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Short Communication

Transformation vector based on promoter and intron sequences of a replacement histone H3 gene. A tool for high, constitutive gene expression in plants

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Abstract

This study explored the possibility of using non-viral, plant-based gene sequences to create strong and constitutive expression vectors. Replacement histone H3 genes are highly and constitutively expressed in all plants. Sequences of the cloned alfalfa histone H3.2 gene MsH3g1 were tested. Constructs of the β-glucuronidase (GUS) reporter gene were produced with H3.2 gene promoter and intron sequences. Their efficiency was compared with that of the commonly used strong 35S cauliflower mosaic virus promoter in transgenic tobacco plants. Combination of the H3.2 promoter and intron produced significantly higher GUS expression than the strong viral 35S promoter. Histochemical GUS analysis revealed a constitutive pattern of expression. Thus, alfalfa replacement H3 gene sequences can be used instead of viral promoters to drive heterologous gene expression in plants, avoiding perceived risks of viral sequences.

Introduction

It has been argued and demonstrated that viral promoters may confer unfavorable characteristics to transgenic plants, including increased recombination (Kohli et al., 1999) and silencing of transgenes (Al-Kaff et al., 2000). Histone genes, existing in multiple copies per genome, have been selected through evolution not to become subject to multi-copy gene inactivation. In plants and animals, histone H3 variants exist that are expressed constitutively. They replace replication-dependent histone forms in terminally differentiated tissues when chromatin is repaired following nucleosome loss caused by gene transcription. Thus, they have been called replacement histones (Marzluff, 1989). Replication-dependent histone H3 is synthesized only in proliferating cells from intron-free genes. In contrast, replacement H3 histones are produced abundantly in most differentiated tissues from intron-bearing genes. In addition to introns within the histone coding region, replacement H3 genes contain an intron in the 5′ untranslated region (Marzluff, 1989). The alfalfa replacement histone H3.2 genes represent the only known example with introns only in the coding region (Waterborg & Robertson, 1996). The synthesis rate of alfalfa H3.2 histone protein from the three H3.2 genes is, on a per gene basis, many times higher than replication-variant histone H3.1 protein synthesis during S phase (Kapros et al., 1995; Robertson et al., 1996). Sequence elements that may prevent chromatin repression have been identified in

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the gene promoter, 5′ untranslated region and introns of plant H3.2 genes (Robertson et al., 1996; Waterborg & Robertson, 1996). In this paper, we demonstrate that these gene domains of one of the alfalfa histone H3.2 genes confer high reporter gene expression in transgenic alfalfa cells and tobacco plants. This identifies them as good alternatives to viral promoters when high, constitutive transgene expression is required.

Materials and methods

DNA manipulation and transformation

The binary vector pBI121 (Clontech), based on pBIN19 (Bevan, 1984), is a GUS reporter construct driven by the 35S CaMV promoter and nopaline synthetase (NOS) terminator. The *Hin*dIII–*Sac*I fragment of pBI121 with the *gusA* coding sequence was replaced with the *Hin*dIII–*Sac*I fragment of plasmid pLP100, derived from pLP17 (Szabados et al., 1990), where the ATG codon of the *gusA* gene overlaps an *Nco*I site. The *Acc*I–*Nco*I promoter fragment of alfalfa clone MsH3g1, positions 1–482 (Robertson et al., 1996), was inserted between the blunted *Bam*HI site of pBI121 and the *Nco*I site, creating pHEX-N. The first intron in the coding region of MsgH3g1, positions 555–668 (Robertson et al., 1996), was amplified by a polymerase chain reaction with primers 5′-CATAGGTAACCACCGTTCACCGCCG-3′ and 5′-GCCTGAACCAAAAAATCGATGAAA-3′. Inserted into the *Nco*I site between the H3.2 promoter sequence and *gusA* coding sequence, plasmid pHEX-110 was created for use in transient expression analyses in alfalfa A2 cells, as described (Kapros et al., 1993). For tobacco transformation, the expression cassette was excised from the residual polylinker sequence as a *Hin*dIII–*Eco*RI fragment and inserted into pBIN19 (Bevan, 1984), creating pBHEX-110. Control plasmid pIDS21, used in transient expression studies, was created by inserting the *Hin*dIII–*Eco*RI fragment from pBI121 into corresponding sites of pUC18 (New England Biolabs). All DNA cloning and manipulation procedures were executed according to standard protocols (Sambrook et al., 1989).

Binary vector constructs were introduced into the *Agrobacterium tumefaciens* strain GV 2260 by the freeze-thaw method (Holsters et al., 1978). Leaf discs of *Nicotiana tabacum* cv SR1 were transformed as described by Fisher and Guiltinan (1995). Regenerated shoots were rooted on hormone-free MS medium containing 100 mg kanamycin and 500 mg augmentin per liter.

GUS activity assays

For the fluorometric GUS assay, alfalfa A2 cells or tobacco plantlets and leaves (> 4 cm in length) were homogenized in GUS extraction buffer (50 mM $NaHPO_4$ pH 7, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% sodium lauryl sarcosine and 0.1% Triton X-100). Protein concentrations were determined by the Bradford assay as described (Kapros et al., 1993). Extracts containing 20 μg protein were added to 200 μl GUS assay buffer, 2 mM 4-methylumbelliferyl β-D-glucuronide in extraction buffer. Reactions for 1–3 h at 37°C were terminated by adding 0.8 ml 0.2 M Na_2CO_3. Fluorescence of methylumbelliferone (MU) was measured with a TKO 100 fluorometer [Hoefer], and expressed in units of 1.0 nmol MU produced per min and per mg of protein. Average values and standard deviation errors were calculated, given the number of independent analyses as 'n'. GUS activities from independent transformants were analyzed by one-tailed Student's t-Test and plotted using Sigma Plot 2000 version 6.10 (SPSS Inc.). Histochemical GUS staining was carried out as described (Kapros et al., 1993).

Results and discussion

In the present work, we have explored the possibility of using sequences from a replacement variant alfalfa histone H3.2 gene as an alternative to the cauliflower mosaic viral 35S promoter to drive gene expression in plants. Transfection of alfalfa A2 cells with the 35S-driven pIDS211 vector produced after 2–3 days lower GUS activity (0.9 ± 0.3 Units, $n = 6$) than pHEX-N (6.4 ± 4.5 Units, $n = 6$) where the *gusA* gene was driven by the promoter of the alfalfa H3.2 gene MsH3g1. Insertion of the first intron of MsH3g1 (114 bp) between the transcriptional and translational start sites in pHEX-110 appeared to increase GUS activity to 29 ± 12 units ($n = 6$). However, sequence differences between pIDS and pHEX vectors may have skewed this preliminary result.

The H3.2 promoter-intron-*gusA* cassette of pHEX-110 was transferred to a pBIN19 vector creating pBHEX-110 to allow direct comparison in stable tobacco transformation with the strong 35S promoter in the pBI121 GUS expression construct, containing the viral nopaline synthase 3′ polyadenylation sequence. Southern analysis and histochemical staining

of primary tobacco transformants revealed insertion of one to four functional *gusA* transgenes per genome from either construct (data not shown). Morphogenic abnormalities were not observed in these plants. Self-pollination yielded seeds that germinated in the presence of kanamycin, producing second-generation transformants. Leaf extracts from nine independent pBHEX-110 transformants and of seven independent pBI121 transformants were analyzed for GUS activity levels as described in Materials and methods. As recognized for 35S-driven GUS activity, a rather high variability of GUS activity between independent transformants was observed (Figure 1A, coefficient of variation: 60%). pBHEX-110-driven GUS activity showed variability of a similar magnitude (coefficient of variation: 78%). Variation in GUS activity levels (Figure 1B) was reflected in the steady-state levels of GUS mRNA in northern analyses (Figure 1C), relative to total RNA (Figure 1D). On average, pBHEX-110 GUS expression appeared stronger than pBI121 activity (Figure 1A). Indeed, statistical analysis confirms that the mean value of GUS activity from pBHEX-110 was significantly larger than that of data from the 35S control pBI121 ($\alpha = 0.05$; $P = 0.014$).

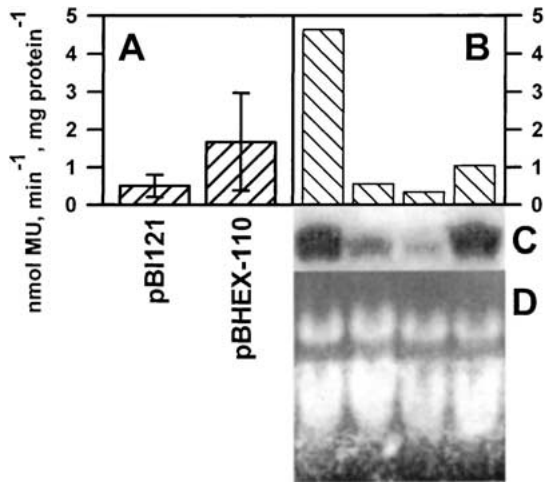

Figure 1. Panel A.: GUS activity in leaves of second generation tobacco plants, transformed by pBI121 or pBHEX-110 GUS expression constructs. GUS activity is expressed as n mol MU produced per minute, per milligram protein extract. Average values and standard deviation errors were calculated based on data obtained from leaves of nine independent pBHEX-110 and seven pBI121 transformants. GUS activity levels in leaves of four independent transformants (Panel B) are compared with *gusA* mRNA levels (Panel C) in total RNA (Panel D), extracted from leaves of the same transformants and analyzed by northern blotting.

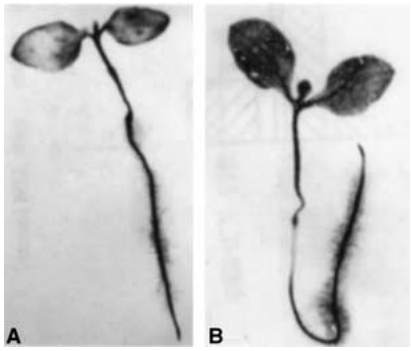

Figure 2. Histochemical detection of GUS activity in second generation tobacco plantlets, transformed by pBI121 (A) or pBHEX-110 (B) constructs.

Histochemical GUS staining of tobacco plantlets was carried out to study the spatial distribution of GUS activity driven by the alfalfa H3.2 sequences. Strong GUS activity was observed in all parts of plantlets transformed with pBHEX-110. Figure 2B shows a representative example. This is consistent with the general, high, constitutive expression of the endogenous histone H3.2 genes. It is quite similar to the distribution of GUS activity, driven by the 35S promoter (Figure 2A).

Transgene silencing is a major problem in plant biotechnology (Al-Kaff et al., 1998). The high expression of endogenous histone H3.2 genes suggests that the 12 histone H3.2 gene copies in tetraploid alfalfa have likely been selected, like repetitive histone genes in general, not to be subject to multicopy gene silencing. This intrinsic characteristic of the H3.2 sequences is important in that it will increase the efficiency of transformation when applied to drive selection marker genes, and the long-term stability of transgenes. It remains to be analyzed whether the pBHEX-110 promoter can confer strong constitutive gene expression in other plant species than alfalfa and tobacco. The ubiquitous presence and conserved function of replacement H3 genes in monocots and dicots (Waterborg & Robertson, 1996) suggests that it can, making it likely for pHEX-110 based expression cassettes to gain application in agrobiotechnology.

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References

Al-Kaff NS, Covey SN, Kreike MM, Page AM, Pinder R and Dale PJ (1998) Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. *Science* **279**: 2113–2115.

Al-Kaff NS, Kreike MM, Covey SN, Pitcher R, Page AM and Dale PJ (2000) Plants rendered herbicide-susceptible by cauliflower mosaic virus-elicited suppression of a 35S promoter-regulated transgene. *Nat Biotechnol* **18**: 995–999.

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl Acids Res* **12**: 8711–8721.

Fisher DK and Guiltinan MJ (1995) Rapid, efficient production of homozygous transgenic tobacco plants with *Agrobacterium tumefaciens*: a seed-to-seed protocol. *Plant Mol Biol Rep* **13**: 278–289.

Holsters M, de Waele D, Depicker A, Messens E, Van Montagu M and Schell J (1978) Transfection and transformation of *A. tumefaciens. Mol Gen Genet* **163**: 181–187.

Kapros T, Stefanov I, Magyar Z, Ocsovszky I and Dudits D (1993) A short histone H3 promoter from alfalfa specifies expression in S phase cells and meristems. *In Vitro Cell Dev Biol* **29**: 27–32.

Kapros T, Robertson AJ and Waterborg JH (1995) Histone H3 transcript stability in alfalfa. *Plant Mol Biol* **28**: 901–914.

Kohli A, Griffiths S, Palacios N, Twyman RM, Vain P, Laurie DA and Christou P (1999) Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. *Plant J* **17**: 591–601.

Marzluff WF (1989) Histone gene expression not coupled to DNA synthesis. *In*: Hnilica LS, Stein GS and Stein LJ (eds.), Histones and Other Basic Nuclear Proteins. (pp. 319–329) CRC Press, Boca Raton.

Robertson, AJ, Kapros T, Dudits D and Waterborg JH (1996) Identification of three highly expressed replacement histone H3 genes of alfalfa. *DNA Seq* **6**: 137–146.

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular Cloning. A Laboratory Manual. 2nd edn. Cold Spring Harbor, NY.

Szabados L, Ratet P, Grunenberg B and de Bruijn FJ (1990) Functional analysis of the *Sesbania rostrata* leghemoglobin *glb3* gene 5′-upstream region in transgenic *Lotus corniculatus* and *Nicotiana tabacum* plants. *Plant Cell* **10**: 973–986.

Waterborg JH and Robertson AJ (1996) Common features of analogous replacement histone H3 genes in animals and plants. *J Mol Evol* **43**: 194–206.