Ph. D thesis

(short version)

Regions controlling the expression of the alfalfa histone H3 gene: studies in transgenic plants

Written by

Zsolt Kelemen

Supervisors
Dr. János Györgyey
Professor Dr. Dénes Dudits

Institute of Plant Biology
Biological Research Centre
Hungarian Academy of Sciences
Szeged, Hungary

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Introduction

While most histone genes display a marked dependence of gene expression on the concurrent synthesis of DNA during the S phase of the cell cycle, a distinct type of histone genes exists in plants as in animals. These type of histone H3 genes are characterized by a cell cycle-independent pattern of expression, the presence of introns in the gene and a few specific amino acid changes in the protein, relative to the common, replication-specific histone H3 forms. It participates in the assembly of new nucleosomes across gene sequences that have lost nucleosomes as a consequence of gene transcription. Since these histone variants replace the more common, replication-dependent histone forms in terminally differentiated tissues, they have been named replacement H3 histones.

In alfalfa, three replacement histone H3 genes exist per haploid genome. These three genes produce two times more new histone H3 proteins than the more than 50 replication-dependent histone H3 genes do together in logarithmically growing suspension cultures of alfalfa A2 cells.

Promoters of all known plant histone genes share many common elements, irrespective of the pattern of gene expression. Promoter elements which drive S-phase specific expression of cell cycle-regulated histone genes in plants are present in the replacement histone H3 genes of alfalfa and Arabidopsis, but S-phase histone H3 genes fail to contain introns. The characteristic intron of replacement histone H3 genes is the one located mainly inside the 5’ untranslated region (UTR) of the gene. The only known exceptions are the alfalfa histone H3.2 genes. However, the 5’UTR of alfalfa H3.2 genes appears to have retained the functional elements of the 5’UTR introns that confer constitutive expression. In contrast to the replication-dependent H3 genes polypyrimidine elements are present in high abundance in the 5’UTR and introns of the alfalfa H3.2 genes and in the introns of the two replacement H3.III genes of Arabidopsis, similarly to the animal replacement H3 genes.

Methods

Plasmid constructs were created using standard recombinant DNA technics. Transgenic tobacco plants were regenerated via *Agrobacterium tumefaciens* mediated transformation procedures. Transient expression assays were carried out on isolated plant protoplast. Transgene expression were analysed by *northern* blot hybridisation on the transcript level. GUS enzymatic activity were determined using fluorometric GUS assays and histochemical GUS staining.

Results

Despite the fact that the *Medicago* histone H3.2 genes contain promoter elements, characteristic to replication-dependent histone genes *northern* analyses have clearly shown a constitutive mode of expression for these genes, with steady state mRNA levels far exceeding those of the replication-dependent H3.1 genes. In order to evaluate whether these mRNA levels reflected promoter strength, we have analyzed tobacco plants carrying a 424 bp promoter fragment of alfalfa replacement histone H3.2 gene msH3g1 in a transcriptional fusion with the GUS reporter gene. As a control, we used pBI121, which contains the GUS gene driven by the cauliflower mosaic virus (CaMV) 35S promoter.

GUS activities were determined in the extracts from old leaves, i.e. longer than 5 cm. Such leaves contain few actively dividing cells, as reflected by the low promoter activity of a cell cycle variant histone H3.1 gene. Here, fluorimetric GUS analysis of the transformants revealed high GUS activities for the H3.2 promoter construct. On average, GUS activity in plants transformed with CaMV-35S promoter construct pBI121 was 5-fold higher. In order to analyze spatial distribution of GUS activity, that is, to test whether the histone H3.2 promoter possesses tissue specific activity, GUS histochemical staining of transgenic tobacco plantlets was carried out. Strong GUS staining resulted in most parts of the plants containing either construct.

In order to analyze the influence of further sequence elements on the expression of the histone H3.2 promoter we have built further expression cassettes beyond the pHEX-N::GUS vector. The pHEX110::GUS construct contains, in addition to the H3 promoter, the 120 bp first intron of the MsH3g1 genomic clone inserted between the start sites of transcription and translation, and the NOS 3′ UTR and polyadenylation sequences. In construct pHEX111::GUS the NOS sequence was replaced by the 3' UTR of the MsH3g1 gene.

To assess the activity of these constructs, alfalfa A2 cells were protoplasted and transformed. As reference, cells were transformed with expression plasmid pIDS211 with the GUS reporter gene driven by the 35S CaMV promoter. In three independent experiments, measuring GUS enzyme levels on days 2 and 3, the replacement H3 promoter in pHEX-N::GUS appeared six-fold stronger than the 35S promoter in the control pIDS211. For highest expression the intron sequence was required, increasing GUS levels an additional four-fold. Replacement of the NOS terminator by the 3′ UTR of the MsH3g1 clone in pHEX111::GUS reduced GUS activity three-fold.

Based on the highest expression of pHEX110::GUS in transient expression, it was further studied in stable transformation of tobacco.Second generation plants were analyzed for GUS expression. Data from HEX110 transformants were compared with the CaMV 35S promoter pBI121 controls. The HEX-110::GUS transformants produced GUS expression that was, on average, three times higher than observed in pBI121 controls.

It was of interest to assess whether the variability in GUS activity reflected steady-state GUS transcript levels. *Northern* analyses were carried out on total RNA from second generation of transformant plants and GUS mRNA signals indeed mirrored the variability in GUS activity levels.

The spatial distribution of GUS activity was determined by histochemical staining of tobacco plantlets for GUS. Strong GUS expression was observed in all parts of plantlets transformed with HEX-110, consistent with the general, high, constitutive expression of the endogenous replacement H3.2 genes. It was quite similar to the distribution of GUS expression, driven by the viral 35S promoter.

The alfalfa histone H3.1 promoter has been demonstrated to be an effective promoter for GUS expression, retaining the replication dependence of the promoter with clear preference for expression in the meristems. Insertion of CT-rich sequence

elements between this promoter and GUS coding sequnce in transformation plasmids caused major quantitative and qualitative changes in GUS activity. The CT-rich sequences were capable of increasing the strength of the H3.1 promoter. In addition, the meristem specificity of the cell cycle-dependent promoter was diminished.

Discussion

We have shown that:

1. – the alfalfa H3.2 promoter itself is a strong, constitutive promoter, drives a high GUS expression in all the parts of the plant.
2. – the insertion of the intron sequence enhanced further the strength of the promoter, the alfalfa replacement H3 promoter-intron cassette has been shown to produce high, constitutive transgene expression in all plant tissues at levels exceeding 35S CaMV promoter strength.
3. – the CT-rich sequences were able to cause the loss of preferential meristematic expression of the S-phase specific alfalfa histone H3 promoter.
4. We have designed high efficiency plant transformation construct that does not contain viral sequences and might prevent gene silencing mechanisms that act by chromatin repression.

PPY/PPU sequences could function through protein recognition of specific base-pair sequences or the local DNA conformation conferred by them, or through the formation of intramolecular triplex DNA structures which appear to affect gene expression directly. The mode, by which these factors might have changed a replication-dependent histone gene into a gene that is continuously active, is still unknown. Nevertheless, we considered the paradigm of GAGA Factor in Drosophila as a possible explanation. GAGA proteins recognize the polypurine core sequence GAGAG, irrespective whether present in the coding or non-coding DNA strand. These motifs have quite a degree of sequence variability, while retaining strong and specific binding as measured by gel mobility shift assays and DNase I footprinting. Analyses by Carl Wu and others have revealed the way GAGA factors act as a transcriptional activator. They have shown that GAGA Factor (1) prevents

nucleosome formation and (2), in the presence of ATP, displaces nucleosomes. GAGA Factor binding to intron-bearing replacement H3 genes in animals has been predicted to reduce the nucleosomal density to 61 percent, fully reversing the characteristic repression of histone genes in cells outside of S phase. A putative plant GAGA-like Factor has been predicted to reduce the nucleosomal packaging of the alfalfa and Arabidopsis replacement histone H3 genes to 65 percent, with the strongest localized derepression near the promoters and across the 5' parts of these genes.

PPY/PPU sequences and their binding factors by themselves, however, do not seem to be sufficient for high promoter activity of the alfalfa H3.2 gene. Deletion of sequences upstream of the TATA box greatly reduced reporter gene expression from the histone promoter, despite the presence of PPY/PPU elements in the 5'UTR. A similar requirement for *cis* promoter elements was also observed in transgenic Arabidopsis experiments. This points to the fact that an open chromatin structure may be a necessary but not sufficient requirement for constitutive gene transcription. 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. Understanding the mode of action of PPY/PPU-binding proteins may provide a better insight into the role of chromatin organisation on gene regulation in plants.

List of publications

Publications used for preparing the thesis

Kapros T., Dudits D., Györgyey J., Mai A. and **Kelemen Zs.**: Növényi génexpressziós vektor család a lucerna H3 hiszton génvariáns (Ms. H3g1) szabályozó DNS szekvencia szakaszainak felhasználásával (hungarian patent; Nr.: P9503352)

Kelemen Zs., Mai A., Kapros T., Fehér A., Györgyey J., Jakob H. Waterborg J.H. and Dudits D. (2002) Transformation vector based on promoter and intron sequences of a replacement histone H3 gene. A tool for high, constitutive gene expression in plants Transgenic Research, accepted

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