Studies on the promoter of the *Arabidopsis thaliana cdc2a* gene

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Abstract The 5′ flanking (promoter) region of the *Arabidopsis thaliana cdc2a* gene was cloned and sequenced. A number of putative regulatory motifs were identified including one Myc and three Myb protein binding sequences plus one abscisic acid and two auxin responsive elements. One of the three Myb protein binding sequences is positioned within an *auxRE*. Promoter–GUS fusions were introduced into plants to study the role of two promoter regions in regulating gene expression. Absence of one Myb binding sequence and the *auxRE* containing a Myb binding sequence resulted in a significant reduction in expression levels as did a deletion involving the Myc and the third Myb binding sequences along with the second *auxRE*. However, no changes in expression patterns were observed. The results were quantified using transgenic root cultures.

Key words: Arabidopsis thaliana; *cdc2a* gene; Promoter; Phytohormone; Response element

1. Introduction

The $p34^{cdc2}$ protein is the catalytic subunit of a protein kinase central to the regulation of the eukaryotic cell cycle [1]. The two main points in the cycle at which $p34^{cdc2}$ function are the G1/S transition and entry into mitosis. The protein kinase becomes active only after complexing with cyclin and both negative and positive regulation of its activity has been demonstrated in yeast [1,2]. Although *cdc2* mRNA and $p34^{cdc2}$ levels remain constant during the yeast cell cycle [3], in mammalian cells the amount of mRNA fluctuates and a positive correlation exists between the level of expression and the proliferative state of cells [4,5].

p34 protein kinases have been highly conserved in evolution and found in all eukaryotes so far examined. cDNA homologues have been isolated from a number of plant species and been shown to complement *cdc2* mutants in *Saccharomyces pombe* or *cdc28* mutants in *Saccharomyces cerevisiae* [6–13].

A functional *cdc2*-homologous gene, *cdc2a*, has been isolated from *Arabidopsis thaliana* [8]. A second *cdc2*-homologous gene, *cdc2b*, whose function is still unknown, has also been found [14]. In situ hybridization showed high levels of *cdc2a* transcripts in tissues that contained actively proliferating cells such as root and shoot meristems and leaf and flower primordia [15]. Similarly, Hemerly et al. [16] found that *cdc2a* expression was strongly correlated with cell proliferation potential during development. The gene was transcriptionally regulated by mitogenic signals such as hormones, light and wounding. Expression was not always linked to cell division and in such cases may reflect competence to divide.

In this report we describe the structure of the *cdc2a* promoter and using promoter–GUS fusions, identify two regions involved in controlling *cdc2a* expression.

2. Materials and methods

An *Arabidopsis thaliana* (eco. Landeberg *erecta*) genomic DNA library cloned in the EMBL3 lambda vector was screened with a full-length (1.4 kb) *A. thaliana cdc2a* cDNA. A positive clone was isolated and subclones covering the promoter and coding regions generated and sequenced for both strands.

A number of chimeric *cdc2a* promoter–GUS gene fusions were constructed to identify promoter regions involved in gene expression (Fig. 1). A 2 kb fragment obtained by annealing the *Eco*RV–*Bam*HI (1.3 kb) and *Bam*HI–*Rsa*I (0.7 kb) fragments was cloned into the pBI101.2 Binary Vector (Clontech). A *Sal*I–*Eco*RV polylinker from the pBluescript plasmid was integrated into the 5′ end of the *Eco*RV site to facilitate cloning. The 'full-length' construct (B2) was 314 bp longer at the 5′ end than that used by Hemerly et al. [16]. The promoter–GUS fusion used by the latter workers was immediately prior to the *cdc2a* ATG. Our construct included the ATG and the junction with GUS was one nucleotide after the second intron.

In the Dm construct the *Eco*RV–*Ava*I fragment was removed, hence the length of the promoter in this construct was almost the same as that used by Hemerly et al. [16]. In the De construct the *Bcl*I–*Bcl*I fragment was deleted. Finally, in the B1 construct the *Bam*HI–*Rsa*I region was deleted. As a result only approximately 68 bp of the *cdc2a* untranslated coding region was included and unlike the other 'full-length' promoter construct (B2), the two introns were absent. A control vector that lacked promoter sequence was also constructed. Each of the constructs was introduced into *Agrobacterium tumefaciens* using a triparental mating system [17]. Transgenic plants were obtained using the root transformation method [18]. For each construct, 8–10 independent transgenic plant lines were grown on kanamycin-containing medium for three weeks before transfer to soil for seed production. T_1 progeny were used for promoter–GUS reporter gene analysis. Integration of the constructs was confirmed by using PCR. DNA extracted from transgenic plants was primed with oligonucleotides representative of the 5′ and 3′ regions of the pBI101 plasmid. Histochemical and quantitative fluorimetric GUS assays were carried out as described by Jefferson [19].

Root cultures were used in order to quantify levels of GUS expression in the different transformants. Root tips (2–3 cm long) from kanamycin (50 μg/ml) selected four week old sterile grown T_1 plants were placed in sterile culture pots containing 50 ml Gamborg's B5 medium (Sigma). Pots were incubated at 22°C with constant shaking for 4 to 6 weeks. The roots were removed from the culture by straining, rinsed with water, blotted dry, frozen under liquid nitrogen and stored at −70°C. The root cultures were homogenized in liquid nitrogen. Ground tissue (0.2 g) was resuspended in 1 ml GUS extraction buffer (50 mM sodium phosphate buffer, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2 EDTA, 0.1% sodium lauryl sarcosyl, 0.1% Triton X-100) followed by 10 min centrifugation in a microcentrifuge. GUS activity in aliquots of the supernatant was assayed at 37°C in GUS extraction buffer using 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate. Methanol (20%) was also included [20]. GUS activity was determined with a spectrofluorimeter (Titertek Fluoroskan II).

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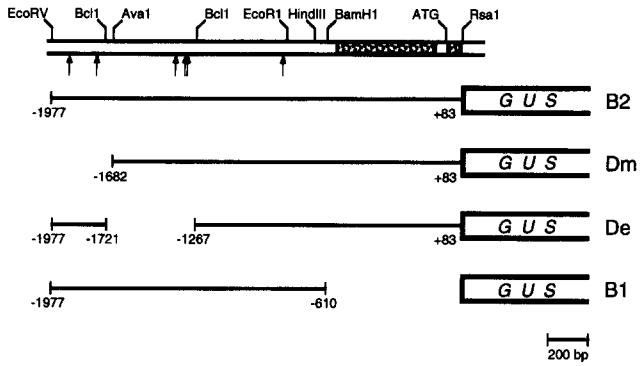

Fig. 1. Restriction enzyme map of the *cdc2a* 5′ flanking (promoter) region and part of the coding region. The blank areas represent the two introns. The four promoter–GUS fusion constructs used to transform plants are also shown. The numbers indicate base pairs 5′ or 3′ of the translation start site. Arrows indicate locations of the potential response elements shown in Fig. 2.
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Fig. 4. Histochemical localisation of GUS activity in transgenic *A. thaliana* plants. (a) Three day germinating seedling, B2 construct. (b) Three day germinating seedling, Dm construct. (c) Wounded leaf, B2 construct. (d) Wounded leaf, De construct. (b) was exposed for slightly longer than (a) to allow weakly stained root regions to be seen more clearly. Dark field optics were used in (c) and (d).
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          EcoRV
-1977  GATATCAGTTCTTTTTTTTTTGATGTTAGTTTTTTAATTATAAACTTTTTGTTAGTTAG
                          Myb
-1917  TTTTGCCAATTCCCCTACTTACAACTGATAACCGTATACCTCTTTTATCAGAAAAACTTT

-1857  ATATATCTAATCTCTCAAAATATACAAATGTGAAAAATATCTGAAAATAGATCAATTCTC
                                        AuxRE/Myb
-1797  ACCAATTATATATATTTGGTTACTTAAAAATGTTAATATATGGCAGTTGCTTGTTCAGAG
                     BclI                                    AvaI
-1737  AATAACTTGAATAAATTGATCACGAGATCGGCTTATGTTTAATATTTTTGAACTCTCGGG

-1677  ATAAAAGTTTTGAAGACATATATATCGTAGAAAAGTTTTGTGTTCTTTAATTAGTTACAA

-1617  AATTTTGAGTTTTTTTTTTCTTTCATAAAATATCAGAATATCAGGGTGATTTATTGAATA

-1557  GTTTATTTTTTCTCGTTCTACTCACGTTATTCTTGCAAGTATTAATTTTGGTTTGTTTAT

-1497  AAAAAAAGTATTTGGAAATTTTATGTGAGCAAGTGCGAAATAAACGAGGATTTTGGGAAG

-1437  TATTTTTTTTTTTGTAAAATCGGTTATATTGAACGTTATACTATATATATTATTATATAA
          Myb                                                   AuxRE
-1377  ATATAACTGCTAAAGACTAAATTTAGATACTAAAATCAGCATTGATGAAGATCCACATGA
                     Myc                                    BclI
-1317  ATAAGACTCGTCTCTTAACTCAACATTTAACATATGATTTTCATATTATTGATCATTTAT

-1257  CTTTGTGTTGGTCCTAGGTGCCAGACTTTATTATTTACATTAAATGTTGAGTTGTTTACT

-1197  GACATATTTTTTAATTAAAACAACTTTCATATTATTGCCCATTTATATTTTGGTTGGTCC

-1137  TCCAAAGACTTACAGGATACATATGTTTCTCCATTATCATTTTTATGATCTAAAACGTCT

-1077  TTTTCTAACTTAGATTCTAAATGGTTACAGCAAACCGGACATACAATTTTGCTTGTCAAA

-1017  TGGATCGTTGAGATAGACAACAATCCAATGTAAAACCAAAACTTGATTTATAATTGGTCT

-957   GCAATGATTTTTTTTTTTGGTTCATATACCATTTTATAAGATAGCAAACCATTTTCATCAT
                                                    ABRE
-897   AAAAGATATATTTTTTTATCATTGAATACCAATCAAACCCGGTATAATAAATAAAATTGT
                     EcoRI
-837   ACCAAATTAATGCGGTTAGGTGAATTCAATTTATTGGAAATTAAAGAAGAGAAAAAAAAT

-777   AACGAACAAAATTTCCTTGACACACAAAAAAAAAAAAAGAAGAAAGAAAAAACCCTTACTC
                                                    HindIII
-717   TCAATTAGTCAATCCCCTTCCCTAGTCCTCCTCTCTTCAAGCTT
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Fig. 2. Sequence of the *cdc2a* 5′ flanking (promoter) region from the *Eco*RV to the *Hin*dIII site. The sequence −832 to −674 bp has already been published [14] but is included here because of differences (4 bases) with the published sequence. The single Myc and three Myb binding sequences plus the one abscisic acid and two auxin response elements are underlined and labelled.

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AuxRE                  CACATGGNCAAGTGTTTCCA
PSIAA4/5  -283   ATTCACATGCTCATGTTTCCTCA---AAATCAACGCTCAAGATTTCGTTCTCAAA
                 :: ::::::   : :  :: ::    :: :::::  : :: :: :  :: ::: :
cdc2a     -1327  ATCCACATGAATAAGACTCGTCTCTTAACTCAACATTTAACATATGATTTTCATA
cdc2a     -1761  ...TATATGGCAGTTGCTT....
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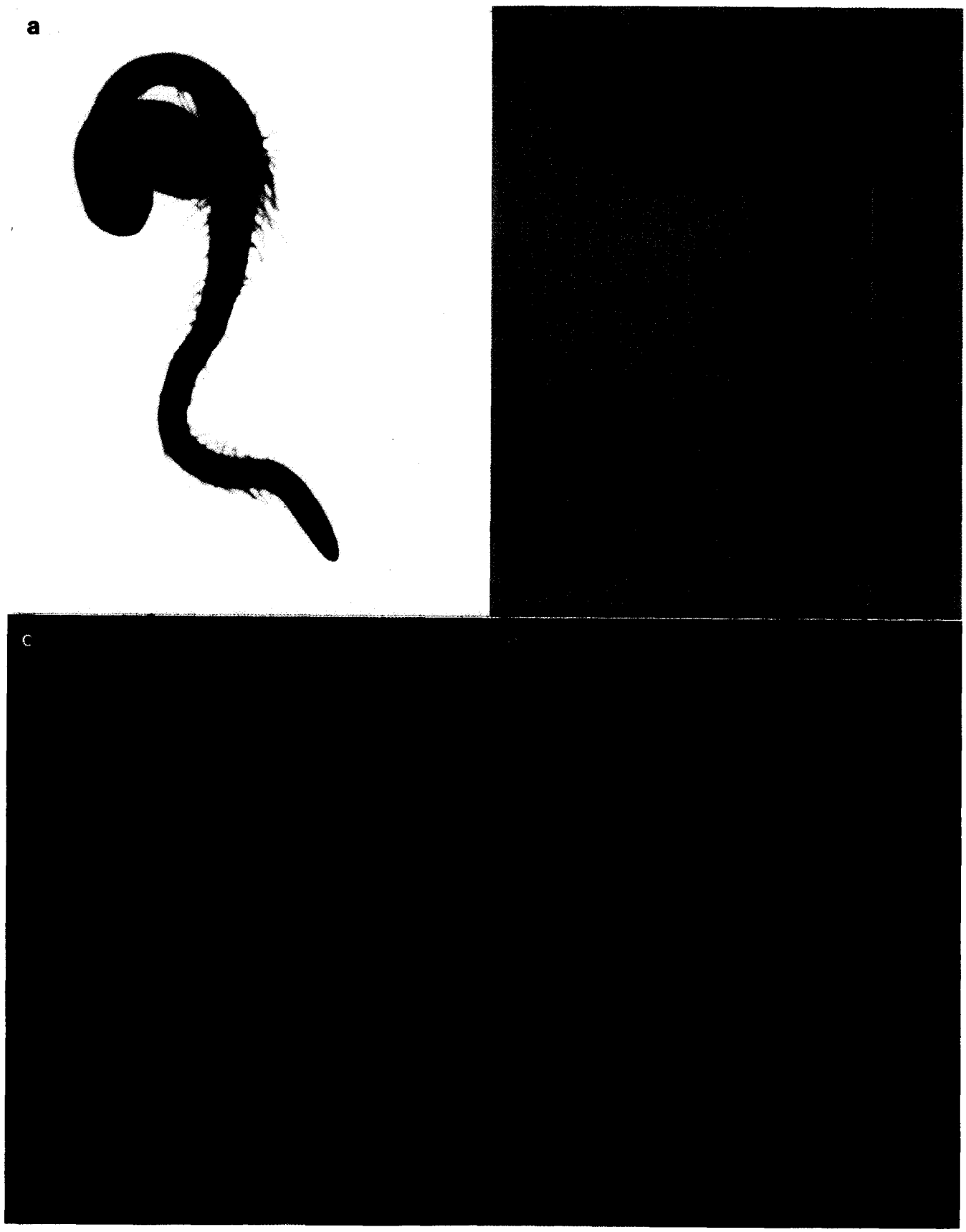
Fig. 3. Auxin responsive element-like sequence present in the promoters of the *A. thaliana cdc2a* gene and the pea *PS-IAA4/5* gene. Part of 'Domain B' of the *PS-IAA4/5* promoter which contains the *auxRE* and resembles a *cdc2a* promoter sequence is also shown. The underlined sequence is the preferred binding site for mouse c-Myb [31].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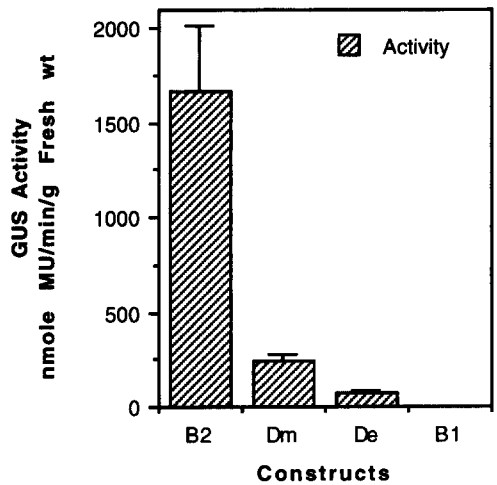

Fig. 5. GUS expression levels in root cultures transformed with four different *cdc2a* promoter–GUS constructs. Each bar represents the mean GUS activity and the standard error of the mean is shown. Four replicates of three independent transformants were used for each of the four constructs.

3. Results and discussion

The restriction map of the DNA sequence is shown in Fig. 1. The nucleotide sequence starting at the *Eco*RV site, −1977 bp from the translation start site is presented in Fig. 2. The *Eco*RV site is −1294 bp from the first nucleotide of cDNA sequence [8]. The TIS of the *cdc2a* gene has not yet been determined. We have sequenced a further 400 nucleotides upstream of the *Eco*RV site but found no open reading frames of any significance (not shown).

A number of putative regulatory elements occur in the promoter region. These include three Myb protein binding sequences. Two of these sequences (CAACTG and TAACTG) are −1896 bp and −1374 bp from the translation start site. The third sequence (CAGTTG) occurs at −1754 bp and is in fact the former sequence in the reverse orientation (i.e. on the opposite DNA strand). The human *cdc2* gene contains two closely spaced c-Myb binding sites in a region extending from nucleotides −410 to −392 upstream of the transcription initiation site and transfection experiments indicate c-Myb activates expression of the *cdc2* gene [21].

Expression of *cdc2a* in *A. thaliana* is regulated by various hormone treatments [16] and the promoter contains both abscisic acid and auxin response elements. A putative ABRE in the *cdc2a* promoter (AACCCGG) which occurs at −862 bp from the translation start site was recently identified in the resurrection plant *Craterostigma plantagineum* and while essential for ABA responsiveness in that plant, additional promoter elements appear to also be required [22]. Two sequences in the *cdc2a* promoter (Fig. 3) resemble a highly conserved sequence found in various auxin-regulated genes including the pea gene, PS-IAA4/5 [23,24]. One of these sequences includes the preferred binding site for mouse c-Myb (Fig. 3). The putative auxin response element is included in the so-called 'Domain B' of the PS-IAA4/5 gene. A 55bp region in the *cdc2a* promoter has 34 nucleotides identical with Domain B (Fig. 3). Domain B is thought to contain an enchancer-like element affecting both the auxin-inducible and constitutive expression of the pea gene. The *auxRE* at −1318 bp in the *cdc2a* promoter contains the sequence AATAAG (Fig. 3) which is part of two auxin-inducible elements of the soybean *9H3* gene promoter [25]. Six base pairs further downstream a *c-myc* element occurs (TCTCTTA) [26]. The B and R proteins of maize possess the basic–helix–loop–helix motif found in Myc proteins and are thought to recognise a *myc* consensus binding site in the promoter of the *Bz1* gene [27].

The spatial and temporal GUS expression was similar in plants transformed with the B2, Dm and De constructs, however the intensity of staining varied. As expected, expression patterns and staining intensity in Dm plants were identical to those described by Hemerly et al. [16] (Fig. 4b). However, increasing the promoter length by 314 bp (B2 construct) led to much stronger expression and this is clearly seen in germinating seedlings (Fig. 4a). The root meristem is intensely stained in both Dm and B2 transformed plants, however staining of the pericycle and particularly the parenchyma of both the vascular cylinder and the cortex is much stronger in the B2 transformants. Similarly, the cotyledons are more intensely stained. De transformed plants stained more weakly than Dm plants although again the expression patterns were not altered.

As previously described [16], GUS activity was induced by leaf wounding (Fig. 4c). The intensity of the response was greatly reduced in De plants (Fig. 4d).

No GUS staining whatever was detected in the roots or cotyledons of B1 transformed plants (not shown). The apical meristem was stained as were the developing first leaves, although staining was weaker than that obtained with the other constructs. However, normal staining did occur in developing flowers.

The results using root cultures show that truncation of the promoter to the *Ava*I site causes a significant reduction in GUS expression in the roots (Dm construct, Fig. 5). Deletion of the *Bcl*I–*Bcl*I fragment (De construct) caused an even greater reduction in GUS expression. Histochemical studies showed the root meristem was strongly stained for GUS activity in all three transgenic root cultures, reduction in expression correlating with weaker staining in the root parenchyma and pericycle (not shown). No GUS expression was detected in roots transfected with the B1 construct (not shown).

The reasons for the unusual GUS expression patterns in B1 plants are not clear. The GUS was fused to an untranslated region of the *cdc2a* gene whereas in the B2 construct a translational fusion to the third exon was made. The B1 expression patterns may be artifactual but raise the possibility that non-translated sequences in the mRNA or introns are involved in regulating *cdc2a* expression. Perhaps an enhancer occurs in one of the introns which in conjunction with the 5′ upstream non-coding sequences is required for normal expression patterns. The 5′ upstream regions of the *GL1* gene, for example, fail to induce trichome expression unless an enhancer from the 3′ non-coding region is also included in the GUS construct [28]. Deletion studies on the *cdc2a* ×610 to +83 region will be required to clarify this point.

The precise role of phytohormones on *cdc2a* regulation will be difficult to ascertain until endogenous levels and rates of synthesis are taken into account when interpreting the effects of exogenous hormones [29]. Intact roots, for example, respond differently from excised roots to hormone treatments [16]. The fact that *cdc2* mRNA appears in pea roots after only ten min-

utes of auxin treatment does suggest auxin is the main hormonal inducer of *cdc2* expression [30]. So it is interesting that sequences resembling known auxin response elements have been deleted from the De construct. However, single Myb and Myc protein binding sequences are also present in the deleted sequence. Although distant from the TIS, the *Eco*RV–*Ava*I promoter region also influences the level of expression and contains two Myb protein binding sequences. We are studying the role of these various elements in *cdc2a* expression.

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