining of the photosynthesis and the whole metabolism. Using the subtraction strategy we succeeded to identify a member of a low-copy number family (3-4 genes) that encodes glutamyl-tRNA reductase (*GTTR*). This enzyme is involved in the synthesis of 5-aminolevulinate (ALA). The chlorophyll synthesis pathway is controlled by light at the level of ALA formation. The *GTTR* transcript level correlates with the capacity for ALA synthesis, indicating that the rate-limitation in the chlorophyll production can be partially due to *GTTR* expression (Bougri and Grimm, 1996).

A further clone is similar to the nuclear gene psbQ coding for a 16 kDa polypeptide. This is one of the polypeptides forming the oxygen-evolving complex that catalyses the water-oxidation reaction (Grover et al., 1999). The gene is light-inducible and expressed only in green tissues, mostly in leaves. We also found a clone coding for the PSI-H – specific subunit of the light harvesting complex I (LHC I) of photosystem I. Lunde et al. (2000) proved that the increased expression of this subunit is associated with dynamic state transitions important for the effective adaptation to different types of stress.

4.1.3. Genes taking part in protein synthesis

We found several genes involved in the protein synthesis that may indicate increased activity of the translation machinery in accordance with an effective drought adaptation.

4.1.4. Genes coding for membrane-associated proteins

Recent evidence has documented that membrane composition can alter the ability of plants to maintain growth under stress conditions, such as high temperature (Crafts-Brandner and Law, 2000). This fact corresponds to the several membrane-associated clones that we found. Four of our clones code for non-specific lipid transfer protein that belongs to a multigenic protein family in plants. *In vitro* they are able to bind all sorts of lipids, but their *in vivo* function remains unclear (Lindorff-Larsen and Winther, 2001). These are very stable proteins, and it has been suggested that they are involved in responses to different types of stress. Also, these proteins may act as transporters of monomers for cutin synthesis (Lindorff-Larsen and Winther, 2001).

Another clone encodes a member of plasma membrane intrinsic proteins (PIPs; water channel proteins; aquaporins) that permit the passage of specific molecules through biological membranes (Li et al., 2000). They are encoded by several gene families. Several genes, whose products are related by their sequences to PIPs, are up-regulated by drought (Mariaux et al., 1998). Regulation of aquaporin transcript level by dehydratation and ABA was reported in the desiccation tolerant resurrection plant *Craterostigma plantagineum* (Matiaux et al., 1998).

Homeostasis keeps relatively constant the plant's internal condition, or allows only limited variation in comparison to the environmental changes (Mundree et al., 2002). A clone from our library is similar to a gene for vacuolar membrane proton pump. During drought, farmers irrigate more their crops and the water acts as a source of Na^+ (Mundree et al., 2002). When the water evaporates, ions such as Ca^{2+} and Mg^{2+} precipitate as carbonates and in most cases Na^+ remains the dominant cation causing salinity stress. The role of the vacuole's ion pumps to maintain the optimal intracellular ion concentration is important for the normal function of the plant cell by keeping the cellular turgor and enssuring optimal pH.

4.1.5. Genes coding for cell wall-associated proteins

The protein fraction of the cell wall includes structural proteins. We found a clone coding for a protein homologous to extensins - members of the family of hydroxyproline-rich glycoproteins (HRG-Ps) that are the most abundant proteins in the cell wall of higher plants (Showalter, 1993). They increase the mechanical resistance of the cell wall and participate in the microtubule organization that plays a role in cell growth. Extensin gene expression has been reported to be regulated by different environmental factors (Showalter, 1993).

4.1.6. Genes coding for cytoplasm-associated proteins

Additionally, we found a clone coding for the light chain of kinesin – an evolutionarily conserved protein among eukaryotes that is involved in the intracellular organelle transport (Lawrence et al., 2001). A further clone is responsible for encoding another cytoplasm localized protein – signal recognition particle 54 kDa protein, shows the increased transport of nuclear encoded proteins to the plastids (Bernstein, 1998).

4.1.7. Genes encoding unclassified proteins

From the randomly selected 78 clones 9 were found to overlap with a cDNA clone coding for a hypothetical protein AC092750. This clone is discovered also in a normalized cDNA library from drought stressed rice seedlings - EST/BI306579 (Reddy et al., 2002), and between the up-regulated transcripts of drought stressed barley leaves - EST/BQ739870 (Ozturk et al., 2002). This clone shows 98 % identiy with 23S rRNA. This could be an artefact, as in the case with rbcL, but sequence similarity does not necessarly mean the same function. Some alignment matches may indicate the presence of a conserved domain or motif or simply a common protein structure pattern (Adams et al., 1991).

We found another 4 clones with homologues to genes coding proteins of unknown function. The designated as *UP_79*, was subjected to expression analysis that showed higher accumulation in control conditions in the tolerant cv. Kobomugi than the cv. Öthalom. PEG treatment caused around 2-fold up-regulation.

Many of the genes represented in the subtracted library dKobomugi (tester cDNAs PEG-treated cv. Kobomugi – driver cDNAs control cv. Öthalom) are highly homologous to known drought responsive genes. These genes give clue about the drought defense system of the tolerant wheat cv. Kobomugi. The survival strategy seems to involve ABA signal pathways for induction of drought-related genes, decreased transpiration to limit water loss, solutes (especially carbohydrates) as osmoprotectants, stabilization of the cell wall and cell membranes, and transport of ions for keeping the homeostasis. The *in silico* data obtained from the subtracted library dPEG (tester cDNAs PEG-driver treated cv. Kobomugi

– cDNAs PEG-treated cv. Öthalom) highlighted the importance of stress-related genes found in the previous subtraction. The genes coding for proline-rich 14 kDa protein, rbcS, rcaA2 and low temperature and salt responsive protein (early drought induced protein) may have important roles in the drought tolerance of cv. Kobomugi. However, these are only *in silico* results and their importance must be further evaluated in plants. Our expression studies on selected differentially expressed genes confirmed that, *rbcS*, and two other genes coding for low temperature and salt responsive protein (early drought induced protein) and unknown protein are induced by drought/osmotic stress. In the rest of the investigated genes, encoding Rubisco activase, rd22 and ubiquitine-conjugating enzyme, the transcript levels were not clearly drought-induced. This might be due to the fact that the plant samples used for cDNA subtraction and expression studies were prepared under different experimental conditions. Alternatively, the subtracted clones should be false.

Drought is one of the major problems facing agriculture. Economic losses from insufficient water supply can be overcome by using marker genes for selection of drought-tolerant cultivars. An alternative is the production of genetically modified drought-tolerant crops. Both approaches can lead to considerable increase in yield, especially in the case of wheat that is main foodstuff for humans and animals.

4.2. Mitosis-specific promoter of the alfalfa cyclin-dependent kinase gene (Medsa;CDKB2;1) is activated by wounding, and ethylene, in a non-cell division-dependent manner

In eukaryotes, the key checkpoints in the progression of the cell cycle, G1/S and G2/M, are controlled by cyclin-dependent kinases (CDKs) (Stals and Inzé, 2001; Vandepoele et al., 2002; De Veydler et al., 2003; Dewitte and Murray, 2003; Inzé, 2005). In plants, the regulation of the mitotic entry (from G2 to M) is governed by the plant-specific B-type CDKs with cell cycle-dependent G2/M-specific expression (Fobert et al., 1996; Segers et al., 1996; Magyar et al., 1997; Mészáros et al., 2000; Joubès et al., 2000; Porceddu et al., 2001; Sorrell et al., 2001; Menges and Murray, 2002; Vandepoele et al., 2002; Kono et al., 2003). Understanding the regulation of G2/M transition is exceptionally important in plants because, at this point, cell cycle progression can diverge towards mitosis or endoreduplication. Therefore, the study about the regulation of this specific CDK family would give a needed information about the mechanism of the switch between mitosis and endoreduplication.

The knowledge about the two classes of B-type CDKs – CDKB1 and CDKB2 has been achieved mainly by investigations on CDKB1 (Yoshizumi et al., 1999; Porceddu et al., 2001; Boudolf et al., 2004a, b; Magyar et al., 2005). Little is known about the role and regulation of B2-type CDKs. The differences in expression pattern, interacting partners, and substrate specificity in comparision with the B1-type CDKs indicate that the two classes are not functionally identical (Fobert et al., 1996; Magyar et al., 1997;

Mészáros et al., 2000; Boudolf et al., 2004a). These observations highlight the significance of collecting more experimental evidence related to CDKB2, too.

4.2.1. Cloning of the upstream region of alfalfa *CDKB2;1*

We focused our efforts on the isolation and characterization of the upstream region of alfalfa *CDKB2;1* gene. Upstream DNA fragments containing the 5' untranslated region and around 300 bp putative promoter region were cloned. Deletions and insertions that could not be due to mis-incorporation by Taq polymerase were observed and, according to their sequence homology, the fragments were divided into 3 groups. We hypothesize that the *CDKB2;1* gene is represented by several copies in the genome, and related promoter variants are also available. Supportingly, in *Arabidopsis* two variants of each B1- and B2-type CDK genes were identified (Boudolf et al., 2001; Vandepoele et al., 2002). The complete sequencing of *Medicago truncatula* genome will help to reveal the number and homology of the genes coding for CDKB2.

4.2.2. *In silico* analysis of the 360 bp-long upstream region of alfalfa CDKB2;1 and Arabidopsis B-type CDK promoters

The *in silico* analysis of the longest (360 bp) cloned DNA fragment, *fpr15*, confirmed the presence of motifs related to the cell cycle regulation of the G2/M-phase-specific *CDKB2;1* gene. Unexpectedly, *cis*-elements suggesting responsiveness to light and wounding were identified, as well. Comparison with the *Arath;CDKB1;1* and *Arath;CDKB2;1* upstream regions revealed a characteristic promoter structure of B-type CDK genes.

4.2.2.1. *Cis*-elements related to G2/M-phase-specific transcriptional regulation

The regulation of G2/M-phase-specific genes may be altered in plant and animal cells. In plants, cell cycle-specific transcription factors of the Myb family were identified to enhance the promoter activity of these type of genes by binding MSA in a cell cycle-dependent manner (Ito et al., 2001, 2005). The online promoter analyzing search engines could recognize only one compressed palindromic MSA-like site (TCCGTTGCACAACGAT) in the *fpr15*. It should be mentioned, that in the cases, where the MSA, or MSA-like, sequences were found to be regulating cell cycle specificity, at least three MSA repeats were required (Ito et al., 1998; Tréhin et al., 1999). In the promoter of tobacco *NACK1* and *NACK2*, two functional MSA motifs are available and they are separated by a spacer of approximatelly 30 to 100 bp (Ito et al., 1998; 2001; Nishihama et al., 2002). Based on these data, we assume that the compressed MSA sequence identified in *fpr15* might not be sufficient to provide cell cycle specificity alone. A case when the MSA was not sufficient to define cell cycle-phase specificity has been reported by Tréhin et al.

(1999). Moreover, Ito (2005) agreed that the reported role of MSA and the Myb factors in tobacco cell cultures has to be proven *in planta* as well. Analyzing Myb knock-out mutants in *Arabidopsis* is in progress and is expected to elucidate the relation between the mechanisms of cell cycle regulation and its developmental control (Ito, 2005).

In contrast to plants, in animal cells G2/M-phase-specific transcription is regulated mainly by two repressor-binding elements: CDE and CHR, as well as the CCAAT activating box (Lucibello et al., 1995; Zwicker et al., 1995a, b; Dohna et al., 2000; Wasner et al., 2003). No information about these motifs has been provided in plants. The *in silico* analysis revealed a putative E2Fb-binding site and a nearby located CCAAT-box in the *fpr15* sequence. The E2F/DP family of TFs plays an important role in controlling gene expression at the G1/S transition in plants and animals (Dyson, 1998; Harbour and Dean, 2000; Chabouté et al., 2000; Menges et al., 2002). An increasing amount of evidence supports the role of E2F transcription factors in the G2/M-phase progression, too (Ishida et al., 2001; Ren et al., 2002; Boudolf et al., 2004b; Magyar et al., 2005). In the regulation of human *cdc2* and *cyclin B1* genes, it has been demonstrated that activating and repressing E2F factors act in combination with factors recognizing CHR and CCAAT *cis*-elements, as well as Myb factors (Zhu et al., 2004). Analysis of the *Arath;CDKB1;1* promoter demonstrated E2F-mediated regulation of this B-type CDK (Boudolf et al., 2004b; Magyar et al., 2005). Boudolf et al. (2004b) provided strong evidence that E2Fa factor activates *CDKB1;1*. On the other hand, Magyar et al. (2005) demonstrated the role of E2Fb for stimulating mitotic cell division, and hypothesized that E2Fa induces indirectly the *CDKB1;1* through E2Fb. Our *in silico* findings suggest binding sites for E2Fa in the promoter region of *Arabidopsis CDKB1;1*, and for E2Fb - in the promoters of both *Arabidopsis* and alfalfa *CDKB2;1*. Currently, there is no experimental information about the effect of E2Fb on CDKB2;1 that would support our *in silico* result. The transcriptional control via E2F factors suggests that, similarly to animals, in plants the G1/S and G2/M cell cycle checkpoints can communicate, and this might be important in the regulation of the switch towards endoreduplication or mitotis (De Veydler et al., 2002; Boudolf et al., 2004b).

4.2.2.2. *Cis*-elements related to light and wound response

Interestingly, *cis*-elements indicating responsiveness to light were found in *fpr15*. Although the analysis of this feature is out of the focus of the present work, it should be noticed that experimental evidence found in the literature supports the role of B-type CDKs in light-dependent aspects of seedling development, probably mediated by brassinosteroids (Yoshizumi et al., 1999).

We focused our attention on wound-responsive motifs recognized in *fpr15*, namely, WUN, WRKY, TCA, as well as ERE related to ethylene response. These elements were shown to be recognized by TF families involved in the regulation of defense genes (Euglem, 2005). We found that the region containing

ERE is conserved between *Medsa;CDKB2;1*, *Arath;CDKB2;1* and *Arath;CDKB1;1*, and includes also a WUN motif. Such a structure indicates a possibility for a cell cycle phase-specific gene to respond to environmental signals.

4.2.2.3. The B-type CDKs have a defined order of promoter *cis*-elements

In the cell cycle-specific regulation of human *cdc2* and *cyclin B1* genes, factors binding the CHR element and CCAAT-box, together with E2F and Myb factors, coordinate the proper timing of expression (Zhu et al., 2004). We found the same *cis*-regulatory elements (CCAAT, E2F- and Myb-binding site) in the *fpr15* sequence, too. It can be assumed that the order and distance of the binding sites may define a special promoter organization important for the cooperative interactions of the TFs, which ensures proper transcriptional initiation.

Furthermore, alignment with the *Arabidopsis CDKB2;1* upstream region showed an almost identical arrangement of the E2Fb, WUN, ERE, MSA, TATA, TCA motifs, although the overall nucleotide sequence similarity of the promoters was low. In the *Arabidopsis CDKB1;1* upstream region, the same elements were present but in a different order. We suggest that the promoter structure involving these regulatory elements might be necessary for the correct function of the B-type CDK gene promoters, and integrates the cell cycle regulation of CDKB with the environmental response.

4.2.3. *Medsa;CDKB2;1* promoter fragment activity in transgenic alfalfa, *Arabidopsis* and tobacco plants

The cloned *fpr15* fragment was linked to *GUS* or *luciferase* reporter genes and characterized in transgenic alfalfa plants, to unravel the propensity of the *Medsa;CDKB2;1* upstream region. In addition, stably transformed *Arabidopsis* and tobacco plants were used to compare the promoter activity in heterologous context.

4.2.3.1. In alfalfa, the 360 bp-long *CDKB2;1* promoter fragment is sufficient to ensure cell division-dependent and cell cycle phase-specific expression of reporter genes

Transgenic plants and cultured cell suspensions carrying the reporter gene constructs produced similar expression patterns of the *fpr15*-driven reporters to the endogenous alfalfa CDKB2;1 kinase. The luciferase/GUS activity was restricted to the actively proliferating tissues and regions of intact plants (callus, shoot and root meristems, petiole-to-leaf junctions, young flowers). Such an expression pattern is characteristic for B-type CDKs, as has been shown by *in situ* hybridisation of the *CDKB1;1* (Fobert et al., 1996; Segers et al., 1996) and *CDKB2;1* (Fobert et al., 1996; Kono et al., 2003) genes coding for these kinases in other species. In addition, the data of Boudolf et al. (2004a) and ours indicate that both

CDKB1;1 and *CDKB2;1* are expressed in stoma cells. However, further investigation is needed to define the functional significance of this expression.

Previously it has been reported that the alfalfa *CDKB2;1* gene is expressed, and the alfalfa CDKB2;1 protein is accumulated, as well as activated, in synchronized cell cultures in a G2/M cell cycle phase specific manner (Magyar et al. 1997; Mészáros et al., 2000). In order to demonstrate *in planta* the characteristic expression of the CDKB2;1 protein, immunohistochemical analysis was performed on root sections from transgenic alfalfa seedlings. We chose to work with roots since they were proven to be an excellent system for cell division cycle investigations (Beeckman et al., 2001; Himanen et al., 2002; Casimiro et al., 2003; Vanneste et al., 2005). The *fpr15*-directed reporters and endogenous CDKB2;1 co-localize in the same cells confirming that *fpr15* could provide similar expression pattern as that of the endogenous *CDKB2;1*. We have not found in the literature any report about *in planta* localization of B-type kinases. The current knowledge about the behaviour of B-type CDKs in roots comes from studies using *promoter:GUS* system or *in situ* hybridization. We performed sections above the elongation zone, in the maturation root zone where initiation of lateral roots starts. During the first stages of lateral root initiation, pericycle founder cells play a principle role (Casimiro et al., 2003). It had been widely accepted that the pericycle cells are G2-arrested, but recent studies revealed that most pericycle cells remain in G1-phase, with only the xylem-pole pericycle cells progressing from G1 to G2 phase (Casimiro et al., 2003). Accordingly, xylem-pole pericycle cells continue to cycle without interuption after leaving the root apical meristem. The immunohisochemical results demonstrated the localization of CDKB2;1 in a characteristic patten inside the stele. Further studies should be performed in order to identify the nature of the root cells where CDKB2;1 accumulates.

Using synchronized cell cultures from transformed alfalfa plants, we demonstrated the G2/M cell cycle phase specificity of the cloned *fpr15* promoter fragment. Taken together the data, we prove that the cloned 360 bp-long upstream region of the alfalfa *CDKB2;1* gene has a promoter activity that is characteristic for dividing cells with G2/M cell cycle phase specificity. The expression pattern of *fpr15*-driven reporter genes was in good correlation with that of the endogenous alfalfa *CDKB2;1* gene.

In a heterologous system as *Arabidopsis*, *fpr15* worked in a very similar way as in alfalfa since GUS activity was seen in proliferating regions. There is also evidence that the *Arabidopsis CDKB2;1* promoter has G2/M activity in tobacco cell cultures (Kono et al., 2003).

4.2.4. Alfalfa CDKB2;1 is responsive to wounding and hormones

In addition to the *cis*-elements implicated in cell cycle regulation, the *fpr15* promoter region contains other potential elements for transcriptional control. Among these, the possibility for alfalfa CDKB2;1 regulation by wounding was further evaluated.

4.2.4.1. Long term wound response of alfalfa CDKB2;1

CDKB2;1 wound response was first demonstrated *in vivo* in leaves of young seedlings grown in sterile conditions. The results showed increased CDKB2;1 promoter activity at the wound site and subsequent protein accumulation between 6 and 12 hours, which indicates long term wound response (Nishitani et al., 2002; Delessert et al., 2004). The supply of hormones and other substances from different parts of the plant towards the damaged region, as well as variations in the physical conditions of the plant, altogether can affect the wounding response in whole plants. Therefore, we chose for detailed studying the CDKB2;1 wound response a simplified system of detached leaves that had been used before in cell cycle research (Meskine et al., 1995; Mészáros et al., 2000; Ouaked et al., 2003). Advantages of this system are the reduced endogenous hormone level and the fact that in mature leaves, generally, growth by cell division does not occur. In these conditions direct activation of cell cycle genes by external stimuli can be explored more directly than in the context of cell cycle progression.

Mechanical injury on leaf explants from transgenic plants clearly induced reporter expression at the wounded site. The reporter activity was increased in time from 24-hours onward. Based on the similar pattern of accumulation of the endogenous alfalfa CDKB2;1 protein in the wounded leaves, it can be assumed that the examined B-type CDK participates in the long term wound response. The data suggest that CDKB2;1 might be part of the defense response directed to repair the damaged tissues and to activate the defense mechanisms that prevent further damage or pathogen invasion (Delessert et al., 2004).

It seems that in young leaves the CDKB2;1 induction, and also degradation, occur with very high rate in comparison with leaf explants. This could be due to the active transport of different compounds that regulate the expression levels. By contrast, in mature leaf explants, the slower CDKB2;1 induction and degradation could be explained with the low growth rate and level of growth regulators. We can speculate that a lack of the cyclin partner renders an inactive CDK state with higher protein stability. Supporting this hypothesis is the fact that in wounded leaves of *Arabidopsis* seedlings mitotic cyclin expression and subsequent cell division were observed 48 hours after the injury (Colón-Carmona et al., 1999). The wound-induced cell cycle activity was not accompanied by growth, and a role in healing was suggested. Alternatively, CDKB2 can be part of a complex involved in recovery processes at the wound site. Such a cell division-unrelated role of B-type CDK has been hypothesized in hypocotyl elongation of dark-grown seedlings, too (Yoshizumi et al., 1999; Boudolf et al., 2004a).

We were interested to check if the wound induction of CDKB2;1 is due to re-activation of cell division. Although the mechanical injury induced *fpr15* activity, dividing cells could not be detected in the wounded leaves by flow cytometry. This means, that the wounding could induce the activity of the promoter via cell proliferation independent pathway. This observation was confirmed by the fact, that

hydroxyurea treatment on wounded leaves did not inhibit the isolated promoter activity. In contrast, the accumulation of the CDKB2;1 kinase in the presence of 2,4-D was likely a consequence of the entry to the cell cycle: it started only at the time when the cells already entered into the cell division cycle, what happened later (from the 3rd day) in comparison to the wound-response (from the 1st day), and the promoter activity could be blocked with hydroxyurea.

Previously it has been shown, that the constitutively expressed during the cell cycle A-type *Arabidopsis* CDK kinase, could also be induced by wounding without cell division. It has been concluded that the expression of this gene by wounding was a consequence of an increased competence for cell proliferation, however this was not proven experimentally (Hemerly et al., 1993). Our data suggest that wound-inducibility in a non-cell cycle-dependent manner can be a common feature of the plant CDK kinases. The promoter structure of these genes seems to integrate gene regulatory mechanisms related to specific developmental processes and effects of environmental stress.

The studies on long term wound response may be used for practical application, such as increasing the tolerance against herbivores, mainly insects, that cause injuries on the plants (Reymond et al., 2000), as well as other stress factors. Overexpression of CDKB2;1 may contribute to increased plant resistance and recovery ability.

4.2.4.2. Effects of defense-related hormones on alfalfa CDKB2;1 expression

Frequently, the induction of wound responses requires the simultaneous action of different signals and regulators. Many inducible defense genes are regulated by a small number of signal pathways depending on signal molecules – ethylene, SA, JA (Reymond and Farmer, 1998). Cross-talks between these pathways appear to be very common and important in the regulation of defense gene expression (Wang et al., 2002). We tested the individual effects of these signal molecules on *CDKB2;1* promoter activity in leaf explants. Jasmonic and salicylic acid could not significantly activate the *GUS* expression, which is in accordance with the finding that they are not directly involved in the wound response at the wound region (Ryals et al., 1996; Wang et al., 2002; Delessert et al., 2004). The TCA-binding sequence identified in *fpr15* was found in the promoter region of different genes known to be induced by one or more forms of stress, including wounding and SA (Goldsbrough et al., 1993). The evidence that SA might stimulate the expression of *Arabidopsis CDKB1;1* during pathogen attack (Vanacker et al., 2001) could not be proved in our system of leaf explants, perhaps because of the possible lack of interaction with other necessary signal pathways. It was hypothesized that pathogenesis (or other SA-inducing conditions) causes the cell cycle to become engaged, which can result in a number of cell alterations, such as cell death, enlargement and division, depending on the cellular context. The phenomenon of cell cycle activation leading to alternative cell fates has been well documented in animals (Evan and Littlewood, 1998).

By contrast, ethephon could turn on the promoter in a similar manner as mechanical injury, which supports the *in silico* finding that predicted putative ethylene-responsive elements in the *fpr15* sequence. It has been demonstrated that ethylene treatment caused G2/M arrest and stimulated endoreduplication, and its removal resulted in progression to mitosis (Dan et al., 2003; Kazama et al., 2004). These results suggest that ethylene might have role at the switch between mitosis and endoreduplication. The accumulation of alfalfa CDKB2;1 kinase due to G2/M progression, or by treatment with ethylene, considered as a G2/M-phase inhibitor, emphasizes the multifuncional role of this kinase. To reach proper kinase activity, CDK kinases need their cyclin partners. Indeed, ethylene treatment could also stimulate the expression of mitotic cyclin in rice (*Oryza sativa*) stem cuttings (Lorbiecke and Sauter, 1999).

Our results indicate that the G2/M cell cycle phase-specific alfalfa CDKB2;1 is involved in ethylene-mediated signal pathway. Since ethylene blocks the G2/M transition but does not repress CDKB2;1, we postulate that the ethylene-mediated G2/M-block affects downstream cell cycle regulators. In accordance with this suggestion, endoreduplication starts only when the mitotic cell cycle stops, which correlates with the loss of mitotic regulators (De Veylder et al., 2001; Foucher and Kondorosi, 2000). In alfalfa, a further link is an activator of the anaphase-promoting complex, CCS52, the anaphase promoting complex being responsible for the ubiquitin-related degradation of mitotic cyclins. It was suggested that the loss of mitotic cyclins is involved in the switch from mitotic cycles to endocycles (Cebolla et al., 1999; Vinardell et al., 2003; Tarayre et al., 2004).

4.2.4.3. Other hormonal effects on alfalfa *CDKB2;1* promoter activity

León *et al*. (2001) reviewed the negative auxin effect on wound-induced gene expression in different species. Upon wounding, recovery of the initial levels of active auxins has been proposed as a mechanism to limit the duration of the response to wounding (Rojo et al., 1998). Further cultivation of leaf pieces on auxin-supplemented medium has been shown to induce indirect somatic embryogenesis (Dudits *et al*., 1991). In accordance, we observed CDKB2;1 activation in putative cell division foci developing into calli. Other hormones as kinetin, ABA and GA did not show significant influence on the activity of *CDKB2;1* promoter in leaf explants. In the case of kinetin and ABA, our results support the literature data reporting that kinetin controls G2/M transition via activating dephosphorylation (Zhang et al., 2005), and ABA has more pronounced role at the G1/S transition (Wang et al., 1998; Swiatek et al., 2002). The lack of GA effect on alfalfa CDKB2;1 could be explained with the fact that ethylene can influence GA action (Fabian et al., 2000). Indeed, we observed increased *fpr15*-regulated reporter activity in wounded leaf explants treated with GA (data not shown), suggesting that additional factors modulate GA effect.

4.3. Summary of the results

4.3.1. Identification of up-regulated drought-related genes in a drought tolerant wheat cultivar

1) We identified differentially expressed genes induced by drought/osmotic stress in photosynthesizing organs of seedlings, and we classified them in the functional categories: osmoprotection, metabolism, protein synthesis, membrane-, cell wall- and cytoplasm-associated, and unclassified proteins.

2) Analysis of the expression profile of genes coding for Rubisco small subunit, low temperature and early drought stress induced protein, and unknown protein, confirmed high induction by drought stress in plantletss.

4.3.2. Regulation of alfalfa CDKB2;1 in environmental context

1) The cloned, 360 bp long, upstream region of alfalfa CDKB2;1 is sufficient to regulate reporter gene expression in cell cycle-dependent and G2/M-phase-specific manner, similarly to the endogenous CDKB2;1 kinase.

2) In heterologous system as Arabidopsis intact plants, the activity of alfalfa CDKB2;1-cloned promoter is characteristic for proliferating plant regions.

3) Wounding activates the cloned and the endogenous alfalfa CDKB2;1 upstream regions in a non cell cycle-dependent manner.

4) Ethylene, known as a wound response mediator and inhibitor of the G2/M transition, also induces the cloned and the endogenous alfalfa CDKB2;1 upstream regions in a non-cell cycle-dependent manner.

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APPENDIX

DESCRIPTION:

Gluthamyl – tRNA – reductase (GTRR)
Lipid transfer protein (LTP)
Lipid transfer protein_38 (LTP_38)
Lipid transfer protein_97b (LTP_97b)
Low temperature and early drought induced protein (lt)
Plasma membrane intrinsic protein (Aquaporine)
Proline-rich protein (pro-rich)
R40_Os (r40)
Rd22-like BURP-domain containing protein (rd22)
Retinoblastoma – B domain (RB-B)[1]
Rubisco activase A2 (rcaA2)
Rubisco activase B (rcaB)[1]
Rubisco small subunit (rcbS)
Translationally controlled tumor protein (TCTP)[2]
Ubiquitine – conjugating enzyme (UCE)
Unknown protein _79 (UP_79)

[1] - no subtraction clones are available
[2] – Q-PCR internal control

Noncoding regions are in lower-case letters, the open reading frame (ORF) is shown in upper-case letters, and the deduced amino acid sequence is presented in one-letter code above. The stop codon is indicated with *.

The subtraction clones are underlined and the part of the cDNA that is sequenced in cv. Kobomugi is shaded.

>Gluthamyl-tRNA-reductase (GTRR):

```
                            M  M  T  G  A  T  S  A  T  A  A  A
1   ccgccgctgctcttgttgttggtctgATGATGACGGGAGCGACTTCAGCCACGGCCGCCGCG  62
      G  A  F  A  A  G  A  K  A  R  G  S  A  A  V  C  P  W  V  V
63    GGCGCATTCGCTGCCGGCGCCAAGGCGCGGGGGTCCGCCGCCGTGTGCCCCTGGGTCGTC  122
      A  G  G  G  R  R  R  S  G  V  V  R  C  D  A  G  G  D  A  Q
123   GCCGGCGGCGGCCGGAGGCGGTCCGGCGTCGTGCGCTGCGACGCCGGCGGGGATGCCCAG  182
      A  A  S  K  L  A  S  I  T  A  L  E  Q  F  K  I  S  A  D  R
183   GCGGCGTCCAAGTTGGCCAGCATCACCGCGCTCGAGCAGTTCAAGATCTCCGCAGACCGG  242
      Y  M  K  E  K  S  S  I  A  V  I  G  L  S  V  H  T  A  P  V
243   TACATGAAGGAAAAGAGTAGCATCGCTGTAATAGGCCTCAGTGTACACACAGCACCAGTG  302
      D  M  R  E  K  L  A  V  A  E  E  L  W  P  R  A  I  S  E  L
303   GACATGCGTGAAAAACTTGCTGTTGCAGAGGAACTATGGCCCCGTGCTATTTCAGAACTC  362
      T  S  L  N  H  I  E  E  A  A  V  L  S  T  C  N  R  M  E  I
363   ACCAGTCTGAATCATATTGAAGAGGCTGCTGTTCTTAGTACCTGCAACAGAATGGAAATA  422
      Y  V  V  A  L  S  W  N  R  G  I  R  E  V  V  D  W  M  S  K
423   TATGTGGTGGCTTTATCGTGGAACCGTGGTATTAGAGAAGTAGTGGACTGGATGTCAAAG  482
      K  S  G  I  P  A  S  E  L  R  E  H  L  F  M  L  R  D  S  D
483   AAAAGCGGAATCCCTGCTTCCGAGCTAAGGGAGCATCTCTTTATGTTGCGTGACAGTGAT  542
      A  T  R  H  L  F  E  V  S  A  G  L  D  S  L  V  L  G  E  G
543   GCCACACGCCATCTGTTTGAGGTATCCGCCGGGCTTGACTCTTTGGTTCTTGGAGAAGGA  602
      Q  I  L  A  Q  V  K  Q  V  V  R  N  G  Q  N  S  G  G  L  G
603   CAAATCCTTGCTCAAGTTAAACAAGTTGTCAGAAATGGGCAAAACAGCGGAGGCTTGGGA  662
      K  N  I  D  R  M  F  K  D  A  I  T  A  G  K  R  V  R  C  E
663   AAGAACATTGATAGGATGTTCAAGGATGCAATCACAGCTGGAAAGCGCGTCCGCTGTGAG  722
      T  N  I  S  A  G  A  V  S  S  V  S  S  A  A  V  E  L  A  M  M
723   ACCAACATATCAGCTGGTGCTGTGTCTGTCAGTTCGGCTGCAGTTGAATTGGCCATGATG  782
      K  L  P  K  S  E  C  L  S  A  R  M  L  L  I  G  A  G  K  M
783   AAGCTTCCAAAGTCTGAATGCTTGTCAGCTAGGATGCTTTTGATTGGTGCTGGCAAAATG  842
      G  K  L  V  V  K  H  L  I  A  K  G  C  K  K  V  V  V  V  N
843   GGAAAATTAGTGGTCAAACATTTGATTGCCAAAGGATGCAAGAAGGTTGTTGTGGTGAAC  902
      R  S  V  E  R  V  D  A  I  R  E  E  M  K  D  I  E  I  V  Y
903   CGTTCTGTGGAAAGGGTGGATGCCATTCGCGAAGAGATGAAAGATATTGAGATTGTGTAC  962
      R  P  L  T  E  M  Y  E  A  A  A  E  A  D  V  V  F  T  S  T
963   AGGCCTCTTACAGAGATGTATGAAGCCGCTGCTGAAGCTGATGTCGTGTTCACAAGCACC  1022
      A  S  E  S  L  L  F  T  K  E  H  A  E  A  L  P  P  I  S  L
1023  GCATCTGAATCCTTATTATTCACGAAGGAGCATGCAGAGGCGCTTCCTCCTATTTCTCTT  1082
      A  V  G  G  V  R  L  F  V  D  I  S  V  P  R  N  V  G  A  C
1083  GCTGTGGGTGGTGTTCGGCTTTTCGTCGACATATCTGTCCCGAGGAATGTCGGTGCCTGT  1142
      V  S  E  V  E  H  A  R  V  Y  N  V  D  D  L  K  E  V  V  E
1143  GTATCTGAGGTGGAGCATGCACGGGTATACAATGTCGACGACTTGAAAGAGGTGGTGGAA  1202
      A  N  K  E  D  R  V  R  K  A  M  E  A  Q  T  I  I  T  Q  E
1203  GCCAATAAGGAAGACCGTGTCAGGAAAGCAATGGAGGCCCAAACAATCATTACCCAAGAA  1262
      L  K  R  F  E  A  W  R  D  S  L  E  T  V  P  T  I  K  K  L
1263  CTGAAACGGTTCGAGGCATGGAGGGACTCACTGGAGACGGTTCCGACCATCAAAAAGCTG  1322
      R  S  Y  A  D  R  I  R  A  S  E  L  E  K  C  L  Q  K  I  G
1323  AGGTCGTACGCCGACAGGATCAGGGCATCTGAGCTCGAGAAGTGCCTGCAGAAGATCGGG  1382
      E  D  N  L  N  K  K  M  R  R  S  I  E  E  L  S  T  G  I  V
1383  GAAGACAATCTCAACAAGAAGATGAGAAGGTCCATCGAGGAGCTGAGCACGGGCATAGTG  1442
      N  K  L  L  H  G  P  L  Q  H  L  R  C  D  G  S  D  S  R  T
1443  AACAAGCTCCTTCACGGCCCACTGCAGCACCTGAGATGCGACGGCAGCGACAGCCGCACC  1502
      L  D  E  T  L  E  N  M  H  A  L  N  R  M  F  N  L  D  T  E
1503  CTGGACGAAACGCTTGAGAACATGCACGCCCTCAACAGAATGTTCAACCTCGACACGGAG  1562
      K  A  V  L  E  Q  K  I  K  A  K  V  E  K  T  Q  S  *
1563  AAGGCGGTCCTTGAGCAGAAGATCAAGGCCAAGGTAGAGAAGACCCAAAGCTGAgaccag  1622
1623  gagacacttgtccgtctgtatatctacttatactgctcccagaatgtcgctacattctaa  1682
1683  tcccaatatttttttctgatcctccacttactgctgagttcttcttgcgccgtgaatta  1742
1743  gccatggcagcccccaaatgtgttgtagagaggagcagcgaagcatgtttgctccaaaac  1802
1803  tgcctgtgtacattatttaccgtggctgctgtggttagagcccaattttcaagctgtact  1862
1863  ataatactctattggatgaaataaacaggaatatatcagtgtgttacctcgtgc  1916
```

The cDNA sequence is a contig obtained by overlapping a cDNA clone - gi|9661723|gb|BE495130.1|BE495130 WHE1274_E02_J04ZS *Secale cereale* anther cDNA library, and TC82557 (www.tigr.org). The search in the database started by using a dKobomugi subtraction clone (dKobomugi_20). The ORF corresponds to glutamyl tRNA reductase (gi|1666079|emb|CAA60055.1|,*Hordeum vulgare* subsp. vulgare).

>Lipid transfer protein (LTP):

```
                              M  A  R  G  A  A  T  Q  L  V
3     aacaccactactactgcaaactcgatagggATGGCTCGCGGTGCAGCTACTCAGCTCGTG  62
      L  V  A  M  V  A  A  M  L  L  V  A  C  D  A  A  I  S  C  G
```

```
63   CTGGTCGCCATGGTGGCCGCTATGCTCCTCGTAGCCTGCGATGCGGCGATATCCTGCGGT  122
     Q  V  T  S  A  L  S  P  C  I  S  Y  A  R  G  N  G  A  N  P
123  CAGGTGACCTCTGCCTTGAGCCCCTGCATCTCCTATGCACGCGGCAACGGCGCCAACCCG  182
     P  A  A  C  C  S  G  V  R  S  L  A  G  A  A  R  S  T  A  D
183  CCTGCGGCCTGCTGCAGCGGCGTCAGGAGTCTGGCCGGTGCAGCCCGGAGCACCGCTGAC  242
     K  Q  A  A  C  K  C  I  K  S  A  A  G  G  L  N  A  G  K  A
243  AAGCAAGCAGCGTGCAAGTGCATCAAGAGCGCTGCTGGTGGGCTCAACGCTGGCAAGGCC  302
     A  G  I  P  S  K  C  G  V  S  V  P  Y  A  I  S  S  S  V  D
303  GCCGGCATCCCTTCAAAGTGCGGCGTCAGCGTCCCCTACGCCATCAGCTCATCTGTGGAC  362
     C  S  K  I  R  *
363  TGTTCTAAGATTCGTTGAtcgaccacttcttctcatcatcgctgcatatagctccagcga  422
423  tctacgctgagtacgttgaggtcatacacatacatatacatatatatgaataagtgctct  482
483  catattatctcactgcgtgacagcgagaggagtacctacgtccggccagctctgcatggc  542
543  aggccacactgttatattgatgtnttggttgttccttcacccttttgagaagactcatga  602
603  gtttgttcttctttgtaccgtgtgcacttttgatatatggatcttacactaaaaaaaaaa  662
663  aaaa  666
```

The cDNA sequence corresponds to a cDNA clone (gi|13114954|gb|BG313151.1| BG313151 WHE2054_D02_H04ZS Wheat salt-stressed sheath cDNA library *Triticum aestivum*) found in the database by using a dKobomugi subtraction clones (dKobomugi_2b, 48 and 382). The ORF corresponds to a lipid transfer protein (gi|1098266|prf||2115353B, *Hordeum vulgare*).

>Lipid transfer protein_38 (LTP_38):

```
1    ccacgcgtccgatcacttagcaaatctagggtctcaccatctccagctgagttcaacacg  60
                           M  A  R  G  A  A  I  P  L  V  L  V
61   attactactgcaaactcgatagggATGGCTCGCGGTGCAGCTATTCCGCTCGTGCTGGTC  120
     A  M  V  A  T  M  L  L  V  A  T  D  A  A  I  S  C  G  Q  V
121  GCCATGGTGGCCACTATGCTCCTCGTAGCCACCGACGCGGCCATCTCCTGCGGTCAGGTG  180
     T  S  A  L  S  P  C  I  S  Y  A  R  G  N  G  A  N  P  T  A
181  ACCTCCGCCTTGAGCCCCTGCATCTCCTATGCCCGCGGCAATGGCGCCAACCCGACTGCT  240
     A  C  C  S  G  V  R  S  L  A  G  A  A  R  S  T  A  D  K  Q
241  GCCTGCTGCAGCGGTGTCAGGAGTCTGGCCGGGGCAGCCCGGAGCACCGCTGACAAGCAA  300
     A  A  C  K  C  I  K  S  A  A  G  G  L  N  A  G  K  A  A  G
301  GCGGCGTGCAAGTGCATCAAGAGCGCTGCCGGTGGGCTCAACGCTGGCAAGGCCGCCGGC  360
     I  P  S  K  C  G  V  S  V  P  Y  A  I  S  A  N  V  D  C  S
361  ATCCCCTCCAAGTGCGGCGTCAGCGTCCCATATGCAATCAGCGCTAACGTCGACTGCTCT  420
     K  I  R  *
421  AAGATTCGCTGAtcaaccactgcccaccatggctgccgtccatagcgccatccatcaccg  480
481  ctgagtatgctgaggtctctaatacatatgaataaatgctctgatctgatctccatgtga  540
541  gggagaaaggagtgcgtacgccgagctagctctgcatggccggcaactattgtactacta  600
601  ctatggttgtttttactttcactaccttgaggagaaccgagactttattgtacgttgta  660
661  ccgcgtacacttccagtatatatatggattatgcttataatcattttcctaaaaaaaaaa  720
721  aaaaaaaaa  729
```

The cDNA sequence is a contig obtained by overlapping two cDNA clones - gi|20436525|gb|BQ240649.1|BQ240649 TaE05014F10R TaE05 *Triticum aestivum* and gi|22030228|gb|BQ806019.1|BQ806019 WHE3573_G09_N17ZS Wheat developing grains cDNA library *Triticum aestivum*. The search in the database started by using a dKobomugi subtraction clone (dKobomugi_38). The ORF corresponds to a lipid transfer protein (gi|1098266|prf||2115353B, *Hordeum vulgare*).
Note: Both LTP and LTP_38 have the same coding region but they differ in their 3′UTR region.

>Lipid transfer protein_97b (LTP_97b):

```
1  gcacgagggagagagagagagagagaactagtctcaacagcaaggctgtggtgtccgccgtg  62
                        M  M  A  G  R  E  A  T  A  L  S  C  G
63   gtcctggcggcggtggtgctaATGATGGCCGGCAGGGAGGCGACGGCACTGTCGTGCGGG  122
     Q  V  D  S  K  L  A  P  C  V  S  Y  V  T  G  K  A  P  S  I
123  CAGGTGGACTCCAAGCTCGCGCCGTGCGTGTCGTACGTGACGGGGAAGGCGCCCTCGATC  182
     S  K  E  C  C  S  G  V  Q  G  L  N  G  L  A  R  S  S  P  D
183  AGCAAGGAGTGCTGCTCCGGTGTGCAGGGGCTGAACGGCCTGGCCCGCAGCAGCCCGGAC  242
     R  K  I  A  C  R  C  L  K  S  L  A  T  S  I  K  S  I  N  M
243  CGCAAGATAGCGTGCAGGTGCCTCAAGAGCCTCGCCACCAGCATCAAGTCCATCAACATG  302
     D  K  V  S  G  V  P  G  K  C  G  V  S  V  P  F  P  I  S  M
303  GACAAGGTCTCCGGCGTGCCCGGCAAGTGCGGCGTCAGCGTGCCCTTCCCCATCAGCATG  362
     S  T  N  C  N  N  V  N  *
363  TCCACCAACTGCAACAATGTCAACTAGttcaatatataacccttccgacgtgcatgcaag  422
423  gacgcgcttgtgtggagcttaatgtctacgttgatggagtgctcatacgatactgagcta  482
483  ctaaataataaaatgaagttgcttgcgtgtgttatttcagtactgtgtcactgtgtgtct  542
```

```
543  gtgtccatgtctgtaccttgcgcacacactgtgtgtgcatatatatttatgcgacatgta  602
603  tgcatgcgtccgtatgcgtgagtgaactgtcaactctttgctgtactctagtgtagccga  662
663  tgtgtacgtgtgatgtgcctgacctacttgttcaggcaattaatgaatagtaattatttt  722
723  cctaaaaaaaaaaaaaaaa  741
```

The cDNA sequence is a contig obtained by overlapping two cDNA clones - gi|25557496|gb|CA741673.1| CA741673 wia1c.pk003.l10 wia1c *Triticum aestivum* and gi|25436444|gb|CA714651.1|CA714651 wdk3c.pk023.n24 wdk3c *Triticum aestivum*. The search in the database started by using a dKobomugi subtraction clone (dKobomugi_97b). The ORF corresponds to a lipid transfer protein (gi|7438283| pir||T05950).

>Low temperature and early drought induced protein (lt):

```
1    catcactctactccacctacctttctcgcgctccttgttcacatcatctcccttgccttgg  61
62    ctttcccttcgacgcttcgagagagagagagagagagagagagagagagagagagaagga  121
                M  A  D  E  G  T  A  N  C  I  D  I  I  L  A  I  I
122   aggaagaagATGGCGGACGAGGGGACCGCCAACTGCATCGACATCATCCTCGCCATCATC  181
      L  P  P  L  G  V  F  F  K  F  A  C  G  I  E  F  W  I  C  L
182   CTGCCGCCGCTCGGCGTCTTCTTCAAGTTCGCCTGCGGGATCGAGTTCTGGATCTGCTTG  241
      L  L  T  F  F  G  Y  L  P  G  I  I  Y  A  V  W  V  I  T  K
242   CTGCTCACCTTCTTCGGCTACCTCCCCGGCATCATCTACGCCGTCTGGGTCATCACCAAG  301
      *
302   TAGgactgcatgcaggaggggctcctctgcctctgccgctaggttatccatggcgaactt  361
362   gttctgtgtctggtacaagccgtttgtctgagatgcgagtcgcggttcatggtgctgcgg  421
422   tgctgcctacgtgtgtgttgatgctgttgtacatctgtttcttgttcgtgcttgatgatt  481
482   agcctcctctctcgtcagttgtcaccttcccttctccagtgtatccatccagttgtgttg  541
542   ttcgtccgggcattttcgtcgtccatgtacttgaactttgattaattaattaatcaatc  601
602   aaaaaaaaaaaaaaaaa  619
```

The cDNA sequence corresponds to a cDNA clone (gi|17146791|gb|BM138024.1| BM138024 WHE0479_F08_K15ZS Wheat *Fusarium graminearum* infected spike cDNA library *Triticum aestivum*) found in the database by using a dPEG subtraction clone (dPEG_55). The ORF corresponds to a putative low temperature and salt responsive protein (gi|23237810|dbj|BAC16385.1| [*Oryza sativa* (japonica cultivar-group)].

>Plasma membrane intrinsic protein (Aquaporine):

```
1     gcttcggcgcactcgcaccttgggcaccaccacacacacccaccctagcttgtcgatcgg  60
                  M  A  K  D  I  E  A  A  P  P  G  G  E  Y  A
61    ctgaggagcgcgacgatggccaaggacatcgaggcggcgccacccggtggggagtacgcg  120
      A  K  D  Y  S  D  P  P  P  A  P  L  F  D  A  E  E  L  T  K
121   gccaaggactactccgacccgccgccggcgccgctcttcgacgccgaggagctgaccaag  180
      W  S  L  Y  R  A  V  I  A  E  F  V  A  T  L  L  F  L  Y  I
181   tggtccctgtaccgcgcggtgatcgccgagttcgtggccacgctcctcttcctctacatc  240
      T  V  A  T  V  I  G  Y  K  H  Q  A  D  P  A  G  P  N  A  A
241   accgtggccaccgtcatcgggtacaagcaccaggcggaccccgccggccccaacgccgcc  300
      D  A  A  C  S  G  V  G  I  L  G  I  A  W  A  F  G  G  M  I
301   gacgcggcctgcagcggcgtgggaatcctcggcatcgcctgggcgttcggcggcatgatc  360
      F  V  L  V  Y  C  T  A  G  V  S  G  G  H  I  N  P  A  V  T
361   ttcgtgctcgtctactgcaccgccggtgtgtcgggtggccacataaacccggcggtgacc  420
      F  G  L  F  L  A  R  K  V  S  L  V  R  A  L  L  Y  I  I  A
421   ttcgggctgttcctggcgcgcaaggtgtcgctggtgcgcgcgctgctctacatcatcgcg  480
      Q  C  L  G  A  I  C  G  V  G  L  V  K  G  F  Q  S  A  F  Y
481   cagtgcctcggcgcaatctgcggcgtcgggctcgtcaaggggttccagagcgccttctac  540
      V  R  Y  G  G  G  A  N  E  L  S  S  G  Y  S  T  G  T  G  L
541   gtgcgctacggcggcggggccaacgagctcagctccggctactccacgggcaccggcctc  600
      A  A  E  I  I  G  T  F  V  L  V  Y  T  V  F  S  A  T  D  P
601   gccgccgagatcatcggcaccttcgtgcttgtctacaccgtcttctccgccaccgacccc  660
      K  R  S  A  R  D  S  H  V  P  V  L  A  P  L  P  I  G  F  A
661   aagcgcagcgcccgtgactcccacgtcccagtgctggctcctctgccaatcggcttcgcg  720
      V  F  M  V  H  L  A  T  I  P  I  T  G  T  G  I  N  P  A  R
721   gtgttcatggtgcacctggccactatcccgatcaccggcaccggcatcaacccggcaagg  780
      S  L  G  A  A  V  I  Y  N  N  E  K  A  W  D  D  H  W  I  F
781   agcttgggagctgctgtgatctacaacaacgagaaggcctgggatgaccactggattttc  840
      W  V  G  P  F  I  G  A  A  I  A  A  L  Y  H  Q  Y  V  L  R
841   tgggtggggccattcatcggcgccgccatcgccgccttgtaccaccagtacgtgctgagg  900
      A  S  A  T  K  F  G  S  S  A  S  F  G  S  R  *
901   GCCAGCGCCACCAAGTTCGGCTCGTCCGCCTCCTTCGGCAGCCGCTAGatcggccggccg  960
961   ccgtctcggtgatggtcggaggcactgaactgtagatatatcagaggacagagggcctcc  1020
1021  agtgtgtgctgctgttcgggtcctggattctgctggtctttgcacgcgtctctgttttcc  1080
1081  gcagctcgccgccgcgtttgctgcttgttttctctaccgatcgagtgtgtcttcgttagt  1140
1141  gtttgcttccattccatgtactatctgtgaacgatgaggatgatgtactgtcatgtatgc  1200
1201  atccaaggtaatttgggggggaaatttgaaatgcaatgtgtacgtacgattcgggttgaaa  1260
```

```
1261  aaaaaaa  1267
```

The cDNA sequence is a contig obtained by overlapping several cDNA clones - gi|23079573|gb|BJ232900.1|BJ232900 BJ232900 Y. Ogihara unpublished cDNA library, Wh_e *Triticum aestivum*; gi|23144597|gb|BJ280669.1|BJ280669 BJ280669 Y. Ogihara unpublished cDNA library, Wh_r *Triticum aestivum*;gi|22142955|gb|BQ838635.1 |BQ838635 WHE2912_H08_P16ZS Wheat aluminum-stressed root tip cDNA library *Triticum aestivum*; gi|23146797|gb|BJ285692.1|BJ285692 BJ285692 Y. Ogihara unpublished cDNA library, Wh_r *Triticum aestivum*; and gi|25192990|gb|CA614693.1| CA614693 wr1.pk177.f5 wr1 *Triticum aestivum*. The search in the database started by using a dKobomugi subtraction clone (dKobomugi_18). The ORF corresponds to plasma membrane intrinsic protein gi|4741931|gb|AAD28761.1|, *Zea mays*.

>Proline-rich protein (pro-rich):

```
                                                                  M
1    tcggcacgaggctagctcactctcaccagcactacctagctagcttagccggcagcaATG  60
     A  S  R  T  F  S  A  A  C  L  L  A  L  L  V  A  N  T  F  L
61   GCGTCCAGGACCTTCTCGGCGGCATGCCTGCTGGCGCTGCTGGTGGCCAACACGTTCCTC  120
     A  G  D  A  C  G  S  C  K  H  K  T  P  P  P  A  S  P  S  P
121  GCCGGCGACGCGTGCGGCAGCTGCAAGCACAAAACCCCGCCGCCGGCCTCCCCGTCCCCG  180
     P  P  P  S  P  S  T  T  P  C  P  P  P  P  S  S  G  G  G  T  S
181  CCCCCACCATCGCCGTCTACGACGCCGTGCCCACCGCCTTCGTCAGGCGGCGGCACGTCG  240
     C  P  T  D  T  L  K  L  G  A  C  A  N  V  L  G  L  V  N  V
241  TGCCCCACGGACACACTGAAGCTGGGCGCCTGCGCCAACGTGCTGGGCTTGGTGAACGTG  300
     G  V  G  K  P  P  S  G  G  G  D  K  C  C  S  L  L  G  G  L
301  GGCGTCGGCAAGCCCCCCAGCGGCGGCGGCGACAAGTGCTGCAGCCTCCTCGGCGGCCTG  360
     A  D  L  E  A  A  V  C  L  C  T  A  L  K  A  N  V  L  G  I
361  GCCGACCTCGAGGCCGCCGTGTGCCTCTGCACCGCGCTCAAGGCCAACGTCCTCGGCATC  420
     V  L  N  I  P  V  K  L  S  L  L  L  N  Y  C  G  K  T  A  P
421  GTCCTCAACATCCCCGTCAAGCTCAGCCTCCTCCTCAACTACTGCGGCAAGACCGCCCCC  480
     K  G  F  Q  C  A  *
481  AAGGGCTTCCAGTGCGCTTAAtccatcagttaatcaggcaaatcagcgcatgcatgcatc  540
541  gtcagtgcatccatgcatgtatgttattttagttccaaatttgatttcatcatgcaataa  600
601  gtcggtcggttgttaagtagtttttcttgcgtgtgttaatttatatgcactggtttggtg  660
661  agattaatttgtcttagtttggctgtgtactacaagagatacatacgactgggtgtgcgt  720
721  gtgtgcgcatgaacagtaaatcgattaagcacgtgtaacacttgtatttgtttgatgaaa  780
781  tgtaatttgcagaatattcattcaaaaaaaaaaaaaaaaaaaa  823
```

The cDNA sequence is a contig obtained by overlapping two cDNA clones - gi|25205991|gb|CA627695.1|CA627695 wdr1.pk0003.f5 wdr1 *Triticum aestivum* (reverse complement) and gi|21997224|gb|BQ788752.1|BQ788752 WHE4153_D12_H23ZS Wheat CS whole plant cDNA library *Triticum aestivum*. The search in the database started by using a dPEG subtraction clone (dPEG_51). The ORF corresponds to a probable proline-rich protein [imported] -*Arabidopsis thaliana* (gi|25307718|pir|| D84887).

>R40_Os (r40):

```
     A  R  G  D  H  P  L  S  L  S  I  *  I  S  G  G  E  E  T  Y
1    gcacgaggggaccacccgctgagttaagcatataaataagcggaggagaagaaacttac  60
     K  D  S  P  S  N  G  E  R  T  G  T  S  P  V  R  L  V  P  Y
61   aaggattcccctagtaacggcgagcgaaccgggaccagcccgGTTCGTCTGGTGCCCTAC  120
     N  P  E  Y  V  D  E  S  V  L  W  T  E  S  R  D  V  G  H  G
121  AACCCTGAGTACGTGGACGAGTCCGTGCTGTGGACTGAGAGCCGCGACGTCGGCCACGGC  180
     F  R  C  V  R  M  V  N  N  I  Y  L  N  F  D  A  F  H  G  D
181  TTCCGCTGCGTGCGCATGGTGAACAACATCTACCTCAACTTCGACGCCTTCCATGGCGAC  240
     K  D  H  G  G  V  H  D  G  T  T  V  V  L  W  E  W  A  K  G
241  AAGGACCATGGCGGCGTCCACGACGGCACCACCGTCGTCCTCTGGGAATGGGCCAAGGGC  300
     A  T  Q  R  W  K  I  L  P  W  *
301  GCCACCCAGCGCTGGAAGATCCTCCCCTGGTAGatatgaagccatccaatcgcggcgttg  360
361  agaagatcgcccggagaaacttcccttgctgccgtctccgtcctccgctccatgtcgtt  420
421  gatcgagtcgttctagttattatctttctgttttcgcgtcggttcctgaatgaccagtaa  480
481  taatataggagtactagttgttgtccatcagcgtgcgcccatgtctgaatctgatcgtat  540
541  cttgctggttgcgcgctgctgctacttctctgtttggtgttctgaaagactttcgagtat  600
601  gttgatgctgagtttcattcagcctttgagtgattctgagtttctgatgaggaatcgatc  660
661  tcccgagctgcattgctgc  679
```

The cDNA sequence is a contig obtained by overlapping two cDNA clones - gi|9303042|gb|BE361583.1|BE361583 DG1_72_H10.b1_A002 Dark Grown 1 (DG1) *Sorghum bicolor* and gi|8090716|gb|AW924890.1|AW924890 WS1_72_E09.g1_A002 Water-stressed 1 (WS1) *Sorghum bicolor*. The search in the database started by using a

dKobomugi subtraction clone (dKobomugi_7). The ORF corresponds to the end coding region of proteins belonging to one family: rice proteins r40c1 (gi|7489571|pir||T03911), r40g2 (gi|7489572|pir||T03960,fragment) and r40g3 (gi|7489573|pir||T03962).
Note: The subtraction clone is placed in a noncoding region which is supposed to be an intron. The presence of stop codon in this part of the cDNA indicates that a splicing process could occur.

>rd22-like BURP-domain containing protein (rd22):

```
1    acccacaatggtaactgggcctggggctcattggacatccaaaagaaccatggagaatcccac   62
                                     M  S  N  M  V  F  L  E  E  A
63       attggccaagacaaccacaaacatggaaccATGTCAAACATGGTGTTCTTAGAAGAGGCC   122
         L  K  P  G  S  T  I  P  C  Y  I  Q  P  S  A  T  L  G  A  P
123      CTCAAACCAGGATCAACTATACCTTGTTACATCCAACCATCGGCTACCTTAGGAGCTCCT   182
         L  L  R  R  D  V  A  D  S  I  P  M  S  T  G  N  F  I  K  I
183      TTGTTACGGCGTGATGTCGCCGACTCTATCCCTATGTCCACGGGGAACTTCATCAAAATT   242
         L  T  M  F  A  P  A  S  N  D  M  A  T  K  I  W  S  T  L  D
243      CTGACAATGTTTGCACCAGCGTCCAACGACATGGCTACCAAAATATGGTCGACGCTGGAT   302
         D  C  E  H  P  R  A  I  K  G  E  T  K  A  C  V  S  S  V  E
303      GATTGCGAGCACCCGCGCGCTATCAAGGGTGAGACGAAGGCATGTGTCAGCTCTGTGGAG   362
         S  M  V  E  F  A  A  S  V  L  G  V  S  T  Y  N  L  A  A  F
363      TCAATGGTGGAGTTTGCTGCATCCGTGCTCGGGGTTAGTACCTACAACCTCGCGGCCTTC   422
         S  S  P  D  V  P  V  D  G  V  M  T  G  R  G  Y  K  V  A  A
423      TCCTCACCGGACGTGCCCGTGGATGGCGTCATGACAGGGAGGGGGTACAAAGTGGCGGCT   482
         V  T  R  V  R  E  A  G  D  T  M  T  C  H  R  G  S  F  P  F
483      GTAACGAGGGTCAGAGAGGCGGGGGACACCATGACTTGCCATCGCGGGAGTTTCCCATTC   542
         A  M  F  M  C  H  A  V  N  P  T  R  V  Y  S  V  T  L  E  G
543      GCTATGTTCATGTGCCACGCGGTGAATCCCACCAGAGTGTACTCGGTGACGCTGGAGGGG   602
         E  D  V  D  A  D  G  A  G  Q  R  M  E  V  L  A  V  C  H  L
603      GAGGACGTTGATGCTGACGGCGCGGGGGCAGAGGATGGAGGTGCTTGCCGTGTGCCACCTG   662
         D  T  S  D  F  H  P  A  K  M  P  L  H  V  K  P  G  D  A  P
663      GACACATCAGACTTCCACCCAGCTAAGATGCCGTTGCACGTCAAGCCTGGAGATGCTCCA   722
         L  C  H  F  I  S  R  D  S  I  L  W  A  P  A  T  P  A  S  A
723      CTCTGCCACTTCATCTCCAGGGACAGCATTCTCTGGGCGCCTGCTACGCCCGCTTCTGCT   782
         H  A  A  A  *
783      CACGCTGCTGCTTAGctacatgtataggtacataaataagaacagtgtgtacctatgtaa   842
843      ggcgtatgcatgcatgtccagagcagtcatgcatgcctcctgcgtgtggctacgtaccta   902
903      cacgtatgtgctctgaataattatttgtgtagtgtgcttgatgtggtgtacggtcgtgtg   962
963      agcgagtatcttacatatatatagtgtgtaccgatgtataggtgtatgcatgtatgtcca   1022
1023     ccgcactcttgcatgcctctctga  1046
```

The cDNA sequence corresponds to TC91444 (www.tigr.org) found in the database by using a dKobomugi subtraction clone (dKobomugi_109). The ORF corresponds to a putative dehydration-responsive protein RD22 precursor *Oryza sativa* (japonica cultivar-group) (gi|15624018|dbj|BAB68072.1|).

>Retinoblastoma – B domain (RB-B)[1]:

```
         V  G  G  N  E  K  C  A  E  V  G  V  H  I  F  F  S  K  I  L
1        GTTGGCGGGAATGAAAAATGTGCTGAAGTAGGGGTTCACATCTTCTTCTCCAAAATCTTG   60
         K  L  A  A  I  R  I  R  N  L  C  E  R  L  R  H  V  E  Q  T
61       AAGTTGGCTGCTATTAGAATAAGGAACCTGTGTGAAAGGCTTCGGCATGTGGAACAGACA   120
         E  R  V  Y  N  I  F  K  Q  I  L  D  Q  Q  T  T  L  F  F  N
121      GAGCGTGTCTATAATATCTTCAAGCAGATTCTTGATCAACAGACAACTTTATTTTTTAAT   180
         R  H  I  D  Q  L  I  L  C  S  L  Y  G  V  A  K  V  S  Q  L
181      CGACACATTGATCAGCTTATTCTTTGTTCTCTCTATGGTGTTGCAAAGGTTTCTCAATTA   240
         T  L  T  F  K  E  I  V  N  N  Y  K  R  E  Q  Q  C  I  P  E
241      ACACTGACGTTCAAGGAGATTGTCAACAATTACAAAAGGGAACAGCAATGTATACCAGAA   300
         V  F  R  S  V  F  V  V  N  T  N  H  N  G  G  L  G  S  R  H
301      GTTTTTAGAAGTGTCTTTGTTGTGAATACAAATCACAATGGAGGACTTGGATCACGCCAT   360
         V  D  I  I  V  F  Y  N  E  V  F  V  P  A  V  K
361      GTTGACATCATTGTTTTTTATAATGAGGTGTTTGTTCCAGCAGTCAAG  408
```

The cDNA sequence represents the B-domain coding region of Retinoblastoma protein. *Zea mays* retinoblastoma related protein RBR1 (rbr1) mRNA, complete cds (gi|9716501:117-2720), was used as a template for finding the *T. aestivum* analogous ESTs.
Note: The cDNA clone for Retinoblastoma B-domain was not presented in our subtraction library but we are interested to study if the Retinoblastoma transcript is drought-inducible in the tolerant wheat cultivars.

>Rubisco activase A2 (rcaA2):

```
1     cttttttttttttttttttatttcgtttatttttttttagaccttcttatcgatagtatc  60
61    taccggctcgaactcgaatttgatcgccagcacagccgcgcgcatccaccgttgtagcaa  120
                            M  A  A  A  A  F  S  S  T  V  G  A  P  A
121   ggtcgaccaagcagcacagagATGGCTGCTGCCTTCTCCTCCACCGTCGGGGCTCCGGCT  180
      S  T  P  T  N  F  L  G  K  K  L  K  K  Q  V  T  S  A  V  N
181   TCTACGCCGACCAACTTCCTCGGGAAGAAGCTCAAGAAGCAGGTGACCTCGGCCGTGAAC  240
      Y  H  G  K  S  S  K  A  N  R  F  T  V  M  A  A  E  N  I  D
241   TACCATGGCAAGAGCTCCAAGGCCAACAGGTTCACAGTCATGGCAGCGGAGAACATCGAC  300
      E  K  R  N  T  D  K  W  K  G  L  A  T  D  I  S  D  D  Q  Q
301   GAGAAGAGGAACACCGACAAGTGGAAGGGTCTTGCTACCGATATCTCCGACGACCAGCAG  360
      D  I  T  R  G  K  G  I  V  D  S  L  F  Q  A  P  T  G  D  G
361   GACATCACCAGAGGGAAGGGCATCGTGGACTCGCTCTTCCAGGCGCCCACGGGCGACGGC  420
      T  H  E  A  V  L  S  S  Y  E  Y  V  S  Q  G  L  R  K  Y  D
421   ACCCACGAGGCCGTCCTCAGCTCCTACGAGTACGTCAGCCAGGGCCTGCGGAAGTACGAC  480
      F  D  N  T  M  G  G  F  Y  I  A  P  A  F  M  D  K  L  V  V
481   TTCGACAACACCATGGGAGGCTTCTACATCGCTCCTGCTTTCATGGACAAGCTTGTTGTC  540
      H  L  S  K  N  F  M  T  L  P  N  I  K  I  P  L  I  L  G  I
541   CATCTCTCCAAAAACTTCATGACCCTGCCCAACATCAAGATCCCACTCATCTTGGGTATC  600
      W  G  G  K  G  Q  G  K  S  F  Q  C  E  L  V  F  A  K  M  G
601   TGGGGAGGCAAGGGTCAAGGAAAATCATTCCAGTGTGAGCTTGTGTTCGCCAAGATGGGC  660
      I  N  P  I  M  M  S  A  G  E  L  E  S  G  N  A  G  E  P  A
661   ATCAACCCCATCATGATGAGTGCCGGAGAGCTGGAGAGTGGGAACGCTGGAGAGCCAGCC  720
      K  L  I  R  Q  R  Y  R  E  A  A  D  M  I  K  K  G  K  M  C
721   AAGCTCATCAGGCAGCGGTACCGTGAGGCTGCAGACATGATCAAGAAGGGTAAGATGTGC  780
      C  L  F  I  N  D  L  D  A  G  A  G  R  M  G  G  T  T  Q  Y
781   TGCCTCTTCATCAACGATCTTGACGCTGGTGCGGGTAGGATGGGCGGAACCACGCAGTAC  840
      T  V  N  N  Q  M  V  N  A  T  L  M  N  I  A  D  A  P  T  N
841   ACCGTCAACAACCAGATGGTGAACGCCACCCTGATGAACATCGCCGATGCCCCCACCAAC  900
      V  Q  L  P  G  M  Y  N  K  R  E  N  P  R  V  P  I  V  V  T
901   GTGCAGCTCCCTGGCATGTACAACAAGAGGGAGAACCCCCGTGTGCCCATCGTCGTCACC  960
      G  N  D  F  S  T  L  Y  A  P  L  I  R  D  G  R  M  E  K  F
961   GGTAACGATTTCTCGACGCTCTACGCTCCTCTGATCCGTGATGGTCGTATGGAGAAGTTC  1020
      Y  W  A  P  T  R  D  D  R  I  G  V  C  K  G  I  F  Q  T  D
1021  TACTGGGCTCCCACCCGTGACGACCGTATCGGTGTCTGCAAGGGTATCTTCCAGACCGAC  1080
      N  V  C  D  E  S  V  V  K  I  V  D  T  F  P  G  Q  S  I  D
1081  AATGTCTGTGACGAGTCTGTCGTAAAGATCGTCGACACCTTCCCAGGACAATCCATTGAC  1140
      F  F  G  A  L  R  A  R  V  Y  D  D  E  V  R  K  W  V  G  S
1141  TTTTTCGGTGCTCTGCGTGCTCGGGTGTACGACGATGAGGTGCGCAAGTGGGTCGGCTCT  1200
      T  G  I  E  N  I  G  K  R  L  V  N  S  R  D  G  P  V  T  F
1201  ACCGGAATCGAGAACATTGGCAAGAGGCTGGTGAACTCGCGGGACGGGCCCGTGACCTTC  1260
      E  Q  P  K  M  T  V  E  K  L  L  E  Y  G  H  M  L  V  Q  E
1261  GAGCAGCCAAAGATGACAGTCGAGAAGCTGCTAGAGTACGGGCACATGCTCGTCCAGGAG  1320
      Q  D  N  V  K  R  V  Q  L  A  D  T  Y  M  S  Q  A  A  L  G
1321  CAGGACAATGTCAAGCGTGTGCAGCTTGCTGACACCTACATGAGCCAGGCAGCTCTGGGT  1380
      D  A  N  Q  D  A  M  K  T  G  S  F  Y  G  *
1381  GATGCTAACCAGGATGCGATGAAGACTGGTTCCTTCTACGGTtagaaaattctccataca  1440

1441  acacccaccatctcttgctgcataggaggaggtAAAGGAGCACAGCAAGGTACTTTGCCC  1500

1501  GATGCCGGAAGGTTGCACCGACCAAAATGCCAAGAACTACGACCCAACGGCAAGGAGCGA  1560

1561  CGAACGGCAGCTGCCTTTACACCTTTTAAGCATGCCACTTTTAAtatctgatgttttacc  1620
1621  tacactaagtccgcatcgcatccaagcgattgctgggcatgttttgttaatatatctcta  1680
1681  aataatattgtgtatacccatttggcaaa  1709
```

The cDNA sequence corresponds to *Hordeum vulgare* rubisco activase (RcaA2) mRNA, complete cds (gi|167090|gb|M55447.1|BLYRCAA2) found in the database by using a dPEG subtraction clone (dPEG_77/dKobo_37). The ORF corresponds to ribulose-bisphosphate carboxylase activase (EC 6.3.4.-) A2 – barley (gi|7438147|pir||T06176).

Note: *rsaA2* transcript contains 50 bp intron (lower-case letters in italic) and is longer than *rcaA1* transcript. However in the beginning of the intron there is a stop codon. That is why the Rca A2 peptide is shorter than the Rca A1 peptide.

In the dKobomugi subtraction library we found clone representing the intron region of *rcaA2*.

>Rubisco activase B (*rcaB*)[1]:

The cDNA sequence corresponds to *Triticum aestivum* rubisco activase B (RcaB) mRNA, complete cds (gi|7960276|gb|AF251264.1|AF251264). The ORF corresponds to *Hordeum vulgare* rubisco activase B (gi|10720253|sp|Q42450|RCAB_HORVU).
Note: The cDNA clone for RcaB was not presented in our subtraction libraries but we consider for interesting to study its expression in parallel with the RcaA1 and 2 expression. However, our efforts for cloning cDNA from wheat were not successful.

>Rubisco small subunit (rbcS):

```
     M  A  P  A  V  M  A  S  S  A  T  S  V  A  P  F  Q  G  L  K
1    ATGGCCCCCGCCGTGATGGCCTCGTCGGCCACCTCCGTCGCTCCTTTCCAGGGGCTCAAG  60
     S  T  A  G  L  P  V  S  R  R  S  N  G  A  S  L  G  S  V  S
61   TCCACCGCCGGCCTCCCCGTCAGCCGCCGCTCCAACGGCGCTAGCCTCGGCAGCGTCAGC  120
     N  G  G  R  I  R  C  M  Q  V  W  P  I  E  G  I  K  K  F  E
121  AACGGTGGAAGGATCAGGTGCATGCAGGTGTGGCCCATCGAGGGCATCAAGAAGTTCGAG  180
     T  L  S  Y  L  P  P  L  S  T  E  A  L  L  K  Q  V  D  Y  L
181  ACCCTGTCTTACCTGCCACCGCTCAGCACGGAGGCCCTCCTCAAGCAGGTCGACTACCTG  240
     I  R  S  K  W  V  P  C  L  E  F  S  K  V  G  F  I  F  R  E
241  ATCCGCTCCAAGTGGGTGCCTTGCCTCGAGTTCAGCAAGGTTGGGTTCATCTTCCGTGAG  300
     H  N  A  S  P  G  Y  Y  D  G  R  Y  W  T  M  W  K  L  P  M
301  CACAACGCATCTCCTGGGTACTACGATGGCCGGTATTGGACAATGTGGAAGCTGCCTATG  360
     F  G  C  T  D  A  T  Q  V  I  N  E  V  E  E  V  K  K  E  Y
361  TTCGGGTGCACTGACGCCACACAGGTGATCAACGAGGTGGAGGAGGTCAAGAAGGAGTAC  420
     P  D  A  Y  V  R  I  I  G  F  D  N  M  R  Q  V  Q  C  V  S
421  CCTGACGCGTATGTCCGCATCATCGGATTCGACAACATGCGTCAGGTGCAGTGCGTCAGC  480
     F  I  A  F  K  P  P  G  C  E  E  S  G  K  A  *
481  TTCATCGCCTTCAAGCCACCGGGCTGCGAGGAGTCCGGCAAGGCCTAA  528
```

The cDNA sequence corresponds to *Triticum aestivum rbcS* gene coding for ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, complete cds, clone: p9-1 (gi|11990896:1-147, 359-739). The ORF corresponds to ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit [*Triticum aestivum*] (gi|11990897|dbj|BAB19812.1|).
Note: For primer designing we matched our clones to the sequence of *Triticum aestivum* DNA for ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit, partial cds, clone CS-B2 (gi|4038720|dbj|AB020958.1|).

>Translationally controlled tumor protein (TCTP)[2]:

```
                                        M  L  V  Y  Q  D  K  L
1    tccttttcgggggagaaatctcaagaggcggcaccATGCTCGTGTACCAGGACAAGCTT  60
     S  G  D  E  L  L  S  D  S  F  P  Y  R  E  L  E  N  G  V  L
61   TCCGGCGACGAGCTTCTGTCGGATTCGTTCCCGTACAGGGAGCTGGAGAACGGCGTGCTC  120
     W  E  V  D  G  H  W  V  V  Q  G  A  V  D  V  D  I  G  A  N
121  TGGGAAGTCGACGGCCATTGGGTCGTTCAAGGAGCAGTTGATGTGGACATTGGAGCCAAT  180
     P  S  A  E  G  G  G  D  D  E  G  V  D  D  Q  A  V  K  V  V
181  CCCTCTGCTGAGGGTGGTGGTGATGATGAGGGTGTCGATGACCAGGCCGTGAAGGTGGTT  240
     D  I  V  D  T  F  R  L  Q  E  Q  P  A  F  D  K  K  Q  F  I
241  GACATTGTTGACACCTTCCGTCTTCAGGAGCAACCTGCTTTTGACAAGAAGCAGTTTATC  300
     S  H  M  K  R  Y  I  K  N  L  S  A  K  L  E  G  D  D  L  D
301  TCTCACATGAAGCGCTACATCAAGAACCTCTCTGCCAAGCTTGAAGGGGATGACCTAGAT  360
     V  F  K  K  N  V  E  S  A  T  K  Y  L  L  S  K  L  K  D  L
361  GTTTTCAAGAAGAATGTTGAGTCTGCCACAAAGTATCTTCTTAGCAAGCTCAAGGACCTT  420
     Q  F  F  V  G  E  S  M  H  D  D  G  G  V  V  F  A  Y  Y  K
421  CAGTTCTTTGTTGGCGAGAGCATGCATGATGATGGCGGCGTGGTGTTCGCCTACTACAAG  480
     E  G  A  A  D  P  T  F  L  Y  F  A  H  G  L  K  E  V  K  C
481  GAGGGAGCTGCTGATCCAACTTTCCTGTACTTTGCACATGGGCTGAAAGAGGTCAAGTGC  540
     *
541  TAAtcgcactgtatgctaaaatctatgcggtcgtcagtatttttgctttgctctatggtt  600
601  gtcagtcgactctccaatgttgtgtcggtaactatcagatgttgagtgtcttgaaaactt  660
661  tctgataattgtgggatttgctttgtaatggtaatcgtgaaattggtgtggttatgtttt  720
721  ggtcaatgcctcggcttttactcgctagaatcaagcgattttgccctgttaaaaaaaaa   779
```

The cDNA sequence corresponds to a cDNA clone - gi|21070378|gb|AF508970.1| *Triticum aestivum* translationally controlled tumor protein.

>Ubiquitin-conjugating enzyme (UCE):

```
1    gaattggcacgagaccaccaccccaccaccaccaaaggggaacgagcgccgccgagcgag  60
61   cacgcacgcacgcccgcctccccccaccggagcacgcgcgccgccccgaaatcccctgtc  120
                                                  M  A  S  S  G
121  gcatccgtcgggcggtctcctccccgtgctcctcacttttctggaATGGCGTCGAGCGGT  180
     D  A  A  G  V  V  V  P  R  N  F  R  L  L  E  E  L  E  R  G
181  GACGCAGCCGGAGTTGTCGTGCCGAGGAACTTTAGACTGCTGGAAGAGCTTGAGAGAGGA  240
     E  K  G  I  G  D  G  T  V  S  Y  G  M  D  D  A  D  D  I  Y
241  GAGAAGGGTATTGGGGATGGAACAGTTAGCTATGGAATGGATGACGCAGATGATATCTAC  300
     M  R  S  W  T  G  T  I  I  G  P  H  N  T  V  H  E  G  R  I
301  ATGCGCTCCTGGACAGGAACAATAATTGGTCCCCACAACACTGTCCATGAGGGCCGGATT  360
     Y  Q  L  K  L  F  C  D  K  D  Y  P  D  R  P  P  T  V  R  F
361  TATCAGTTGAAGCTTTTCTGTGACAAGGACTATCCCGACAGGCCACCGACTGTTAGGTTT  420
     H  S  R  I  N  M  T  C  V  N  A  E  T  G  L  V  D  Q  R  K
421  CACTCAAGAATCAACATGACCTGTGTAAATGCCGAGACTGGATTGGTGGACCAAAGGAAG  480
     F  S  L  L  S  N  W  R  R  E  Y  T  M  E  N  I  L  I  Q  L
481  TTTAGCCTGCTGTCCAACTGGCGCCGTGAGTACACAATGGAGAACATCTTGATACAGCTT  540
     K  K  E  M  A  T  S  H  N  R  K  L  V  Q  P  P  E  G  T  F
541  AAGAAAGAGATGGCGACATCACACAACCGGAAATTAGTGCAGCCTCCGGAGGGGACCTTC  600
     Y  *
601  TACTGAaggagcacttgccgtgtcgcacgtgtcattcccaagaaccctcgcggttcccat  660
661  tttagatatcgcataagatcgcaacatttggtgcaaacgattatgtataactttgacggt  720
721  gtccttcacatggttcttgtgtgtcttgtctcgttatatctcctgggtacgcctgtgttg  780
781  taatgatacgctggagcttcagctattgtttttaaaagtaccattacgttgcttcgttct  840
841  aaaaaaaaaaaaaaaaaaaa  861
```

The cDNA sequence corresponds to TC87648 (www.tigr.org) found in the database by using a dKobomugi subtraction clone (dKobomugi_50). The ORF corresponds to ubiquitin-conjugating enzyme (gi|18404032|ref|NP_565834.1|, *Arabidopsis thaliana*).

>Unknown protein_79 (UP_79):

```
                    M  D  S  Q  A  P  A  G  D  D  R  R  S  T
1    cagttgagctaataggcgATGGATTCCCAGGCGCCGGCCGGCGACGACCGCCGGAGCACC  60
     H  L  N  K  A  V  D  E  Q  S  P  R  Q  S  A  P  S  K  Q  A
61   CACCTCAACAAGGCTGTGGACGAGCAATCTCCTCGACAATCGGCGCCCAGCAAGCAGGCA  120
     G  A  P  C  V  Y  L  K  K  G  T  C  A  E  G  S  M  R  H  D
121  GGTGCTCCGTGCGTCTACCTCAAGAAAGGCACGTGCGCGGAGGGAAGCATGCGGCATGAC  180
     A  H  G  H  A  P  H  L  A  A  S  H  A  H  *
181  GCCCATGGCCACGCGCCCCACCTTGCCGCAAGCCATGCTCACTGAgcgtaccgtatagca  240
241  gcagccagcagcagagacaatactatgtattgccgcaataccagtactacgtactttcta  300
301  cgtatttctgctccgtcatgtcttcttctcttccgctgcttgctgctagtatctatctaa  360
361  ggccgccccatatggccgtatatacagtaaaaagattgtaataaataatgacccactctg  420
421  tgtgtggtccctgtgattgcggtcacggtcggtcttgcgggctcctcgagctcgtcgctc  480
481  gtgctgcgtagattcctgccggctccttctcggagcggcgaggttaggggtttcttgtcg  540
541  tgtgtgtgtacacgtcggcgagattatttggtgtctggttcaagggcttcgacggcgacg  600
601  actgcatctcatatgcatgaagacttcctggcagtcatcaataagatcgggccaactgtg  660
661  ctccggtatgaacagttcgttcggtggtccatagaccttgatgtaatattttattatgtt  720
721  tggggtgctttgtacttctggcgagcctttataataaagataatttagttactaaaaaaa  780
781  aaaaaaaaaaaaa  794
```

The cDNA sequence is a contig obtained by overlapping two cDNA clones - gi|14314837|gb|BG907161.1|BG907161 TaLr1157F05R TaLr1 *Triticum aestivum*, and gi|9416028|gb|BE418182.1|BE418182 SCL022.C02R990722 ITEC SCL Wheat Leaf Library *Triticum aestivum*, found in the database by using a dKobomugi subtraction clone (dKobomugi_79). Search with this clone in *T. aestivum* - TIGR database (www.tigr.org) resulted in TC57949. The ORF does not correspond to any known protein in the database and is categorized as unknown.

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Pettkó-Szandtner A, Mészáros T, Horváth G, Bakó L, Csordás-Tóth É, Blastyák A, **Zhiponova M**, Miskolczi P, Dudits D (2006) Activation of an alfalfa cyclin-dependent kinase nhibitor by calmodulin-like domain protein kinase. (*in press*)

Zhiponova MK, Pettkó-Szandtner A, Stelkovics É, Neer Z, Bottka S, Dudits D, Fehér A, Szilák L (2006) Mitosis-specific promoter of the alfalfa cyclin-dependent kinase gene (*Medsa;CDKB2;1*) is activated by wounding, and ethylene, in a non-cell division- dependent manner. (*in press*)

SUMMARY IN ENGLISH

Introduction

Owing to their sessile lifestyle, plants have to respond to local environmental conditions such as light, temperature, water, nutrients, gravity, pathogen attack, wounding by changing their physiology and redirecting their growth. The perceived stimuli are communicated across the plant body through chemical messengers, hormones, which affects diverse aspects of plant growth and development. Since the rigid cell wall does not allow mobilizing of specialized cells for stress response, plants developed the capacity of making cells competent for the activation of defense responses. The mechanisms for increased tolerance to environmental stress involve physiological changes, or expression of genes that results in modification of molecular and cellular processes.

The information about the perceived stress in the cells is transmitted through a signal transduction pathway. The signalling molecules can regulate the gene expression. The transcription factors recognize multiple regulatory sequences within the promoter regions of the genes ensuring nuanced response to different signals. Studying the gene expression regulation at transcription level may improve the understanding about the regulatory networks acting during stress adaptation.

In the present study, I aimed the examination of the effect of stress factors on gene expression in higher plants. In the first part of our work, I investigated the expression of genes up-regulated in response to drought/osmotic stress. My approach was based on gene substraction; a set of genes was isolated from drought sensitive and was substracted from the gene set of drought tolerant wheat (*Triticum aestivum* L.) cultivar. This project was done in collaboration with the Department of Plant Physiology of the University of Szeged with the purpose to collect information about the molecular mechanism of drought tolerance of wheat. These data can serve as molecular markers for fast identification of drought tolerant species, as well as for the establishment of drought tolerant transgenic lines at a later stage.

In the second part of our work, we studied how a G2/M regulated gene is involved in the wound stress response. We focused on analyzing the regulation of B-type cyclin dependent kinase (B-type CDK, or CDKB) in alfalfa (*Medicago sativa* L.). The CDKBs are key regulators of the G2/M checkpoint of the cell division cycle progression in plants. They are characteristically regulated at the transcriptional level and promoter studies are a useful approach to define different signal pathways that can affect the expression of these cell cycle kinases. There are two CDKB classes – CDKB1 and CDKB2 (the expression of CDKB1 precedes the expression of CDKB2). In an earlier work, Magyar et al. (1997) demonstrated that two CDK genes, assigned as c*dc2MsD* and *cdc2MsF* (according to the recent nomenclature *Medsa;CDKB1;1* and *Medsa;CDKB2;1*, respectively) accumulate at the G2/M cell cycle-

phase transition. In the present work, we wanted to clone and characterize the upstream region of the Medsa;CDKB2;1 kinase. Up to now, no detailed investigations have been performed on promoters of B2-type CDKs, which highlights the requirement and the interest to improve our understanding about their regulation.

Results

I. Differential gene expression in response to drought/osmotic stress in wheat

In order to study the differentially expressed genes under drought stress in wheat, we constructed **subtractive libraries** using both non-treated and PEG-treated seedlings **from** two **wheat cultivars**, the mild drought tolerant cv. Öthalom and the drought tolerant cv. Kobomugi. Clones from the resulting cDNA populations were sequenced and subjected to database screening. According to the characteristics of the proteins coded by the homologous genes, classification into several categories was performed.

Many of the genes are highly homologous to known **drought-responsive genes** and give ideas about the drought/osmotic stress defense system of the tolerant wheat cv. Kobomugi. The survival strategy seems to involve ABA signal pathways for induction of drought related genes, decreased transpiration to limit water loss, solutes (especially carbohydrates) as osmoprotectants, stabilization of the cell wall and cell membranes, and transport of ions for keeping the homeostasis. The genes coding for proline-rich protein, Rubisco small subunit, Rubisco activase and low temperature and salt responsive protein (early drought induced protein) were highlighted to have putative role in the drought tolerance of cv. Kobomugi. However, these are only *in silico* results and their importance must be further evaluated by investigations on plants. Our expression studies on selected differentially expressed genes confirmed several genes e.g. Rubisco small subunit, low temperature and salt responsive protein (early drought induced protein) , and an unknown protein, are indeed under drought/osmotic stress regulation.

II. Activation of alfalfa B2-type CDK (Medsa;CDKB2;1) by wounding, and ethylene, in a non-cell division-dependent manner

We focused on the isolation and characterization of the upstream region of alfalfa *CDKB2;1* gene. A 360 bp DNA fragment was cloned and its *in silico* analysis revealed the presence of putative motifs related to the cell cycle regulation, light responsiveness and wounding. Comparison with the upstream regions of Arabidopsis B-type CDKs revealed a characteristic promoter structure and we hypothesized that the order of regulatory elements might be necessary for the correct function of these genes.

In order to unravel the propensity of the alfalfa *CDKB2;1* promoter, it was linked to reporter genes (GUS, and luciferase) and characterized in transgenic plants. It was demonstrated that the reporter activity

was restricted to the actively proliferating tissues and regions of intact plants. More detailed studies using synchronized alfalfa cell cultures **confirmed G2/M cell cycle phase-specificity of the promoter**. The expression data of the reporters were in good correlation with that of the endogenous alfalfa *CDKB2;1*. In addition, we compared the promoter activity in heterologous systems such as stably transformed *Arabidopsis*. In Arabidopsis, the cloned promoter worked in a very similar manner as in alfalfa with reporter activity characteristic for proliferating regions.

Based on the *in silico* results, the **wound inducibility** of alfalfa CDKB2;1 was further **evaluated**. The CDKB2;1 wound response was demonstrated in leaves of young seedlings and in a simplified system of detached leaves. The wound induction occured in a non-cell division-dependent manner. Treatment with ethylene precursor, ethephon, could turn on the promoter in a similar manner as mechanical injury without cell division activation. Ethylene is known as one of the wound response mediators, and on the other hand, it can cause a G2/M arrest and stimulate the endoreduplication. We suggest that ethylene might have role at the switch between mitosis and endoreduplication. The accumulation of alfalfa **CDKB2;1 kinase** due to G2/M progression, or by treatment with ethylene, considered as a G2/M-phase inhibitor, emphasizes the **multifuncional role** of this kinase.

SUMMARY IN HUNGARIAN

Bevezetés

A növényi élet helyhez kötött sajátosságából következik, hogy a környezeti hatásokra, mint fény, hőmérséklet, szárazság, patogének, sebzés a növény csak a fiziológiás kondícióinak megváltoztatásával tud reagálni. A növényben a stressz- szignál hormonok, kémiai jelmolekulák útján jut el a sejtekhez. Mobilizálható sejtek hiányában a növények sejtjeinek rendelkezniük kell a védekezés képességével, ami egy komplex rendszer és magában foglalja a transzkripciós/ gén-regulációs szabályzást is.

A stressz a sejtekben szignál-átviteli utakat aktivál, ami válasz fehérjék megjelenésében manifesztálódik. A transzkripciós faktorok - olyan szabályozó fehérjék, amelyek egy adott cél-fehérje termelését irányítják - felismerve a génkifejeződést reguláló DNS-en lévő kötő helyeiket, a stressz következtében aktiválódnak és irányítják a fehérje kifejeződését. A gének promoterének (szabályozó régiójának) a tanulmányozása közelebb vihet bennünket az adott szignálra adott sokrétű válasz megértéséhez.

A doktori értekezésben célul tűztem ki, hogy különböző stresszek (szárazság, sebzés) által indukált gén-expressziót tanulmányozom magasabb rendű növényekben. Az értekezés első részében megvizsgálom, hogy milyen gének biztosíthatják a búza szárazságtűrését. Gén-szubsztrakciós (kivonásos) eljárással adok választ, hogy a szárazságtűrő búza miért viselheti el a vízhiányt. Az eljárás azon alapul, hogy a szárazságtűrő fajta génkészletéből kivonjuk a szárazságra érzékenyebb génkészletét. Ezt a munkát a Szegedi Egyetem Növényélettani csoportjával kollaborációban végeztük. A kivonás eredményeként kapott gének visszaellenőrzést követően markeréül szolgálhatnak a növénynemesítőknek a szárazság tűrő egyedek gyorsabb azonosításhoz.

Dolgozatom második részében azt vizsgálom lucernában, hogyan szabályozódik egy a sejtciklus G2/M fázisában termelődő ciklin függő B-típusú kináz (CDKB). Feltárom a regulációs folyamatok komplexitását, bebizonyítva, hogy a sebzés, és az etilén is hat a gén expressziójára.

A növényi sejtciklus sajátossága, hogy nemcsak a ciklinek, hanem a CDKB csoportba tartozó kinázok is rendelkeznek sejtciklus fázisától függő expresszióval. Két tagjuk ismert a CDKB1 és CDKB2, amit *cdc2MsD* és *cdc2MsF* -ként azonosítottak korábban csoportunkban, (az érvényben lévő nomenklatura szerint *Medsa;CDKB1;1* és *Medsa;CDKB2;1*) (Magyar és mtsai, 1997). Doktori értekezésemben célul tűzöm ki, hogy izolálom a mitotikus *Medsa;CDKB2;1* szabályozó upstream régióját, és *in silico* analizálom. Riporter géneket (GUS és luciferáz) kapcsolok a promoter után, és transzgenikus növényekben tesztelem a promoter szabályzását, majd adott növényekből sejtszuszpenziós

kultúrát hozok létre és tartok fenn, azért, hogy szinkronizálás után megállapíthassam az expresszió pontos sejtciklus-fázisát.

Eredmények

I. Génexpressziós különbségek vizsgálata szárazság tűrő és érzékeny búza fajtákban

A szárazság különböző géneket, géncsoportokat indukál. Polietilén-glycollal (PEG) ozmotikus stresszt indukáltunk szárazság érzékeny Öthalom, és ellenálló Kobomugi búza fajtákban, majd kivontuk az indukált génállományból a nem indukáltat, illetve a különbséget képeztünk a két faj génkészlete között is indukált, és nem indukált állapotban. A kapott klónoknak meghatároztuk a DNS szekvenciáit, *in silico* azonosítottuk a géneket. A géneket több csoportba osztottuk funkciójuk, és sejten belüli lokalizációjuk szerint. A vízhiány többek között ABA (növényi hormon) szignál utat használva szabályozza a párologtatás, az ozmotikumot biztosító fehérjék, ioncsatornák, sejtfalszintézis, sejtmembrán alkotók génjeinek kifejeződését. A kapott eredményeink ezt alátámasztják. Azonban a **differenciál génkészletet** csak *in silico* analizáltam, teljes körű *in vivo* expressziós vizsgálatokat csak néhány, általunk fontosabbnak vélt géneknél végeztem el, úgy mint RuBiSCO kis alegysége, egy alacsony hőmérsékleten termelődő sressz fehérje, és egy magas só koncentrációra termelődő stressz fehérje esetén.

II. A mitotikus lucerna CDKB2 kináz kifejeződését a sebzés és az etilén is aktiválja

Izoláltuk a *Medsa;CDKB2;1* gén előtti 360 bp szakaszt. A fragment *in silico* analízise egy komplex szabályozást valószínűsített, feltételezett kötőhelyeket lehetett beazonosítani, amik a sejtciklus regulációban, fényszabályozásban, és a sebzés indukált válaszban játszanak szerepet. Arra vállalkoztam, hogy bizonyítom, hogy a klónozott szakasz valóban működhet promoterként, biztosíthatja a sejtciklus-specifikus génkifejeződést, továbbá a sebzést követően, sejtciklus független módon bekapcsol.

Transzgén növényeket hoztunk létre a klónozott fragment után kapcsolt vagy GUS, vagy luciferáz **riportergén**ekkel. Immunhisztokémiai festéssel egyértelműen nyomonkövethetővé vált, hogy a **360 bp fragment promoter**ként működik, és specifikusan csak az **osztódó szövetek fejezik ki** a riporter fehérjéket, az endogén *CDKB2;1* kinázhoz hasonlóan. Megfelelő növényekből sejtszuszpenziós kultúrákat indítottam. **Szinkronizált sejtszuszpenzió** segítségével bizonyítottam, hogy a riporter gének transzkripciója az endogén *Medsa;CDKB2;1* kinázzal egy időben következik be, ami az irodalmi adatokkal megegyezően a G2-M fázis határán történik.

Heterológ rendszerben, *Arabidopsis thaliana*ban is az osztódó sejtekben volt látható a riportergének megjelenése.

Ezután igazoltam, hogy a *Medsa;CDKB2;1* kináz **sebzés**t követően **indukál**ódik és a promoter sebzéssel való aktiválása a **sejtciklustól függetlenül** következik be. A sebzés indukálta szignál utak egyike az etilénen keresztül történik. Az etilén prekurzor ethephon hozzáadása szintén a promoter bekapcsolását eredményezte. Azonban az etilén szerepét nem csupán a sebzésben betöltött szerepe, hanem, pl. sejtciklus blokkoló tulajdonsága is érdekessé teszi. Az irodalmi adatok szerint az etilén a G2-M fázis határán állítja meg a sejtciklust. Ez feltételezi a CDKB2;1 kinase komplex szerepét a sejt életében, hiszen a sejtciklus G2/M fázisa, és ennek blokkolása is a kináz megjelenését eredményezi.