

Short polypyrimidine/purine sequences modulate tissue-specific control of a cell cycle variant alfalfa histone H3 gene promoter

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Abstract

Plant and animal cells produce a form of histone H3 protein that is deposited in the chromatin in a cell cycle-independent manner. Genes for such replacement histone H3 are highly and constitutively expressed despite the presence of replication-associated histone promoter elements. Previously, we have shown that polypyrimidine/polypurine (PPY/PPU) sequences in replacement histone H3 promoters and introns may be required for this constitutive expression. Here, we report that gel mobility shift experiments detect proteins in plant nuclear extracts that can specifically interact with double and single stranded PPY/PPU elements located in the 5' untranslated region (UTR) of alfalfa replacement histone H3.2 genes. GUS reporter gene analyses of transgenic tobacco seedlings demonstrate that insertion of parts of these sequence elements into the 5'UTR of a replication variant alfalfa histone H3.1 gene results in H3.1 promoter activities that are no longer confined to tissues where replication of DNA occurs. Similar spatial distribution of activity was observed when the replacement variant histone H3.2 promoter was used as a control. We speculate that the PPY/PPU-rich elements of replacement H3 genes may alter chromatin structure and may have been a critical factor in the emergence of replacement H3 histone genes.

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1. Introduction

While the expression of most histone genes is coupled to DNA synthesis, a distinct type of histone H3 gene exists in plants and animals. It differs from the common, replication-specific histone H3 genes, which in animals lack mRNA polyadenylation, by a cell cycle-independent expression, the presence of introns, a polyadenylated transcript, and a few specific amino acid changes in the protein [1]. Since this histone H3 gene is also active in differentiated tissues, its protein product replaces the more common, replication-

dependent histone H3 forms in the assembly of new nucleosomes across transcriptionally active gene sequences. Because of this feature, this variant has been named a replacement histone [2].

In plants, the abundant replication variant histone H3 genes (H3.1) show high expression levels in cycling cells such as meristems. Replacement variant histone H3 genes (H3.2) are transcribed in most plant tissues [3,4], and show developmental stage-specific expression in alfalfa somatic embryos [4]. This “constitutive” expression occurs despite the fact that promoters of all H3.2 genes sequenced so far contain sequence elements that link expression of histone genes to DNA replication [3,5,6]. The main difference between the H3.1 and H3.2 genes seems to be the presence of introns in the H3.2 variants [1,3,5,6]. The characteristic intron is located inside the 5' untranslated region (UTR) of the gene [1]. Recently, we have shown that insertion of PPY/PPU-rich introns from an Arabidopsis H3.2-type gene into the 5'UTR of an Arabidopsis histone H3.1 gene completely

Abbreviations: PPY/PPU, polypyrimidine/purine; UTR, untranslated regions; GUS, β -glucuronidase

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abolished meristem-specific promoter activity [7]. This result has suggested that 5'UTR introns, or sequence elements contained within, cause constitutive expression, typical of the H3.2 type genes in plants. The alfalfa histone H3.2 genes appear to be exceptions since they contain no intron in the 5'UTR, yet their expression is also constitutive [4,6].

Instead of an intron, the 5'UTR of all alfalfa H3.2 genes known contains one to three repeats of a conserved PPY/PPU element (Fig. 1), with limited sequence similarity to PPY/PPU stretches in the H3.2 Arabidopsis 5'UTR introns. Embedded in each of these alfalfa sequence blocks is one copy of the motif CTCTC/GAGAG, known to be the cognate sequence for the GAGA binding protein in soybean and for GAGA factor in *Drosophila* [8–11]. In the present work, using competition gel shift analyses, we have shown that in crude nuclear extracts of alfalfa and broccoli, proteins exist that specifically interact with CT-rich sequence elements in histone H3.2 genes. Also, insertion of CT motifs into the 5'UTR of a cell cycle variant alfalfa H3.1 gene results in GUS expression that is no longer confined to regions that consist of cycling cells in tobacco roots. A hypothesis is discussed how PPY/PPU-binding proteins may modulate gene expression in plants.

2. Materials and methods

2.1. Preparation of plant nuclear extracts

Plant nuclear extracts were prepared as described [12] and were provided in part by Drs. Julio Saez-Vasquez and Craig S. Pikaard at the Biology Department, Washington University, St. Louis, MO. The protein concentration was measured, using a Bradford BioRad Protein Assay kit with bovine serum albumin as standard.

2.2. Competition gel mobility shift analysis

The oligonucleotide probe in this study was prepared by annealing 30-mer oligonucleotide 5'-TTTCCCTCTCTTCGAGCTTCTCTCTCCGA-3' with its 27-mer complement, lacking the three 3' A's, and filling in the 5' protruding end with Klenow polymerase in the presence of [³²P]-dATP. As a specific competitor, annealed oligonucleotides, prepared as described above without labeling, were used. As a non-specific competitor, we used two different double stranded oligonucleotides. nsp1 consisted of the sequence

5'ACACGCCACGTCATCGATCCGCGTGTCCGAT3', nsp2 of the sequence 5'TTTCCCGCGCTTCGAGCTTTCGCGCGCCGA-3' (complementary strands are not shown). This latter competitor was identical to the probe except that replacement of pyrimidine T's by G's interrupts alternating CT motifs at positions shown above.

The competition mobility shift assays were carried out as described [13]. Nuclear extracts from alfalfa and broccoli with 4 µg protein in 4 µl were pre-incubated on ice for 10 min with 1.5 µg of poly(dI-dC) (Roche Molecular Biochemicals, Indianapolis, IN) as a generalized competitor in the presence of 25 mM Tris-HCl pH 7.6, 50 mM KCl, 0.5 mM EDTA, 2.5 mM DTT, 5% glycerol and 1 mg/ml acetylated bovine serum albumin (New England Biolabs, Beverly, MA) in a total volume of 10 µl. Following pre-incubation, labeled probes were added, supplemented with specific or non-specific competitor oligonucleotides, as indicated, and incubated for 15 min on ice. The pattern of oligonucleotide mobility shift changes, induced by nuclear extract factors, was analyzed by electrophoresis at 4 °C in 4% polyacrylamide gels (ratio of acrylamide to bisacrylamide monomers of 80:1) in 0.5× TBE [14], gel drying and autoradiography.

2.3. Plant expression cassette

All DNA cloning and manipulations reported here were performed according to standard protocols [14]. DNA constructs were created by inserting varying numbers of the double stranded oligonucleotide 5'-gtacCTCTC-ggCTCTCgtac-3' into the *Kpn*I site located between the AIH3.1-1 gene promoter [15] and the start code of GUS in the AIH3.1-1-GUS pLP100 construct [16]. Subsequently, the *Hind*III-*Sac*I fragments containing these expression cassettes were removed from the pLP100 plasmid and were used to replace the corresponding fragment in pBI101 [17]. As a control, the promoter of the replacement variant histone H3.2 gene clone msH3g1 [6] was used also in pBI101. The nucleotide sequence of each of these constructs was verified by dideoxynucleotide sequencing [14]. In the present work, the AIH3.1-1 promoter is simply referred to as the H3.1 promoter.

2.4. Tobacco transformation

Binary vector constructs were introduced into *Agrobacterium tumefaciens* strain GV 2260 [18] by the freeze-thaw method [19]. Leaf discs of *Nicotiana tabacum* cv SR1 were

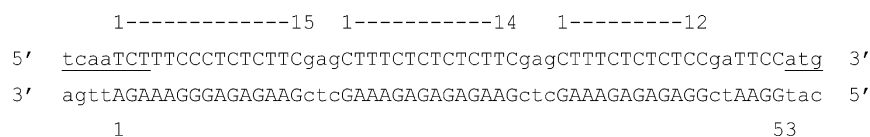


Fig. 1. The 5'UTR of alfalfa histone H3.2 cDNA clone pH3c110 (GenBank U09460). The transcription cap site and ATG start codon sequences at the 5' and 3' termini are underlined. The polypyrimidine/purine elements are capitalized and numbered.

transformed and plants regenerated as described previously [16]. The number of gene copies incorporated into the genomes was assessed by standard Southern analyses with a GUS-specific probe.

2.5. Tissue staining for GUS activity

Tobacco seed germination and culture of seedlings were performed under sterile conditions. Histochemical GUS staining was carried out overnight at 37 °C in tissue culture plates as described [20]. After staining, seedlings were fixed in 3% formaldehyde–100 mM sodium phosphate buffer, pH 6.8 for 3 h at room temperature. Tissues were cleared with several washes of 70% ethanol [21]. Photomicrography was performed using a LeicaMZ APO stereomicroscope equipped with a NikonDXM 1200 camera, and a Zeiss Photomicroscope II connected to a Canon PowerShot G5 digital camera.

3. Results and discussion

Our phylogenetic analysis [1] and earlier studies in transgenic Arabidopsis and tobacco have suggested that PPY/PPU sequences, which occur at high frequencies in replacement histone H3 genes, are involved in the control of gene expression, possibly at the transcriptional level [7]. PPY/PPU sequences can exert their effect in various ways. CT-rich sequences are prone to the formation of intramolecular triplex DNA structures [23], which can affect gene expression directly [22–24]. CT repeats adopting non-B DNA structures may serve as alternative transcription start sites [25]. Our attempts to detect the occurrence of triplex DNA in the full-length 5'UTR histone H3 cDNA clone pH3c110 clone (Fig. 1) by digestion with single strand-specific nucleases have consistently failed [6]. Ribonuclease protection assays also failed to detect alternative transcription sites in the H3.2 promoter in alfalfa cells (results not shown). Thus, it seemed important to analyze whether PPY/PPU sequence motifs in plant histone H3.2 genes are targets for specific DNA–protein interactions. To this end, competition gel mobility shift assays were carried out using crude nuclear extracts from an alfalfa (*Medicago varia* Rambler A2) cell line [4]. In addition to A2 callus suspension culture, we also used nuclear extracts from broccoli plants (*Brassica oleracea*) for the detection of PPY/PPU-binding proteins. Broccoli inflorescence has been shown to be an abundant source for producing high quality plant nuclear extracts [12]. In our competition gel shift experiments, α [³²P]-dATP labeled double stranded probe was used which consisted of a major portion of the 5'UTR of the alfalfa H3.2 clone called msH3g2 (see Section 2) [6]. The specific competitor was identical to the probe, but was unlabeled. As a non-specific competitor, first we used an oligonucleotide termed nsp1. It was the same length as the probe, but had very little sequence similarity (Fig. 2).

The result of a representative competition mobility shift assay is shown in Fig. 2A and B. The nuclear extracts of both alfalfa and broccoli contain proteins that form at least three groups of complexes. This suggests that multiple factors or complexes of proteins could interact with the 5'UTR of the alfalfa histone H3.2 promoter in vivo. The differences in binding pattern with the broccoli nuclear extract and extracts from nuclei of dedifferentiated alfalfa callus cultures suggest that plant species or tissues are likely to differ in PPY/PPU-specific binding proteins and/or in their associated complexes. If specific double stranded competitor oligonucleotides, identical in sequence to the labeled probe, were present in 200-fold molar excess, complex formation with labeled probe was prevented (Fig. 2, lanes e and n). The same amount of non-specific oligonucleotide nsp1 (see Section 2) had no effect, an indication that the protein–DNA interactions detected are specific (Fig. 2, lanes f and o).

Polypyrimidine-binding proteins have been described that bind to promoter CT sequences in chicken with high specificity for single stranded DNA and no detectable affinity for double stranded templates [25]. In contrast, GAGA factor of *Drosophila* has been shown to bind both double and single stranded DNA with the highest affinity for the polypurine GA-rich strand [26]. To test whether PPY/PPU-binding factors targeting histone H3 genes in plants also interact with single stranded DNA, we used each strand of the UTR probe separately as a specific competitor in gel mobility shift experiments. When the CT-rich DNA strand was included in the reaction, only complex group 2 was effectively prevented (Fig. 2, lane c). Also, some components of group 3 were affected (Fig. 2, lane c). However, the complementary, GA-rich, single stranded DNA (Fig. 2, lane d) was as effective as the double stranded specific competitor (Fig. 2, lane e) in preventing formation of all three groups. In the broccoli extract, both CT and GA strands could compete out groups 4 and 6, as well as components of group 5 (Fig. 2, lanes l and m, respectively). The GA strand was somewhat more effective competitor for protein DNA complexes in group 5.

The 5'UTR of alfalfa replacement H3.2 genes represented by the probe used in our experiments contain short alternating CT repeats [6]. In order to evaluate whether these sequence motifs are target sites for specific DNA protein interactions, we used 200X molar excess of a mutated CT oligo, called nsp2, in which the CT repeats of the probe DNA was changed to alternating CGs. As shown in Fig. 2, lanes i and r, nsp2 prevented formation of some of the DNA protein complexes in both alfalfa and broccoli extracts, while others were barely affected, as marked by asterisks in Fig. 2, lanes i and r.

The above experiments suggest that, under the experimental conditions used, nuclear proteins from the two plant sources have the capability of specifically interacting with the PPY/PPU-rich 5'UTR of a replacement variant H3.2 gene, both as a single or double stranded DNA. Furthermore, some of these proteins specifically target short alternating

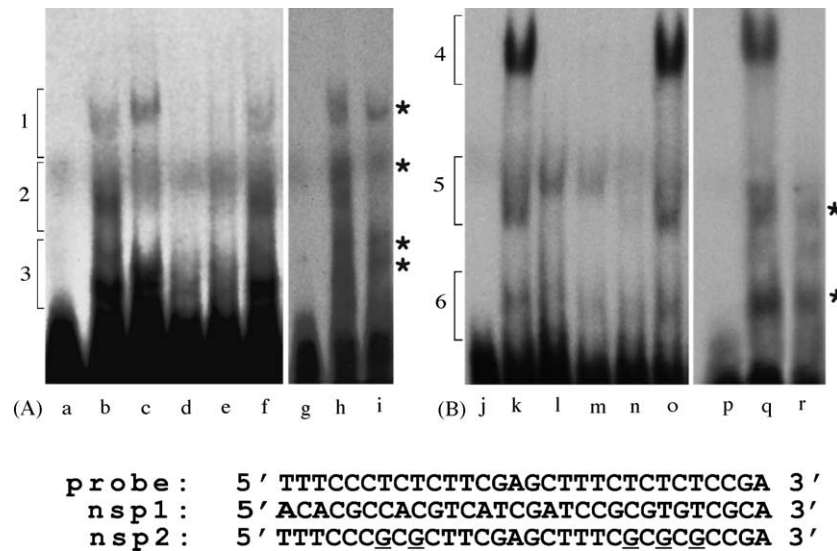


Fig. 2. Gel mobility shift assay with 5'UTR competitors. Radio-labeled, double stranded DNA oligonucleotide containing most of the 5'UTR motif of the msH3g1 clone, was electrophoresed with alfalfa (Panel A) and broccoli nuclear extracts (Panel B) in the absence of competitor oligonucleotides (lanes b, h, k, q). Binding reactions contained 200-fold molar excess of double stranded unlabeled DNA identical to probe (lanes e and n). Groups of mobility shift complexes that were reproducibly detected are numbered 1–3 for alfalfa and 4–6 for broccoli. The majority of unbound oligonucleotide probe ran below the boundary of the figure. The same, labeled probe was also was electrophoresed without extract (lanes a, g, j, p); with a 200-fold molar excess of competitor nsp1 (lanes f and o); the CT-rich single strand (lanes c and l) and AG-rich single strand of 'probe' (lanes d and m). In lanes i and r, nsp2 is used as a mutated CT oligo competitor.

CT motifs. The size of DNA binding complexes detected in our gel shift experiments shows large variations. However, UV cross-linking followed by size fractionation by ultrafiltration have indicated that the binding complexes are less than 100 kDa in size (results not shown).

In order to assess how DNA binding proteins that target short CT repeats in the 5'UTR may affect histone gene expression *in vivo*, we inserted varying numbers of the oligonucleotide 5'CTCTCgCTCTC3' into the 5'UTR of an alfalfa replication variant histone H3.1 promoter immediately upstream of the ATG code for the β -glucuronidase (GUS) reporter gene [15,17]. Previously, activity of this histone H3.1 promoter has been shown to be confined to meristematic tissues in transgenic tobacco plants [16]. The H3.1 and recombinant H3.1 promoter GUS expression cassettes with one to three tandem oligonucleotide insertions were placed in the binary plant transformation vector pBI101, and were introduced into tobacco by *Agrobacterium*-mediated transformation. As a control, we used the promoter of the replacement variant histone H3.2 gene clone msH3g1, in transcriptional fusion with GUS, also in pBI101.

In order to study the location of promoter activities of these constructs, we performed histochemical GUS staining on at least 10 seedlings from each of the 4 or 5 independent tobacco lines for 1, 2 and 3 numbers of insertions, respectively. In each experiment, seedlings were stained at same time, under the same conditions, and for the same duration of time. Plantlets harboring a given plasmid showed similar staining patterns with some variations. In the case of the H3.1 GUS chimera, the expression pattern was identical to that found previously [16], consistent with the nature of replication variant histone genes in plants. In the root, the

H3.1 promoter produced strong GUS staining primarily in the region of cell division, including the apical meristem and cell layers above it (Fig. 3A/3). Weaker, but consistent GUS staining also occurred in the vascular cylinder in the elongation and maturation zone. In the shoottip, the lower part of the apical dome showed the strongest GUS staining (Fig. 3A/1). Blue precipitate was barely noticeable in the stem except for slight staining in the vascular tissues (result not shown). GUS expression in the cotyledons, and leaves was also observed with relatively low color intensities (Fig. 3A/2). Interestingly, guard cells showed marked GUS expression (see inset in Fig. 3A/2).

Insertion of the CT motifs into the H3.1 promoter caused a major change in the location of GUS expression. The GUS staining pattern for these constructs was similar, however, the promoters with two and three copies of the CT motif showed stronger intensities. A representative sample of plant lines with three oligonucleotide insertions is shown in Fig. 3, column B. In addition to areas where the unmodified H3.1 promoter was active, the recombinant H3.1 promoters also produced strong GUS staining in the roots' differentiation region (Fig. 3B/3) all the way up to the root–shoot transition zone (result not shown). Also, in the leaves, and cotyledons, pronounced blue precipitate formation was observable with strong expression in guard cells (Fig. 3B/2). In the shoot apex, the GUS expression pattern was different from that in the H3.1 seedlings. The entire apical dome showed strong blue staining (Fig. 3B/1). No major increase in intensity or change in topology of GUS staining was observed in the lower parts of the stems (result not shown). The expression pattern from the recombinant H3.1 promoter was similar but not identical to that from the H3.2 promoter in the same

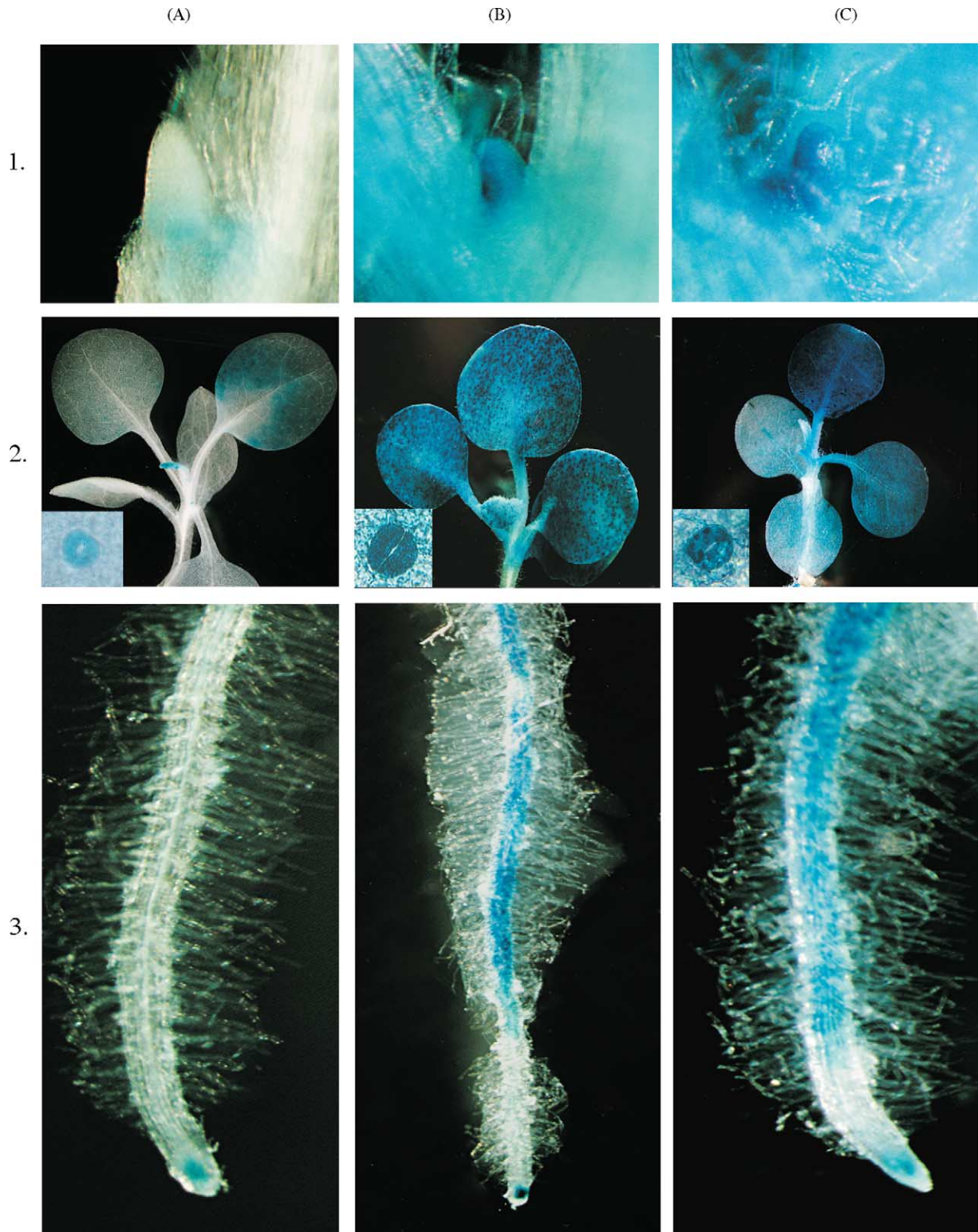


Fig. 3. An oligonucleotide containing the 5'CTCTCggCTCTC motif were inserted in tandem into the 5'UTR of a cell cycle-controlled alfalfa histone H3.1 gene. The unmodified H3.1 (column A), the recombinant H3.1 (column B), and, as a control, the replacement variant H3.2 gene promoter from clone msH3g1 (column C) were fused with the β -glucuronidase gene and transformed into tobacco. Histochemical GUS staining was performed on transgenic seedlings to analyze spatial distribution of GUS activity driven by the histone promoters. In Panel B, a seedling containing three tandem oligonucleotide insertions in the H3.1 promoter is shown: (1) shoot apex; (2) cotyledons and leaves; (3) lower part of roots. Insets in line 2 show guard cells in cotyledons.

vector background (Fig. 3, column C). Also, the latter promoter produced consistently faster accumulation of the blue precipitate particularly in the roots of tobacco seedlings (result not shown).

The experiments presented here support the notion that control of the expression of the replacement variant alfalfa H3.2 genes may be associated with PPY/PPU sequence repeats located in the 5'UTR.

How PPY/PPU-binding factors might change a replication-dependent histone gene into a gene that is continuously active is currently a matter of speculation. We consider the possibility that PPY/PPU-binding proteins in plants might act similar to GAGA factor in *Drosophila*, which recognizes the polypurine core GAGAG [10,26] and is capable of preventing nucleosome formation, and in the presence of ATP, of displacing nucleosomes [9,27]. This process would provide access for transcription factors to their DNA binding sites and reverse the strong repressive folding of nucleosomes. Currently, we have no direct evidence that PPY/PPU factors in plants cause chromatin derepression, but our results in transgenic plant experiments are consistent with this possibility. PPY/PPU sequences and their binding factors by themselves, however, do not seem to suffice to recreate the high promoter activity of the alfalfa H3.2 genes. Deletion of sequences upstream of the TATA box greatly reduced reporter gene expression from the alfalfa H3.2 promoter, despite the presence of PPY/PPU elements in the 5'UTR (result not shown). A similar requirement for cis promoter elements was also observed in transgenic *Arabidopsis* experiments [7]. This points to the fact that an open chromatin structure may be a necessary but not sufficient requirement for strong, constitutive gene transcription.

While database searches have failed to find plant homologues to *Drosophila* GAGA factor, unrelated proteins that bind to $(GA)_n/(CT)_n$ sequences appear to be present in many plant species. One such protein involved in binding to promoter elements of the soybean heme and chlorophyll synthesis gene *Gsa1* has been described [8]. At present, it is not known whether this soybean protein is related to that which binds to plant replacement H3.2 genes. The possibility exists that the same DNA binding module is utilized for the regulation of both types of genes. At any rate, the abundance of PPY/PPU sequences in the plant genomes, and the demonstrable effect these motifs can exert on promoter activities, warrant further investigations into the mechanism by which PPY/PPU-binding proteins may modulate gene expression in plants.

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