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QUARTERLY

Structure of yellow lupine genes coding for mitotic cyclins

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Cell cycle progression in eukaryotes is controlled by complexes of p34 protein kinases and cyclins. For the first time in plants, we have established the sequence of four yellow lupine mitotic cyclin B1 genes. Their coding regions and expression pattern were also characterised recently. Structure of all the four lupine genes is similar: they consist of nine exons and eight introns, analogously located, except Luplu;*CycB1;*3 lacking 7th intron. Analysis of 5'-regulatory sequences of two of them showed that both comprise M-specific activators (MSA), common to plant genes induced in late G2 and early M. Putative repressor binding sites CDE/CHR found in animal G2-specific promoters can also be detected in lupine genes. Controlling region of Luplu;*CycB1;*4 gene that is highly activated by IAA, contains up to 7 auxin response elements, while insensible to IAA Luplu;*CycB1;*4 gene have no such motifs. Further studies should be undertaken to determine precisely the functions of putative regulatory elements in the expression of lupine mitotic cyclins.

Progression through the cell cycle and key molecules regulating cell division are conserved in various groups of eukaryotes, although their development and morphogenesis considerably differs. Cell cycle, comprising phases G1 (gap 1), S (DNA synthesis), G2 (gap 2) and M (mitosis), is controlled at G1/S and G2/M transition points [1]. Animal cells leave proliferative state in G1 and after terminal differentiation become quiescent, while plant cells can differentiate both in G1 and G2 [2]. G2/M transition is regulated by a complex known as MPF (maturation promoting factor) [3, 4], consisting of cdc2 protein kinase and cyclin. MPF activates a cascade of protein kinases and phosphorylates directly cellular components, leading to chromosome condensation, nuclear envelope breakdown and

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Abbreviations: CDE, cycle-dependent element(s); CHR, cell cycle genes homology region(s); DRE, DNA replication-related element; MSA, M-specific activator(s); MPF, maturation promoting factor; USF, upstream stimulatory factor.

cytoskeleton rearrangement (review: [5]). Similar complex, SPF, acts at G1/S transition [6].

Distinct classes of cyclins are expressed successively at specific stages of the cell cycle and regulate progression through respective phases, e.g. cyclin B controls the entry into mitosis, cyclin D (and animal cyclin E) is involved in the passage from G1 to S-phase and cyclin A is active during the replication of DNA [7–9]. Term "cyclin" originates from cyclic variation in the protein level in proliferating cells [10]. Now, proteins are classified as cyclins if they contain a conserved region, cyclin box, responsible for association with cdc2 kinase. The cell cycle degradation of these regulatory proteins depends on the presence of a destruction box in mitotic cyclin, that is a target for ubiquitin [11] or PEST element in G1 cyclins [12].

The accumulation of cyclin B protein that is required to activate MPF, of other cyclins, correlates well with its mRNA level [13]. In many animal genes specifically expressed in G2 phase (cyclin A, *cdc2*, *cdc25*) CDE/CHR elements (cell cycle-dependent element and cell cycle genes homology region) are involved in transcriptional repression in all other cell cycle phases [14, 15]. Besides, G2-specific expression of mammalian genes is mediated by upstream stimulatory factor (USF) which binds to the core promoter [16]. Plant cyclin B promoters contain putative sites for binding Myb transcription factors [17]. M-specific activators (MSA) present in regulatory sequences of various plant genes activated in G2 and M phases were shown to be necessary and sufficient for their expression restricted to these cell cycle stages [18].

Over 80 cyclins have been identified in 20 plant species thus far. On the base of sequence comparison, they were classified in 5 groups of mitotic cyclins: A1, A2, A3, B1, B2 and 3 groups of G1 cyclins: D1, D2, D3 [19]. Although some reports suggested that plant cyclin genes may contain introns, no genomic sequence has been published. We have previously characterised four yellow lupine cDNA clones coding for putative mitotic cyclins of B1 type: Luplu;*CycB1;*1 (EMBL/GenBank accession number U24192), Luplu;*CycB1;*2 (U24193), Luplu;*CycB1;*3 (U24194), Luplu;*CycB1;*4 (U44857) [20–22]. Here we present structural organisation of four lupine genes (AF126105–AF126108) and describe putative regulatory elements present in the promoters of two of them.

MATERIALS AND METHODS

Screening of genomic library. 3×10^5 clones of yellow lupine (*Lupinus luteus cv. Ventus*) genomic library in λEMBL-3 vector (Clontech Laboratories, Inc.) were transferred to nitro-cellulose membranes and screened using the partial lupine cyclin Luplu;*CycB1;*2 cDNA [21] as a probe. The probe was labelled with [α-^{32}P]ATP using Boehringer Mannheim Random Primer Labelling Kit. Hybridisation was carried out at 60°C in the solution recommended by Clontech.

Restriction mapping was performed according to Maniatis *et al.* [23]. Genomic clones digested with *Sal*I, *Eco*RI, *Hin*dIII, *Sal*I-*Eco*RI and *Sal*I-*Hin*dIII restriction enzymes were hybridised to the whole Luplu;*CycB1;*2 cDNA, *Eco*RI-*Hin*dIII 5′-part of Luplu;*CycB1;*4 cDNA and 3′-terminal *Hin*dIII-*Xho*I fragment of Luplu;*CycB1;*4 cDNA. Fragments of genomic clones were subcloned into pBluescript vector (Stratagene).

Isolation of lupine DNA. Genomic DNA was extracted from lupine seedlings as described by Dellaporta *et al.* [24]. Genomic DNA (25 ng) was used as a template for PCR amplification.

PCR amplification. Genomic clones of lupine cyclins were identified by PCR, using oligonucleotides (Table 1) complementary to unique sequences in 3′ end regions of previously characterised cDNAs [20–22, 25]. Templates for sequencing cyclin genes were obtain

by PCR amplification of respective fragments of the genomic clones or lupine genomic DNA, with primers designed according to known cDNA sequences. Amplification was performed with 0.2 units of Taq polymerase (Boehringer Mannheim) in a buffer recommended by the supplier. Reaction consisted of DNA melting for 5 min at 94°C and 30 cycles: 30 s at 95°C, 30 s at 55°C, 2 min at 72°C and final extension 5 min at 72°C. One tenth of the sample was analysed in 1.5% agarose gel.

Inverse PCR (I-PCR). Templates for sequencing of cyclin promoters were prepared by I-PCR. 10 μg of lupine genomic DNA was digested with 20 units of *Hin*dIII (*Hin*dIII is an internal site in each of the four lupine cyclin gene). After thermal inactivation of an enzyme (15 min at 75°C) DNA was circularised with 10 units of T4 DNA ligase (Promega) in a final volume of 250 μl. Ethanol precipitated DNA was resuspended in 10 μl of water and one tenth was used as a template for PCR amplification of a region located upstream known cDNA sequence. Reaction was performed with Expand Long Template PCR System (Boehringer Mannheim), in conditions recommended by the producer, with elongation at 68°C for 8 min. The first primer (5′→3′) hybridised close to *Hin*dIII site inside cyclin gene, while the second (3′→5′) hybridised near known 5′end of cDNA (primers were divergent in linear gene sequence). Cyclin regulatory sequences were amplified with 5′bR and btF primers for Luplu;*CycB1*;2 and 5′dR and 4F for Luplu;*CycB1*;4 (Table 1).

DNA sequence analysis. Sequences of genomic clones were determined on both strains by dideoxy chain-termination method [26] using ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). As templates for sequencing, we used either fragments of cyclin genomic clones subcloned into pBluescript, or PCR amplification products of lupine DNA with oligonucleotide starters specific for cyclins (Table 1). Promoter regions obtained by I-PCR were reamplified prior to sequencing. All the PCR products were purified with QiaGen PCR Purification Kit (QiaGen).

RESULTS AND DISCUSSION

Isolation and sequencing of cyclin genomic clones

Lupine cyclin Luplu;*CycB1*;2 cDNA was used to screen yellow lupine genomic library. Six clones that we isolated contained cyclin B1 genes, as determined by Southern blotting (data not shown). For all the genomic clones, numerous DNA bands hybridised to the entire cDNA probe as well as to the 5′- or 3′-part of cDNA, what suggested that the genes contained multiple introns. To establish the identity of detected genomic clones, we used PCR primers designed against 3′-regions of four previously characterised cyclin cDNAs [20–22, 25]. Genomic clones represented three of the cDNAs: Luplu;*CycB1*;1, Luplu;*CycB1*;3 and Luplu;*CycB1*;4. Since the genomic clone for Luplu;*CycB1*;2 was not isolated from the library and the three other genes had to be divided into numerous subclones, we decided to amplify succeeding, overlapping fragments of cyclin genes by PCR, with the use of genomic clones or DNA extracted from yellow lupine as a template, and primers synthesised according to cDNA sequence.

Promoter regions of Luplu;*CycB1*;2 and Luplu;*CycB1*;4 genes were isolated by inverse PCR (I-PCR), owing to amplification of sequences upstream ATG after circularisation of DNA.

The sequences of the four cyclin B1 genes were determined and compared to the respective cDNA sequences, which allowed to establish the genomic structure (Fig. 1). The genomic sequences, available in the EMBL/GenBank with accession numbers AF126105–AF126108, represent the first genes coding for cyclins characterised in plants.

Table 1. Oligonucleotides used for template amplification and sequencing of cyclin genomic clones.

F (*forward*): 5′→3′ primers, R (*reverse*): 3′→5′ primers. * Primers used for identification of genomic clones.

Name	Primer for:	Sequence
5′aF	*CycB1*;1	TAGCGGTGGCGACTAAT
5′aR	*CycB1*;1	TTGATTAGTCGCCACCGCT
ai1F	*CycB1*;1	ATGGGTTCGATACTTCAA
DB	*CycB1*;1	CTTAAGGATATTGGTAAT
apR	*CycB1*;1	GCACATTAGCAACAGGAT
CB-R	*CycB1*;1, (2)	CACTTCTATTAACCAATC
CB-F	*CycB1*;1, 2	TTGACACTCAATATAGTTGAT
atR	*CycB1*;1	ACAATCCCTTAGTTGTTC
3′aF*	*CycB1*;1	AGCTTCATTTTCTTGATGGGT
3′aR*	*CycB1*;1	GATTCGCCCAATATCATTCA
5′bF	*CycB1*;2, 3, 4	AAATGGCATCAAGACCC
5′bR	*CycB1*;2	TCAACGATTTCGACAGGCT
5′bR2	*CycB1*;2	GCTTGTTGGGGTTGAAGT
biF	*CycB1*;2	GCGAACAAGAACAAGGAACAA
biR	*CycB1*;2	TCATTTCTGCTATCCCCTGC
btF	*CycB1*;2, 3, 4	TGGACTTTGACTGTGCC
3′bF*	*CycB1*;2	GGCTCTAGAGTTTGAGGGGA
3′bR*	*CycB1*;2	ACAACAATCATCAATAATGCCA
5′cR	*CycB1*;3, (4)	GCACCACCCACATTAGGAA
5′cR2	*CycB1*;3	GCCATTACTCGAACTTGT
ciF	*CycB1*;3	CCCGCCGAGAAGAAAGTT
ciR	*CycB1*;3	CCTCCTCGGCACAACCTT
3′cR*	*CycB1*;3	CATTGACTTGAGTTGTCCTGG
3′cR*	*CycB1*;3	CATTGACTTGAGTTGTCCTGG
5′dF	*CycB1*;4	GCTCCCTCTTCATCTTC
5′dR	*CycB1*;4	CGCTTCTCAACCTCAACGG
5′dR2	*CycB1*;4	CCTTATGTGATCGGTGAG
diF	*CycB1*;4, 3	TGCTGATGGAGTTGTTGCTAAA
dpR	*CycB1*;4	TTAATTTCAATGACTTCTTG
4F	*CycB1*;4, 2, 3	AGATTATTGACATTGATG
4R	*CycB1*;4, 2, 3	AGCCATGTTTTCCAACTC
3′dF*	*CycB1*;4	AGGGGGAGGGATTGATTTA
3′dR*	*CycB1*;4	ACAAAACAATAACTTCCACACATG
λ-L	λ EMBL-3	ACTGCGCAACTCGTGAAAGGTAAG
λ-P	λ EMBL-3	TAAAAGCAGAAGTCCAACCCAGATAACG

Structure of the genes coding for lupine cyclins

Lupine cyclin B1 genes consist of nine exons and eight introns, except Luplu;*CycB1*;3 gene lacking the intron n°7 (Fig. 1). The exon/intron junctions have sequences similar to the consensus determined for dicotyledonous plants (Table 2) [27–28]. As in other plant genes, the most conserved are two first and two last nucleotides in the intron as well as two last nucleotides in the exon. Based on these consensus sequences, the exact positions of introns in cyclin genes were established.

The sequences of coding region of the cyclin genes are identical to respective cDNA se-

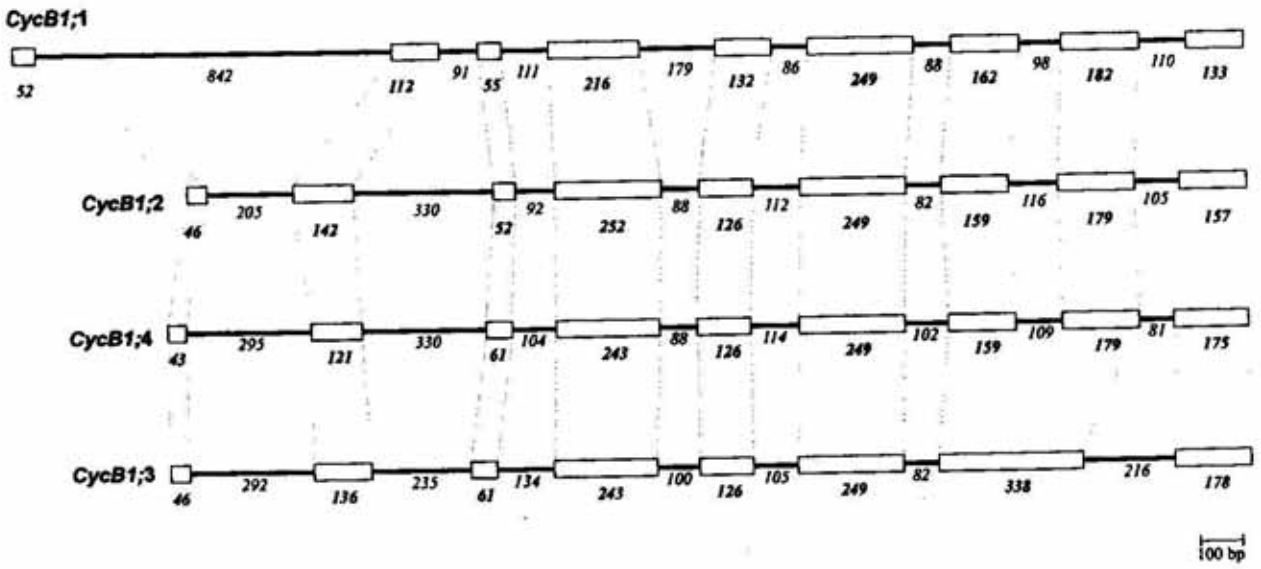

Figure 1. Structural organisation of lupine B1 cyclin genes.

Exons are shown as rectangles, introns – lines. Numbers stands for length (in bp) of particular DNA fragments. Introns are distributed in analogous way in coding sequences.

quences that we characterised recently [20–22]. Particular introns are located in analogues positions in each gene but they differ in length and sequence. Most of the introns are short (80–120 bp), slightly longer in 5′-regions (200–300 bp), except the first intron of Luplu;*CycB1*;1 gene which comprises 840 bp. In the 3′-part of this intron, a 106 bp sequence is repeated 4 times. As in other eukaryotic genes, all the intron sequences are rich in A-T. Arrangement of introns is apparently not related to functional or structural domains of a cyclin, e.g. one intron is located upstream α4 helix in the first cyclin fold while two introns: one in $\alpha 2'$ helix and another in $\alpha 5'$ helix are present in the second cyclin fold, assuming that the structure of lupine cyclin B is similar to that of animal and human cyclins A and H [29–30].

The organisation of plant cyclin genes was not known before, although some authors suggested the presence of introns, since they observed longer PCR products of genomic DNA amplification than cyclin cDNA amplification. Deckert reported that soybean cyclin B1 gene possess one intron [31], but probably this sequence represents a pseudogene as it lacks 5′-region, including the start codon. Interestingly, the gene coding for human B1 cyclin has similar organisation as the lupine genes: it comprises 9 exons and 8 introns [16]. However, introns in the human gene are much longer and lay in different positions than in lupine genes.

Analysis of promoter regions

The sequences of 5′-regulatory regions of two cyclin genes: 545 bp of Luplu;*CycB1*;2 and 1526 bp of Luplu;*CycB1*;4 isolated by I-PCR are shown in Fig. 2. Both genes lack a consensus TATA box, although A-T rich regions are present. Significantly changed variants or the absence of this motif are typical for many cell cycle gene promoters, both in animals [15–16] and in plants [17–18].

In vertebrates, SP1 and NF-Y factors are elements of regulation of cell cycle genes that activate transcription constitutively, while other elements, both inducers and repressors, confer restriction of gene expression to respective stage of the cell cycle [15–16]. In lupine

Table 2. Sequences of exon/intron junctions in lupine cyclin genes.

Nucleotides consistent with consensus underlined, absolute consensus in bold.

	Luplu;*CycB1*;1	Luplu;*CycB1*;2	Luplu;*CycB1*;3	Luplu;*CycB1*;4
Intron 1	AGgtat–tagGT	AGgtat–tagGT	AGgtgg–tagGT	AGgtat–tagAT
Intron 2	AGgtgc–cagGA	AGgtac–tagGA	AGgtac–tagGA	AGgtac–tagGA
Intron 3	AGgttt–cagAA	AGgtga–cagAA	AGgtca–cagAA	AGgtca–tagAA
Intron 4	AGgtaa–cagGC	AGgttg–cagGC	AGgttg–cagGC	AGgtca–tagGC
Intron 5	AGgtaa–tagGA	AGgtat–aagAA	AGgtgt–aagAG	AGgtat–aagAA
Intron 6	AGgtgt–tagGT	AGgtgc–cagGT	AGgtaa–cagGT	AGgtac–cagGT
Intron 7	AGgtat–tagAT	AGgtga–cagTT	–	AGgtga–cagTT
Intron 8	AGgtta–tagGG	ATgtaa–cagGG	ATgtaa–tagGG	ATgtaa–cagGG
Consensus	EXON/ intron /EXON AG/gtaagt– –tg(c/t)ag/GT			

cyclin promoters, consensus NF-Y or SP1 binding sites are absent. However, it is likely that cell cycle regulation of plant genes is mediated by a distinct mechanism, since single stimulatory element, MSA, was shown to confer transcriptional oscillation in synchronised tobacco cells [18].

As expected, sequences characteristic for genes induced in G1, such as E2F binding site [32–34] or DNA replication-related elements (DRE) [33] were not detected in promoters of lupine cyclin genes, what confirms that they are expressed at the beginning of mitosis but not in G1/S transition.

Sequence consistent with upstream stimulatory factor (USF) binding site (CACGTG) necessary for activation of human cyclin B1 gene in G2 [16] is located 441 bp upstream ATG of Luplu;*CycB1*;4 and may be engaged in its specific induction at this stage of cell cycle.

Animal and human genes activated in G2 contain cycle-dependent elements (C/G)GCGG (CDE) and cell cycle genes homology regions TTGAA (CHR) in their regulatory se-

5'-region of Luplu;*CycB1*;2 · 545 bp

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                                                          CCTAC      -541
AACTTTTGTTTTATGCACTAATATATTAGATGATAGTAAAATGATTTATATGACAACATT      -481
TCCACTCTCACCAAAAAACAATTTCTGTGAGTTAATTTATAACTTATAAGATAGTTTTGA      -421
TACATGTTATTATTATCTAATAAATATATTTTTAAATTTACCAAGTAGAAACTATTACTA      -361
TTTTATTATAAACTCAACATTTTCATAATTTTATTTATACTAAGAATTTGACTTGAGTAT      -301
AAATACTATAAAAATTTAATATAACATTATTTATTTGCTATTATAATTTGATTTGCTATA      -241
                                  nodule
TGTCTATTTTAGAACTTCCATAACATCTCTTCATACATTTTCATTCTTTGTCGTTTCTTT      -181
                MSA                CHR nodule      MSA
GTTTTTGGATAACAGCCGTGCGATATTGAAAGATCCAACGGTAAGAAAGTAGGTAAAAAT      -121
          MSA  CHR          MSA
TCGACCGTTGAAAGGAACCGTTGCTTCCTTCAACTATTTCACTGTTAAATCTCTCATCAA       -61
          nodule                                    nodule
ATCTCATTCACTCTTCACTGTGAGAACCAGAGAGTGAATCAAGAGAGAGAATTCTTCGAA        -1
ATG                                                                  +3
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5'-region of Luplu;*CycB1*;4 1526 bp

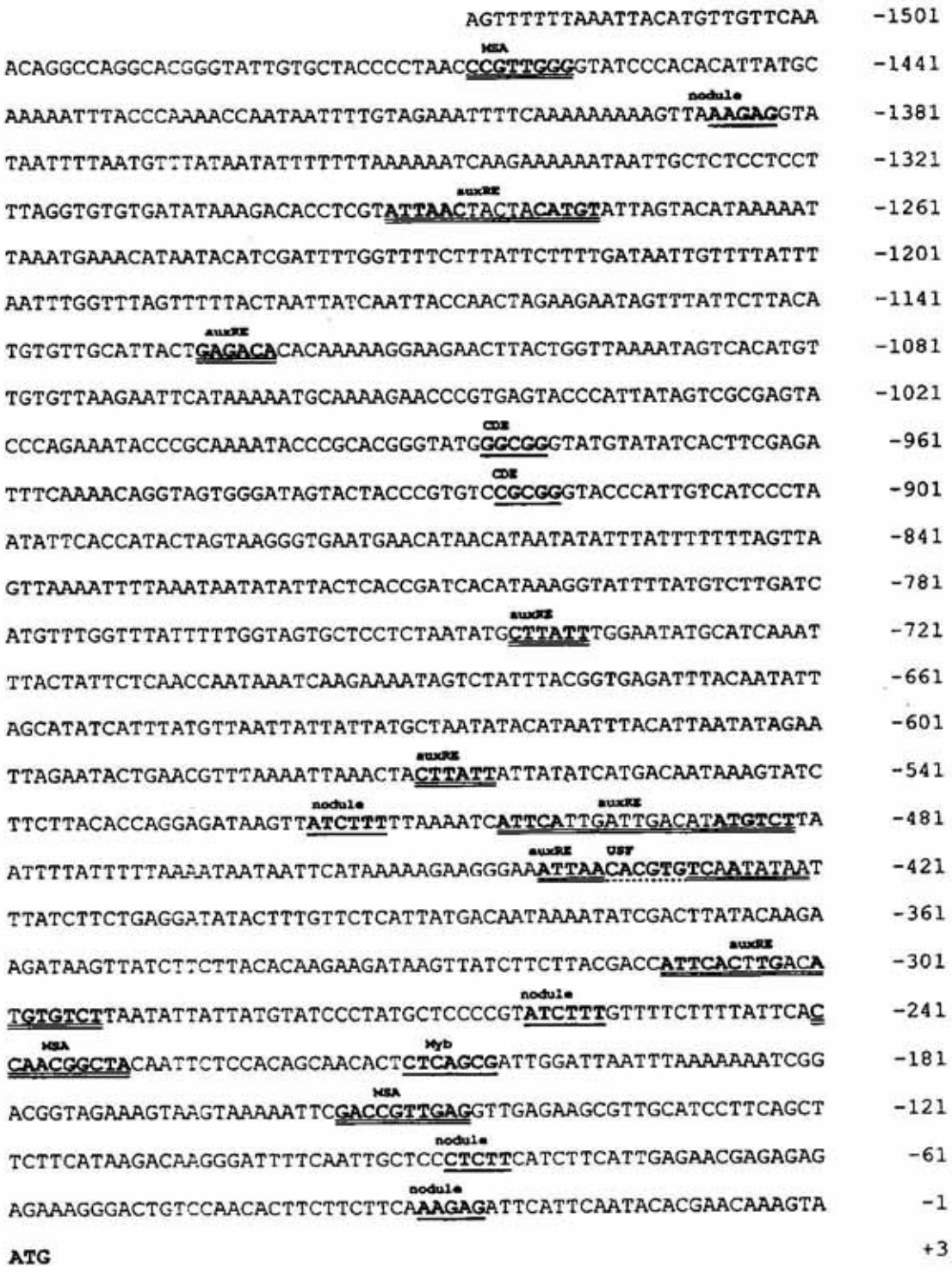

Figure 2. Sequence of Luplu;*CycB1*;2 and Luplu;*CycB1*;4 promoters.

Putative regulatory elements are in bold and underlined. MSA and auxRE are double underlined; USF, marked with dashed line. MSA, M-specific activator; Myb, sequence recognised by plant Myb homologue; auxRE, auxin response elements: TGTCTC—AATAAG and ATT(C/A)ACATGG-(C/A)(A/G)TGT(T/C)(T/C)(C/A), nodule, nodule specific elements: CTCTT and AAGAT, CDE, cycle dependent element; CHR, cell cycle genes homology region; USF, upstream stimulatory factor binding site.

quences [14-15, 34]. Binding of repressor proteins to CDE/CHR elements results in inhibition of gene expression in all the cell cycle phases except G2 and early M. Two elements consistent with CDE were found in Luplu; *CycB1;*4 promoter, however they are probably too far from ATG (over 900 bp, compared to 20-30 bp upstream start transcription in animals) to play important role in transcription regulation of lupine cyclins. 5'-Uncoding region of Luplu;*CycB1;*2 comprises two CHR motifs not far from ATG, in the vicinity of G-C rich stretches, similarly as in animal cell cycle genes. If these sequences bind repressor in Luplu;*CycB1;*2 gene, it may suggest distinct regulation of various lupine cyclin genes, since Luplu;*CycB1;*4 does not contain any CHR. Analysis of two other known plant B1 cyclin promoters (*Nicotiana silvestris* [17] and *Catharantus roseus* [18]) shows the presence of CHR close to start transcription site.

As it was determined recently, the fusion of 5'-region that control plant cyclin B expression with a coding sequence of reporter gene leads to protein synthesis specifically in G2 [35-36] and regulatory elements typical for plant cyclin genes were identified [17-18]. Consensus sequence that resembles animal c-Myb and v-Myb transcription factor binding sites found in promoters of plant genes induced in G2/M is (T/C)C(T/C)**AACGG**(T/C)(T/C)A [18]. Shown necessary and sufficient for cyclic transcription induced only in mitosis, this element was called M-specific activator (MSA). MSA acts irrespective of orientation and usually is repeated a few times. Probably such cassettes take part also in the control of cyclin expression in yellow lupine. In Luplu;*CycB1;*2 regulatory sequence four MSA elements (including two inverted) are present close to each other, not far from ATG, as in other plant promoters. Luplu;*CycB1;*4 gene possess two MSA sequences (in different orientation) in a region where they are found in other plants and one additional motif located far upstream ATG (-1430).

Plant homologue of Myb found in *Arabidopsis* [37] is likely to regulate gene expression in G2/M, but it recognises sequence CTCAGCG, different from that determined for MSA. Plant Myb homologue putative binding site was detected in Luplu;*CycB1;*4 promoter. Myb family in plants is more extensive and differentiated than in vertebrates [38] what probably reflects more numerous variants of cyclins or other cell cycle regulators and more complex control mechanisms of cell division in plants.

In animal cells transcription restricted to G2 requires both activators (e.g. USF [16]) and repressors (CDE/CHR [14-15, 34]) while in plants MSA seems to ensure cyclic expression. Moreover, MSA cassettes do not appear in animal genes that participate in cell division, and many vertebrate regulatory elements are not detected in plants, what points to distinct mechanism of expression regulation dependent cell cycle in different organisms. However, control of cyclin expression *via* repression may also take place in plants, with participation of elements such as CHR in lupine. It seems that switching on and off the transcription of genes so important in a proper cell division process cannot base only on one element i.e. MSA.

The four lupine cyclin B1 genes were shown to be induced during root nodule development [25]. Three repeats (in different orientation) of CTCTT motif found in most of nodulin genes [39] were detected in both Luplu; *CycB1;*2 and Luplu;*CycB1;*4 promoters. However, they seem to be located to close to ATG. Another element common to nodule-specific genes: AAAGAT [39] is present in normal orientation in Luplu;*CycB1;*2 and twice in inverted orientation in Luplu;*CycB1;*4, but its context differs from optimal. Although consensus binding site for NAT2 factor that participates in transcription of many nodulins was not found in regulatory sequences of lupine cyclin genes, possibly their A-T stretches may serve as targets for this factor [39]. To

reveal the role of mentioned elements in specific activation of cyclin expression in nodules, a functional analysis of lupine cyclin promoters should be undertaken.

Several plant cell cycle genes, such as coding for cdc2a kinase in *Arabidopsis* [40] and B1 cyclin in *N. silvestris* [17] which are induced by auxin, were shown to possess auxin response elements (auxRE) in their regulatory regions. Typical auxRE consists of two conserved parts separated by a few nucleotides: TGTCTC—AATAAG [41]. The second part of this motif is present in promoter of mitotic cyclin in tobacco [17] and *C. roseus* [18]. One element identical to the first half of auxRE (TGTCTC) and two inverted AATAG sequences were found in lupine Luplu;*CycB1;*4 promoter. Regulatory regions of genes induced by auxin often contain another conserved element: ATT(C/A)ACATGG-(C/A)-(A/G)TGT(T/C)(T/C)(C/A), present (with some changes) also in kinase *cdc2a* gene in *Arabidopsis* [40] and cyclin B1 gene in *N. silvestris* [17]. Similar sequence is repeated four times in Luplu;*CycB1;*4 promoter. Central part of this cassette is modified in lupine, what seems to be typical to various auxRE motifs which appear to consist of two separated parts. It is worth to note that Luplu;*CycB1;*4 expression is highly induced by indole-acetic acid (IAA), while Luplu;*CycB1;*2 gene, which does not contain any auxRE in its controlling region, is rather insensible to auxin [25]. Such a conformability of cyclin expression pattern with presence or lack of auxin response elements allows to suppose that these motifs may play essential role in transcription activation of lupine B1 cyclin genes.

The actual roles of particular control elements in the lupine genes encoding cyclins still remain to be evaluated This will require functional analysis of promoters (in fusion with reporter genes) in transgenic plants and determination of binding sites of protein factors (*via* gel shift or footprint).

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