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Tissue-dependent enhancement of transgene expression by introns of replacement histone H3 genes of *Arabidopsis*

Nicole Chaubet-Gigot[1], Tamas Kapros[2], Martine Flenet[1], Katherine Kahn[2,3], Claude Gigot[1,4] and Jakob H. Waterborg[2,*]

[1]*Institut de Biologie Moléculaire des Plantes, CNRS, Université Louis Pasteur, 12 rue du Général Zimmer, 67084 Strasbourg Cedex, France;* [2]*Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Room 414 BSB, 5100 Rockhill Road, Kansas City, MO 64110-2499, USA (***author for correspondence; e-mail: waterborg@cctr.umkc.edu);* [3]*present address: Sainsbury Laboratory, Norwich Research Park, Colney NR4 7UH, UK;* [4] *Deceased*

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Abstract

Intron-bearing replacement histone H3 genes in *Arabidopsis* and other plants are highly and constitutively expressed. We demonstrate that the introns located within the 5′-untranslated regions (5′-UTR) of the two *Arabidopsis* replacement H3 genes will abolish the cell cycle dependence of an endogenous histone H4 promoter. We demonstrate that these introns, functionally combined with their endogenous promoters, could produce the high and constitutive expression of the replacement H3 genes observed *in planta*. They strongly increase gene expression whatever the promoter, from the strong 35S CaMV promoter to complete and resected promoters of cell cycle-dependent and replacement histone genes. Quantitative analysis of the extent of reporter gene enhancement in different parts of developing transgenic plantlets, ranging from 2-fold to 70-fold, supports the notion that *trans*-acting factors are responsible for this effect. Such factors appear most abundant in roots.

Introduction

Expression of histone genes is typically linked to DNA replication in the S phase of the cell cycle. In recent years we have described two histone H3 gene variants in *Arabidopsis thaliana* (Chaubet *et al.*, 1992) and three histone H3 gene variants in alfalfa (Robertson *et al.*, 1996) that are expressed constitutively. They produce the so-called replacement histone H3 proteins, whose function is the repair of the nucleosomal chromatin structure across transcribed genes (Waterborg, 1993). Transcription of chromatin in plants has been shown to lead to transient loss of nucleosomes. Constitutive production of replacement histone H3 proteins allows creation of new nucleosomes in non-S phase cells and maintenance of a stable chromatin structure (Waterborg, 1993). The replacement of replication-specific by constitutive forms of histone H3 has coined the phrase ‘replacement histone’.

As in animals, replacement histone H3 genes in plants contain introns, typically one within the 5′-untranslated, transcribed region (5′-UTR) of the gene and one or more within the protein coding sequence (Kanazin *et al.*, 1996; Waterborg and Robertson, 1996). These may function to insulate the limited number of replacement H3 genes from the much more abundant, intronless replication histone H3 genes, which tend to maintain sequence homogeneity through homologous recombination. It has been argued that the highly conserved protein sequence differences in replacement histones are essential to allow assembly of nucleosomes in cells outside of S phase (Waterborg and Robertson, 1996).

It has been demonstrated that the S-phase-specific and meristematic expression of replication-dependent histone genes is determined by a limited set of sequence elements that are observed in a variety of

patterns, relative to each other, typically within 250 bp upstream of the TATA box (Chaboute *et al.*, 1987; Brignon and Chaubet, 1993; Kapros *et al.*, 1993; Chaubet *et al.*, 1996; Ohtsubo *et al.*, 1997; Robertson *et al.*, 1997; Shen and Gigot, 1997; Taoka *et al.*, 1998). A subset of these elements is highly conserved at both the sequence and position level. An octameric motif CGCGGATC, or a degenerate copy of this element containing a one-base mismatch, is followed 10 to 30 nucleotides downstream by a CCGTCC (1–2 mismatches allowed) motif and 8 to 10 nucleotides further downstream by a nonameric motif whose consensus sequence is CCATC-CAAC in monocots and CAATC-CAAC in dicots (the - may represent one additional nucleotide). Extensive compilation of plant histone promoters has even led to extend this consensus sequence by GGCPu in monocots and CPuPyPy in dicots (Brignon and Chaubet, 1993). It has been recognized that these elements do allow replication-dependent (Lepetit *et al.*, 1992) and tissue-specific gene expression (Terada *et al.*, 1995). Interestingly, the (extended) nonamer motif is not found in the promoter of replacement H3 genes. In this paper we examine the tissue and cell cycle activity of these promoters and explore the possibility that the introns within replacement H3 genes impose a constitutive pattern of expression on elements that confer cell cycle control. We chose to use the promoter of the *Arabidopsis* histone H4 gene H4A748, which drives expression of a β-glucuronidase (GUS) reporter gene in an S-phase- and meristem-specific pattern (Atanassova *et al.*, 1992; Chaubet *et al.*, 1996). We show that addition of the 5′-UTR intron of either replacement histone H3 gene of *Arabidopsis* to this cell cycle-dependent promoter results in meristem-independent expression in *Arabidopsis*. We demonstrate that the constitutive expression of the replacement H3 genes of *Arabidopsis* results from both the presence of the intron and the nature of the promoters which lack some *cis*-elements found in replication-dependent promoters.

In alfalfa, the three replacement H3 genes are continuously transcribed 3–5 times higher than each of the 56 cell cycle-dependent H3 genes during S phase, producing during logarithmic growth twice as much mRNA as all 56 cycle-regulated H3 genes combined (Kapros *et al.*, 1995; Robertson *et al.*, 1996). The relative rate of transcription of the 2 replacement H3 genes of *Arabidopsis* (Chaubet *et al.*, 1992), relative to the 5–7 replication-dependent genes (Chaboute *et al.*, 1987), has yet to be measured. However, it must also be high, judged by the observation that at steady state more than 55% of all H3 protein in *Arabidopsis* is produced by the two replacement H3 genes (Waterborg, 1992). Consistently with this deduction, we observed that the level of reporter gene expression is strongly enhanced by replacement H3 intron sequences when driven by the replication-dependent histone promoter, the endogenous replacement H3 promoters or the strong, constitutive 35S cauliflower mosaic virus (CaMV) promoter.

Materials and methods

Plasmids

Chimeric histone H4 promoter-GUS (β-glucuronidase) fusions were generated by ligating a 900 bp *Xho*I promoter fragment of the *Arabidopsis H4A748* histone H4 gene with the *Sma*l site of the binary vector pBI101.1 (Jefferson *et al.*, 1987), designated ‘pBI101’in Figure 1, after blunt-end formation with the Klenow fragment of DNA polymerase I, as described (Atanassova *et al.*, 1992). This construct with 837 bp promoter sequence upstream of the TATA box was designated plasmid ‘748’. Truncated histone H4 promoters, shortened to 219 bp (designated ‘NarI’) and 93 bp (designated ‘EcoRV’), were obtained using isolated *Nar*I-*Xho*I and *Eco*RV-*Xho*I fragments, respectively, as described (Atanassova *et al.*, 1992).

The 420 bp fragment of the CaMV 35S promoter, corresponding to 7016 to 7434 of CM1841 (Gardner *et al.*, 1981), was excised from plasmid pRT100 (Topfer *et al.*, 1987) by *Bam*HI, incubated with Klenow and ligated into plasmid pBI101, digested with *Sma*I. The resulting plasmid was digested with *Hin*dIII and recircularized to remove repetitive polylinker sites derived from pRT100 and the M13 sequence in pBI101, creating the plasmid designated ‘35S’.

Promoter and intron cassettes, derived from the *Arabidopsis* replacement histone H3 genes, were constructed using a 3.6 kb *Pst*I fragment of cosmid C22, cloned into pUC19, containing ca. 230 bp cosmid sequence followed by nucleotides 1–3368 of the tandem repeat of the two replacement histone H3 genes (accession number X60429; Chaubet *et al.*, 1992).

The promoter of gene 1 was excised as a 606 bp fragment from the pUC19-*Pst*I clone by *Bsa*BI and *Dde*I, cutting at positions 85 and 690 (see accession number X60429), respectively, filling the *Dde*I end by Klenow. This fragment from 544 upstream of the

TATA box to +63 bp, the known start site of transcription (Chaubet *et al.*, 1992). It was inserted into pUCI9, digested with *Xba*I and filled by Klenow, excised as a 622 bp *Sal*I-*Bam*HI directional fragment, bordered at both termini by 8 bp of vector DNA, and ligated upstream of the GUS coding sequence into pBI101, after digestion with *Sal*I and *Bam*HI, to create the construct designated 'Pg1'.

The intron located within the 5′-UTR of gene 1 from 692 to 1100, abutting the ATG start codon (see accession number X60429), was excised from the pUC19-*Pst*I plasmid by double digestion with *Eco*RI and *Pst*I and the 1388 bp *Eco*RI fragment from position 643 to 2030 was purified by solute-displacement DEAE ion-exchange HPLC (Waterborg and Robertson, 1993), digested with *Dde*I and *Mse*I and the 398 bp fragment from position 688 to 1087 was purified by DEAE HPLC (Waterborg and Robertson, 1993). Oligonucleotides taattgttgaacagatccc and gggatctgtcaacaaat were annealed and ligated with T4 ligase, recreating the sequence up to position 1102, extended by a blunt half *Sma*I site, and the *Dde*I terminus was filled by Klenow. The fragment was ligated into *Sma*I-digested pUC19 and clones were selected that produce a directional 421 bp *Bam*HI-*Sma*I cassette of the intron bordered by 7 and 5 non-intron nucleotides, respectively. It was ligated immediately upstream of the GUS coding sequence into plasmids pBI101 and 748, following digestion with *Bam*HI and *Sma*I, to create plasmids designated 'i1' and '748+i1', respectively. The 622 bp *Sal*I-*Bam*HI promoter fragment of replacement histone H3 gene I was ligated upstream of the intron into plasmid i1, after digestion with *Sal*I and *Bam*HI, to create the construct designated ''Pg1+i1'.

The promoter of gene 2 was excised as a 307 bp fragment from the pUC19-*Pst*I clone by *Ase*I and *Alu*I, cutting at positions 2126 and 2433 (see X60429), respectively, filling the *Ase*I end. This fragment contains the full promoter of gene 2 because it contains the last 10 3′-UTR nucleotides observed in the longest known cDNA of gene 1 (Chaubet *et al.*, 1992), i.e. 255 bp upstream of the TATA box, and ends 13 bp beyond the transcription start site of gene 2, as determined from cDNA clones (F. Grellet, Perpignan, personal communication). It was inserted into pUC19, digested with *Bam*HI and filled with Klenow, excised as a 323 bp *Sma*I-*Bam*HI directional fragment, bordered at both termini by 8 bp of vector DNA, and ligated upstream of the GUS coding sequence into pBI101, sequentially treated with *Sal*I, Klenow and *Bam*HI, to create the construct designated 'Pg2'.

The intron located within the 5′-UTR of gene 2 from position 2449 (F. Grellet, Perpignan, personal communication) to 2911 (Chaubet *et al.*, 1992), abutting the ATG start codon (see X60429), was excised from the pUC19-*Pst*I plasmid by double digestion with *Eco*RI and *Pst*I and the 1337 bp *Eco*RI-*Pst*I fragment from position 2031 to 3368 was purified by solute-displacement DEAE ion-exchange HPLC (Waterborg and Robertson, 1993), digested with *Cfo*I and the 878 bp *Eco*RI-*Cfo*I fragment from position 2031 to 2908 was purified by DEAE HPLC (Waterborg and Robertson, 1993). Palindromic 18-mer oligonucleotide cagatcccgggatctgcg with an internal *Sma*I site was annealed and ligated at 50-fold excess by T4 DNA ligase to the *Cfo*I end to recreate the intron sequence to 2913, two bp beyond its 3′ end. The blunt 484 bp *Alu*I-*Sma*I fragment (2433–29l3, extended by a half *Sma*I site), was isolated after agarose gel electrophoresis and ligated into pGEM7 (Promega), digested with *Sma*I. The intron was excised as a 510 bp *Bam*HI-*Sma*I directional cassette and ligated immediately upstream of the GUS coding sequence into plasmids pBI101 and 748, following digestion with *Bam*HI and *Sma*I, to create plasmids designated 'i2' and '748+i21', respectively. It was also ligated into plasmid 748, sequentially treated with *Xba*I, Klenow and *Bam*HI, to create a variant of plasmid 748+i2 in which the intron direction was reversed, designated as '748+2i'. The *Bam*HI-*Sma*I fragment was filled by Klenow and ligated into plasmids 35S, NarI and EcoRV, digested with *Sma*I, selecting plasmids with the normal orientation of the intron, designated as '35S+i2', 'Nar+i2' and 'RV+i2', respectively. The same blunted *Bam*HI-*Sma*I fragment was ligated into plasmid 748, sequentially treated with *Hin*dIII and Klenow, selecting a product with the normal orientation of the intron, upstream of the 748 promoter, designated as 'i2+748'. The 323 bp *Sma*I-*Bam*HI promoter fragment of replacement histone H3 gene 2 was ligated upstream of the intron into plasmid i2, following sequential treatment with *Sal*I, Klenow, *Bam*HI and calf intestinal phosphatase, to create the construct designated 'Pg2+i2'.

Transformation

Constructs were transferred from *Escherichia coli* strain DH5α into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating with the helper plas-

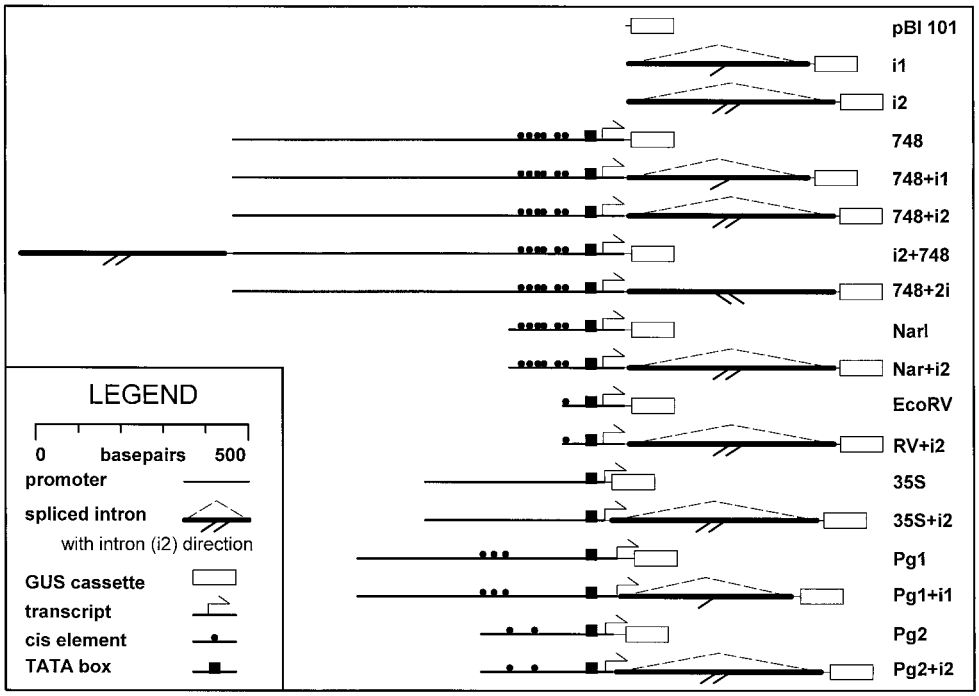

Figure 1. Transformation constructs. As detailed in Materials and methods, plasmid constructs were based on the promoter-less expression vector pBI101 which contains the bacterial β-glucuronidase sequence (GUS, white box). The name of each construct is given at the right side. For details on the source and orientation of sequences, see plasmid construction in Materials and methods. Known binding sites for transcription factors are marked by circles on the solid line, representing promoter DNA sequences. Intron sequences are marked by the bold line, by one-directional half-arrow for the intron of gene 1 (i1) and two for the intron of gene 2 (i2), and by splicing markers if RNA processing of transcripts is predicted from the normal orientation of the intron, downstream of the start site of transcription. All sequences are aligned by the TATA box (square) and the start of transcription is marked by the arrow.

mid pRK2013 (Bevan, 1994; Atanassova *et al.*, 1992). *Arabidopsis thaliana* ecotype C24 was transformed with *Agrobacterium* using the root transformation method (Valvekens *et al.*, 1988). The number of insertions per kanamycin-resistant transformant was not directly measured but was estimated typically to be more than 1, as observed before with typically 1 to 3 inserts per transformant following the same transformation protocol used here and based on segregation of kanamycin resistance in offspring of selfed F_1 plants, transformed with constructs i1, i2, 748, 748+i1, 748+i2, NarI or 35S. In 56 tests, 13 $\pm$ 3% of resulting offspring (830 plantlets) had become kanamycin-sensitive.

GUS assays

GUS enzyme activity was quantitated fluorometrically according to Jefferson *et al.* (1987) by measuring the kinetics of appearance of methylumbelliferone (MU) produced by cleavage of methylumbelliferyl-β-D-glucuronide in crude extracts from 3–4 buds, half of a root system or 3–4 full-size rosette leaves of mature F_1 plants. GUS activity is expressed as pmol MU produced per minute per milligram of protein. Protein concentration in crude extracts was determined by the dye binding method of Bradford (1976) with a kit supplied by BioRad Laboratories and with bovine serum albumin as a standard.

GUS histochemical staining was carried out using the basic procedure described by Jefferson *et al.* (1987) as described in detail elsewhere (Chaubet *et al.*, 1996). As for the quantitative GUS assay, analyses were not performed on primary regenerants but on selfed F_1 offspring, early after germination before the apical meristem between the cotyledons begins to expand (named: stage 1), when the first 2 leaves have clearly emerged (named: stage 2) and on mature, flowering plants. *Arabidopsis* organs (hand-cut into small pieces) or whole seedlings were wet in 70% ethanol and incubated for 45 min at room temperature in a prefixation solution (0.3% formaldehyde, 0.3 M mannitol, 10 mM MES pH 5.6). After several washes in

50 mM sodium phosphate buffer pH 7.0, tissues were vacuum-infiltrated and incubated at 37 °C for a few minutes, some hours or overnight in 50 mM sodium phosphate buffer pH 7.0 containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc, 0.05 mg/ml) and 0.5 mM potassium ferricyanide/ferrocyanide. The reaction was stopped by several washes in 50 mM sodium phosphate buffer and plant material was dehydrated through an ethanol series.

Results

Replacement histone H3 5′-UTR introns do not contain cryptic promoters

Arabidopsis has two histone H3 variant genes which are expressed in a constitutive, replication-independent manner (Chaubet *et al.*, 1992). These H3 genes exist in a tandem repeat. In this paper, the upstream and downstream genes of the repeat are named genes 1 and 2. In addition to introns within the coding sequences, each gene contains one rather long intron within its 5′-UTR sequence, abutting the ATG start codon (Chaubet *et al.*, 1992). The intron cassettes created from these 5′-UTR introns (see Materials and methods) are named i1 and i2, respectively. They were used to test for the presence of cryptic promoters in the promoter-less transformation plasmid pBI101 which contains the β- glucuronidase (GUS) reporter gene (Figure 1). GUS activity levels in mature F_1 i1- and i2-transformed plants were raised only 4- and 2-fold above the very low level of GUS expression from pBI101 (Figure 2). Histochemically, GUS activity was undetectable in plantlets transformed by pBI101 (data not shown), by plasmid i1 (Figure 3r) or i2 (Figure 3t). Very low levels of GUS expression could be detected at the apex of some i1 plantlets at stage 2 (Figure 3s) and in adult tissues. GUS activity remained below 1% of that observed for GUS driven by histone H4 promoter H4A748 in the construct named 748. These observations showed that the 5′-UTR introns did not contain cryptic promoters that could produce high, constitutive replacement histone H3 gene expression.

Promoter activity of H4A748, modified by intron sequences

The histone H4 promoter H4A748 has been demonstrated to be an effective promoter for GUS expression, retaining the replication dependence of the promoter with a clear preference for expression in meristems (Atanassova *et al.*, 1992). This translates quantitatively to a 6–9-fold higher reporter gene expression in meristem-rich buds than in leaves (Figure 2D) (Chaubet *et al.*, 1996). GUS activity is clearly strong in apical (Figure 3a–c) and root meristems (Figure 3a, d) in lateral root primordia, and in flower buds and carpels of mature plants, as reported before (Atanassova *et al.*, 1992).

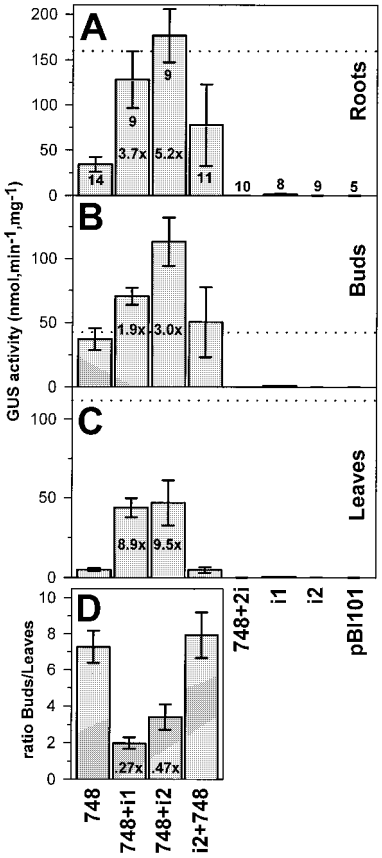

Figure 2. Intron modulaton of GUS expression from a cell cycle-regulated *Arabidopsis* histone H4 promoter. GUS enzyme activity in roots (A), buds (B) and leaves (C) of mature F_1 plants, transformed by plasmids listed along the lower axes, are shown as averages with standard deviation error bars for individual plants. The number of plants analysed for each is noted in panel A. The ratio of GUS activity in buds versus leaves is shown in D. Intron-dependent changes, relative to column 1, are noted by x signs. For comparison purposes, the tissue-specific level of GUS expression, driven by the general 35S promoter (see column 1, Figure 6), is indicated by dotted lines.

Insertion of the i1 or i2 intron between this H4 promoter and GUS in transformation plasmids 748+i1 and 748+i2, respectively (Figure 1) caused major quantitative and qualitative changes in GUS expression. Within buds where meristem-based transcription

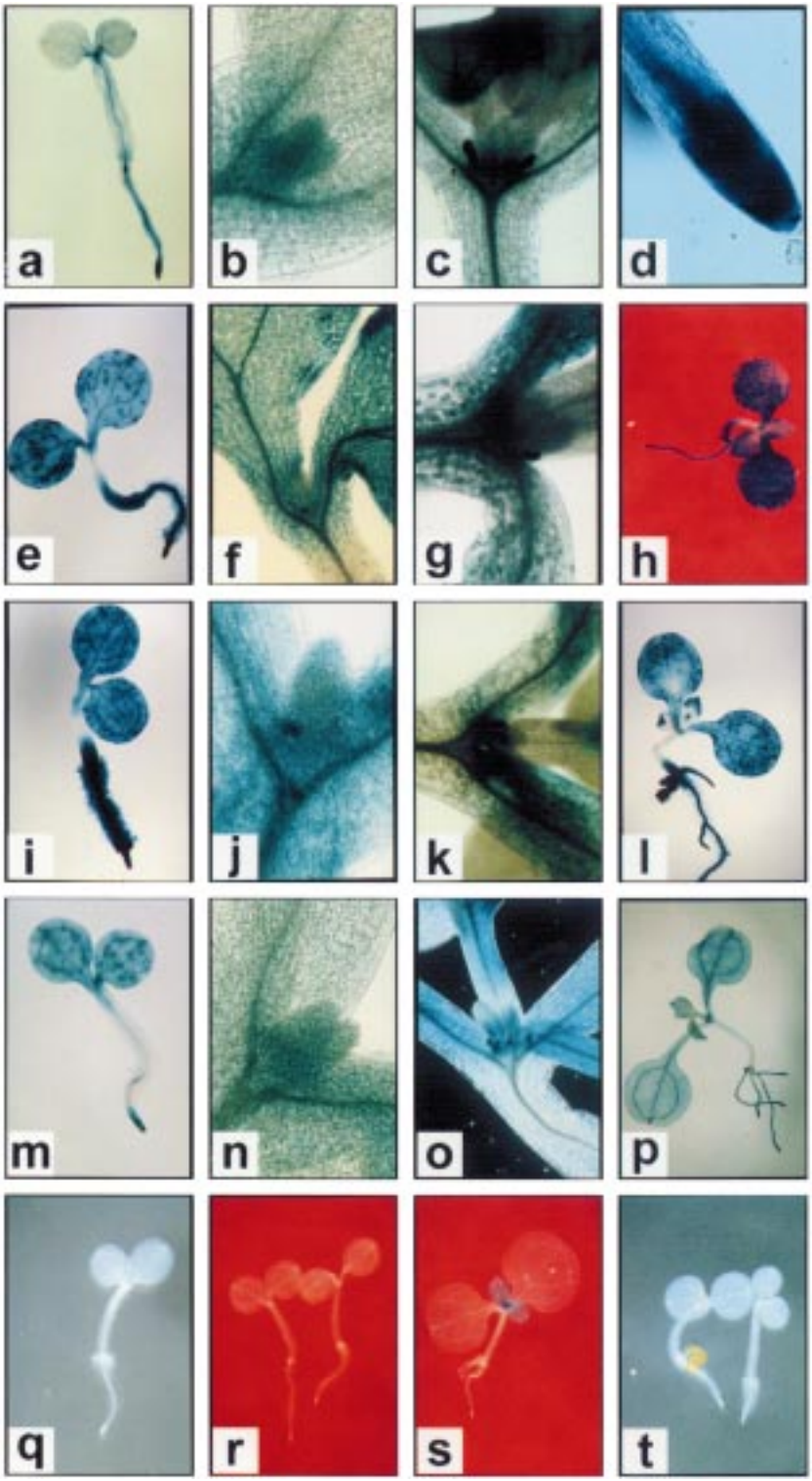

Figure 3. Histochemical localization of GUS expression from an intron-modified histone H4 promoter. GUS activity in plantlets transformed by plasmids 748 (a–d), 748+i1 (e–h), 748+i2 (i–l), i2+748 (m–p), 748+2i (q), i1 (r–s) and i2 (t). Stage 1 (columns 1–2) and stage 2 (columns 3–4) plantlets are shown with details of apical meristems without (column 2) and with leaflets and stipules (column 3). Panel d shows typical GUS expression in root tips when controlled by a meristem-specific promoter.

factors allowed high expression from the H4 promoter, GUS activity increased just 2–3-fold (Figure 2B). In roots, with a lower proportion of meristematic tissues, GUS activity increased 4–5-fold (Figure 2A). In leaves, the low basal expression of GUS by the H4A748 promoter (Lepetit *et al.*, 1992) increased most, about 10-fold, but overall GUS activity levels clearly remained below those of buds and especially of roots (Figure 2C). To facilitate comparisons between tissues and constructs, figure panels of GUS activities show by dotted lines the level of GUS expression driven by the constitutive 35S CaMV promoter. Relative to this measure, intron-stimulated expression, already high in buds, reached twice 35S-driven levels (Figure 2B), in roots increased to levels similar to 35S (Figure 2A) but remained lower than 35S in leaves (Figure 2C). These changes caused a decrease in the buds/leaves ratio of GUS expression (Figure 2D) to levels typically seen for meristem-independent, constitutive reporter gene expression (Atanassova *et al.*, 1992; Chaubet *et al.*, 1996).

The loss of preferential meristem expression of GUS by insertion of either replacement H3 5′-UTR intron was also clear when GUS expression was analysed histochemically. GUS expression was enhanced, similarly by i1 (Figure 3 e–h) and by i2 (Figure 3i–l), but GUS activity did not become uniform in all tissues. Very high expression was observed in roots, including root hairs, and in the lower half of the hypocotyl in stage 1 (Figure 3e, i) and stage 2 (Figure 3h, l) plantlets. Cells in leaves were typically uniformly stained for GUS (Figure 3f and g, j and k) except for the punctate pattern of high GUS expression in stomatal guard cells and enhanced GUS staining along the leaf vasculature. Only the upper half of hypocotyls had low GUS expression (Figure 3e, h, and i, l). These results suggested that the intron cassettes, or more likely factors that interact with the intron sequences, determine the level of GUS expression. Such factors would be more abundant in root tissues and in selected cell types such as stomatal guard cells. It is unlikely that factors interacting with the H4A748 promoter sequences can enhance GUS expression further, as judged by the absence of intensified GUS expression in the apical meristems of 748+i1 (Figure 3f) or 748+i2 (Figures 3j) transformants. This observation makes it likely that the increase observed in buds (Figure 2B) results from increased expression in non-meristematic bud tissues.

Based on the observation that the results obtained with intron cassette i1 were essentially identical to those with i2, it was decided to perform most further tests with one intron only. The i2 cassette was chosen. When the i2 sequence was inserted upstream of the H4A748 promoter in construct i2+748 (Figure 1) it had no significant effect on the level of GUS expression (Figure 2A–C), meristem preference (Figure 2D) or tissue localization (Figure 3m–p). GUS expression was as observed with the unmodified H4A748 promoter (Figure 3a–d). This eliminated the formal possibility that the i2 intron sequence contained position-independent enhancer elements that could enhance gene transcription *in cis*. When the i2 sequence was inserted in an inverted orientation between the H4A748 promoter and the GUS gene in construct 748+2i (Figure 1), GUS activity was abolished (Figure 2A–C) in all tissues (Figure 3q).

The intron-based increase of GUS expression can be limited by histone promoter elements

The first set of experiments noted the lack of GUS stimulation in the meristems of 748+intron transformants (Figure 3f, j). Thus, the transcription factors in cycling cells that can bind to H4A748 promoter elements appear not to be able to stimulate GUS expression from a 748+intron promoter. Could they limit GUS expression? To evaluate this possibility, truncated versions of the H4A748 promoter were tested. It had been shown previously that the shortened NarI promoter with 126 bp upstream of the TATA box (Atanassova *et al.*, 1992) is still a reasonably competent promoter to drive GUS expression (Figure 4) and retains meristem preference (Atanassova *et al.*, 1992) albeit with a reduced GUS expression ratio of buds/leaves at 5 ± 1 (results not shown). In leaves without meristem-associated transcription factors, the activity from the short NarI promoter is raised by the i2 intron to the same level as seen for the longer 748 promoter, enhanced by i2 (Figure 4C). In contrast, in buds (Figure 4B) and roots (Figure 4A) with meristems and replication-associated transcription factors, GUS expression increased an additional 3–4-fold over the level produced by the 748+i2 promoter. This confirmed that limited availability of transcription factors for H4 regulatory promoter elements, largely identified as positive (Atanassova *et al.*, 1992), could limit GUS expression. The pattern of tissue expression in Nar+i2 transgenic plantlets (Figure 5e–h) was qualitatively identical to that of 748+i2 (Figure 3i–l).

Additional truncation of the promoter produces construct EcoRV, retaining only the TATA *cis*-element

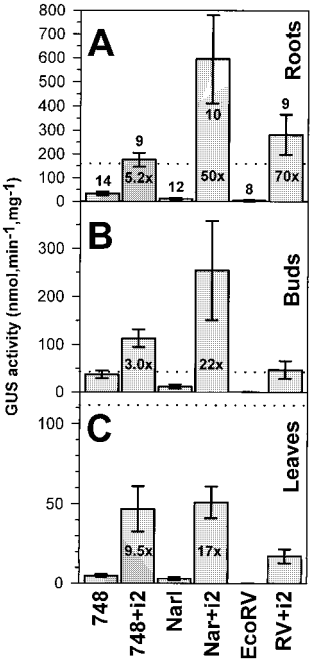

Figure 4. Intron modulation of GUS expression from shortened histone H4 promoters. GUS enzyme activity in roots (A), buds (B) and leaves (C) of mature F_1 plants, transformed by plasmids listed along the lower axes, are shown as averages with standard deviation error bars for individual plants. The number of plants analysed for each is noted in panel A. Intron-dependent changes, relative to the preceding columns, are noted by x signs. For comparison purposes, the tissue-specific level of GUS expression, driven by the general 35S promoter (see column 1, Figure 6), is indicated by dotted lines.

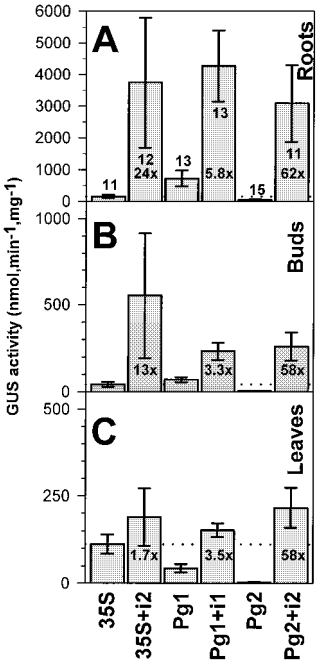

Figure 6. GUS expression from 35S CaMV and *Arabidopsis* replacement H3 promoters. GUS enzyme activity in roots (A), buds (B) and leaves (C) of mature F_1 plants, transformed by plasmids listed along the lower axis, are shown as averages with standard deviation error bars for individual plants. The number of plants analysed for each is noted in panel A. Intron-dependent changes, relative to the preceding columns, are noted by x signs. GUS expression levels by the 35S plasmid are also shown by dotted lines.

of the 748 promoter (Figure 1) (Atanassova *et al.*, 1992). This EcoRV construct essentially fails to produce GUS (Figure 4 and 5d). Insertion of intron 2 between this very weak promoter and the GUS reporter gene strongly increased GUS expression (Figure 4) but it failed to produce expression levels obtained by an effective but uninhibited promoter like NarI. Histochemical analysis of RV+i2 plantlets showed some distinct features. The typical high root and root hair expression was absent, as was the low GUS expression in the upper half of stage 1 hypocotyls seen with all effective promoters when combined with i1 or i2 sequences (Figures 3e, i, 5e, 7e, m, u). Overall, addition of i2 to the ineffective RV promoter (Figure 5d) increased GUS expression with remarkably little variation between plantlet tissues (Figure 5i–k) and without any effect on the low buds/leaves GUS activity ratio of 3 ± 1 (results not shown).

Intron stimulation of constitutive 35S CaMV and endogenous replacement H3 promoters

One gets the idea from the two sets of experiments described above that the i1 and i2 intron sequences facilitate the function of effective promoters, allowing high expression in many tissue types, overriding the meristem preference of the H4A748 promoter while likely retaining the limiting effect of meristem transcription factors. This suggested that promoters which are less subject to limiting controls by tissue factors might be enhanced even more by the intron sequences. The strong 35S CaMV promoter, which typically shows a relatively low degree of tissue specificity, was chosen to test this idea.

The distribution of GUS expression, driven by the 35S promoter, differs less than a factor of 2 between leaves and buds or roots (Figure 6). Limited variation in reporter gene expression was also seen histochemically (Figure 7a–d) with low levels in the upper part of hypocotyls (Figure 7a, d) and a punctate pattern of leaf expression with high expression at stomata (Fig-

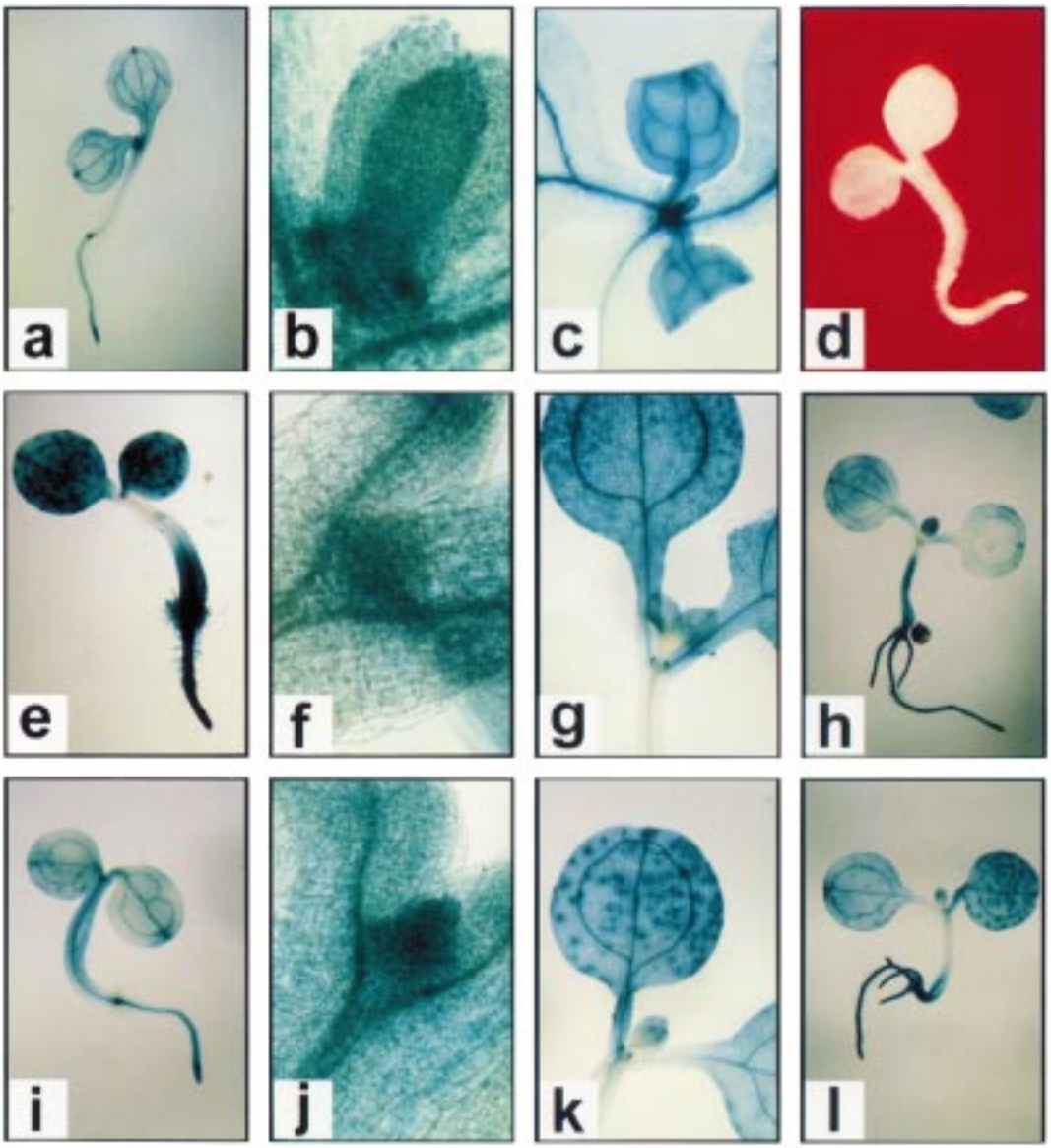

Figure 5. Tissue localization of GUS expression from shortened histone H4 promoters. GUS activity in plantlets transformed by plasmids NarI (a–c), RV (d), Nar+i2 (e–h) and RV+i2 (i–l). Plantlet stages and details as in Figure 3.

ure 7b). Combining the strong 35S promoter with the i2 sequence increased GUS activity levels by more than 10- and 20-fold, respectively, in buds (Figure 6B) and roots (Figure 6A) with only a limited effect on leaf expression (Figure 6C). This was also seen in the histochemical analysis of this activity, which showed no changes in the spatial patterns of GUS expression, except for a lighter punctate staining in cotyledons (Figure 7e–h).

The proper promoter of replacement H3 gene 1 (Pg1) was shown to be quite strong, exceeding 35S function in roots (Figure 6A) and buds (Figure 6B) with slightly less activity in leaves (Figure 6C). The level of GUS expression, driven by Pg1, was similar in leaves and buds but 10-fold higher in roots of mature F_1 plants. This difference is clearly less in plantlets with remarkably even distribution of GUS staining across all tissues (Figure 7i–l). Thus, the strength of the Pg1 promoter with fewer *cis* elements than the H4A748 promoter (Chauben *et al.*, 1992, 1996; Robertson *et al.*, 1997) appears less affected

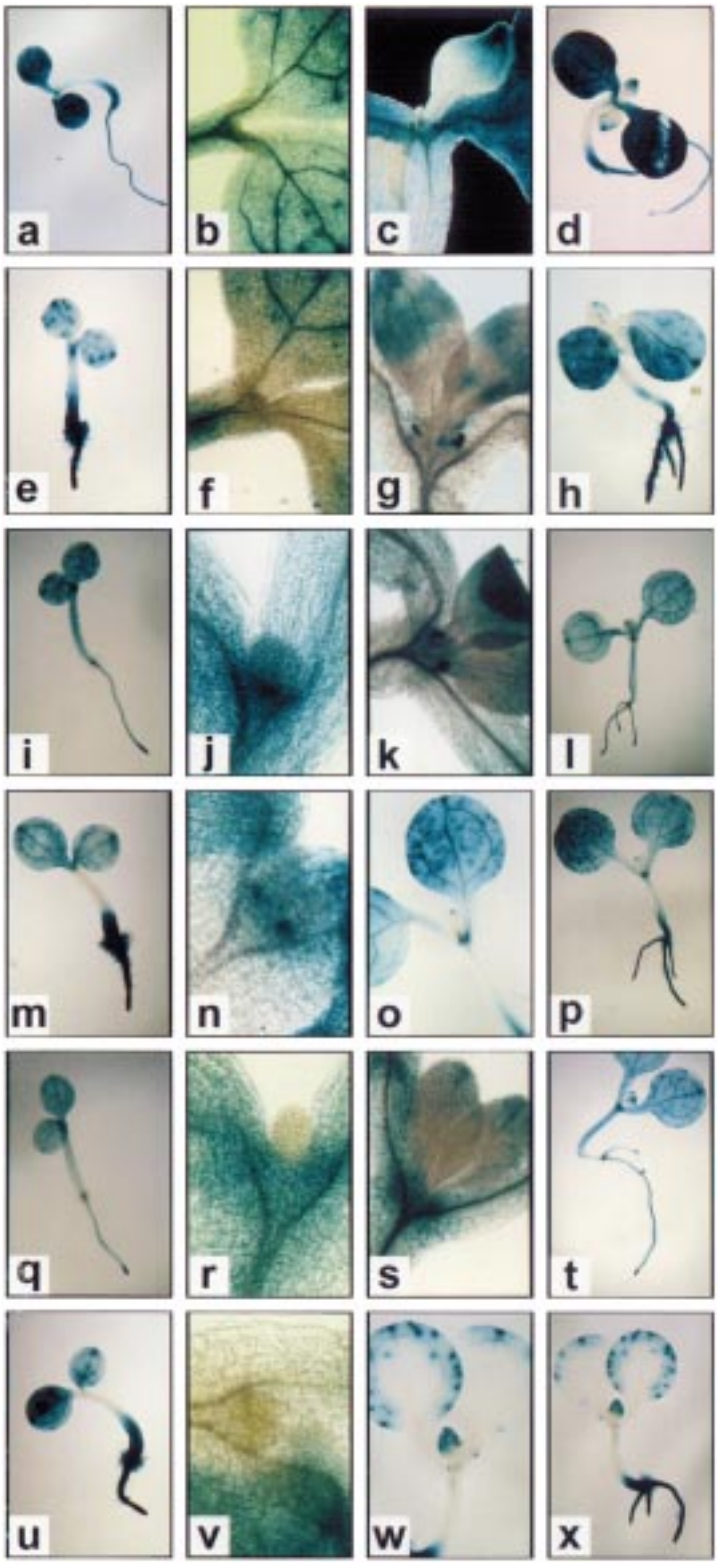

Figure 7. Tissue expression of GUS from modified 35S and endogenous replacement H3 promoters. GUS activity in plantlets transformed by plasmids 35S (a–d), 35S+i2 (e–h), Pg1 (i–l), Pg1+i1 (m–p), Pg2 (q–t) and Pg2+i2 (u–x). Plantlet stages and details as in Figure 3.

by differences in cell cycle-dependent transcription factors.

The combination of the strong Pg1 promoter with its endogenous intron i1 enhanced GUS expression 3–6-fold, resulting in levels of GUS expression very similar to those obtained from the 35S promoter combined with intron i2 (Figure 6). In both cases the expression in roots and root hairs is extremely high with relatively low GUS expression in the upper part of the hypocotyl (Figure 7m–p). This pattern suggests that the factor(s) that allow the very high promoter activity in roots from either promoter (Figure 7e, h, m, p) are present in limiting amounts in hypocotyls, only sufficient for low levels of expression (Figure 7i, l).

The promoter of gene 2, Pg2, by itself is a weak promoter in all parts of the plant (Figure 6) with very light but even staining for GUS activity across all tissues of young plantlets (Figure 7q–t). In combination with its endogenous intron i2, Gus expression was stimulated 60-fold, reaching levels equal to those seen with the strong Pg1 promoter with intron i2 in all tissues (Figure 6) and with similar tissue distributions (Figure 7u–x).

Discussion

We have demonstrated that the 5′-UTR introns of the two constitutive replacement histone H3 genes of *Arabidopsis* are capable of increasing the promoter strength of weak and strong promoters including the strong 35S CaMV promoter. In addition, the meristem specificity of the cell cycle-dependent histone H4 promoter A748 of *Arabidopsis* was diminished. Apparently this was not caused by interference with cell cycle-specific factors in meristems. GUS expression was equally intense in apical meristems when driven by the A748 H4 promoter alone (Figure 3b, c) or by this promoter in combination with intron i1 (Figure 3f, g) or i2 (Figure 3j, k). However, the expression in non-meristematic cells of buds was clearly increased, leading to an overall 2–3-fold increase in GUS activity (Figure 2B) and a characteristic drop in the ratio of GUS expression in buds versus leaves (Figure 2D) (Atanassova *et al.*, 1992). The increase of GUS activity in leaves to 50 nmol per minute per mg protein, half the level observed for the 35S promoter (Figure 2C), may represent a limit imposed by limiting transcription factors required for A478-based promoters in this tissue.

Even the most basic, limited promoters are stimulated by the 5′-UTR introns of the replacement H3 genes. For example, the RV promoter, a histone H4 promoter resected to remove almost all transcription factor binding sites (Figure 1), reducing it to just more than a TATA box, was stimulated to the level of the 35S promoter activity in buds (Figure 4B) and even higher in roots (Figure 4A). Similarly, the Pg2 histone H3 promoter, which by itself is almost incapable of driving GUS reporter gene expression, is stimulated in all tissues to levels above those of strong 35S or Pg1 promoters without introns. It reaches levels which are also attained by these strong promoters if combined with an intron (Figure 6). However, clear differences exist between these two weak promoters. While the Pg2+i2 combination becomes as active as the 35S+i2 system in every plant tissue (Figure 6), the RV+i2 strength never reaches these levels. Its GUS activity is like that of 35S alone in buds and roots (Figure 4A, B) and much lower than that in leaves (Figure 4C). Thus, it gains a large part of A748 promoter strength but, like the A748 promoter, remains limited. Apparently, addition of a stimulatory intron cannot overcome the inherent limited availability of required meristematic transcription factors which are low in leaves. Their limiting effect remains even within the shortened RV promoter (Figure 4C).

Intron-mediated enhancement (IME) of gene expression has been described for reporter genes in dicots and monocots alike, typically with up to 10-fold stimulation in dicots while enhancement in monocots can exceed 100-fold (Callis *et al.*, 1987; Mascarenhas *et al.*, 1990; Tanaka *et al.*, 1990; Luehrsen and Walbot, 1991; Rethmeier *et al.*, 1997; Rose and Beliakoff, 2000). IME is described as a post-transcriptional mechanism which, through nuclear stabilization of transcripts, leads to increased reporter gene expression (Rethmeier *et al.*, 1997; Rose and Beliakoff, 2000). It appears to require an intron positioned between promoter and coding sequence (Callis *et al.*, 1987; Snowden *et al.*, 1996) and the orientation of the intron must be normal, compatible with RNA splicing, even though completion of splicing reactions appears not to be required (Luehrsen and Walbot, 1991; Rose and Beliakoff, 2000). Constructs i2+748 and 748+2i (Figure 1) are the only intron-containing constructs tested which fail to meet these two requirements. GUS expression was unaffected by the intron sequence in the first case (Figures 2, 3m–p) and abolished in the second one (Figures 2, 3q). Analysis in *Arabidopsis* has suggested that recognition of intron processing signals

by the nuclear splicing machinery suffices and that the sequence content of introns is irrelevant (Rose and Beliakoff, 2000). However, it is quite clear that some introns enhance reporter gene expression while others do not (Mascarenhas *et al.*, 1990; Luehrsen and Walbot, 1991; Rose and Beliakoff, 2000). Gene enhancement in excess of increases in mRNA levels has been described (Mascarenhas *et al.*, 1990). Also, introns have been observed to affect other processes, such as translation (Gallie and Young, 1994), or act as promoters (Warnecke *et al.*, 1999) or enhancers (Gidekel *et al.*, 1996; Bhattacharyya and Banerjee, 1999). We have excluded that the replacement H3 introns have intrinsic promoter activity (Figure 2, 3r–t) or that the i2 sequence can act as a position-independent enhancer sequence in the i2+748 construct (Figures 2, 3m–p).

One major characteristic effect of the replacement H3 introns presented here has not been observed in systems where post-transcriptional IME was described. IME enhances reporter gene expression in general, without tissue or cell type modulation. In our system, expression of GUS was not enhanced by introns i1 or i2 in meristem cells (Figure 3). At the same time, GUS levels in root tissues were 20-fold higher than in buds or leaves, reaching the same level whether driven by strong promoters like the CaMV 35S promoter or the histone H3 Pg 1 promoter, or driven by the weak Pg2 promoter (Figure 6). Loss of promoter control over GUS expression was also obvious in non-meristem tissues when intron i2 was combined with the strong but cell cycle-dependent histone H4 A748 promoter, in the less restricted NarI construct or in combination with the weak RV promoter (Figures 4 and 5). Especially the weak promoters demonstrate clearly that a direct increase in transcription must occur as a result of the presence of intron sequences. The strong increase in GUS expression from weak promoters like RV and Pg2 can only result in high GUS expression if the rate of transcription has become significant. Without transcripts to act upon, post-transcriptional mechanisms like IME could not enhance reporter expression. The primary transcript produced from 35S+i2, Pg2+i2, 748+i2, Nar+i2 and RV+i2 constructs are all identical (Figure 1). Thus, one would not expect differential processing and transcript stabilization, comparing results obtained in the same plant tissue. The differences observed in changes in GUS expression based on the presence of intron sequences (Figures 4 and 6) must reflect changes in gene transcription. Taking into account that the intron sequences i1 and i2 affected the A748 promoter in a similar fashion (Figures 2 and 3) the similarity of GUS expression from Pg1+i1 and Pg2+i2 in all tissues (Figures 6 and 7) supports the notion that transcription rate from these two promoters has been equalized by intron sequences. However, not all transcriptional controls were abolished by intron insertion. Meristem control of GUS expression was retained (Figure 3). The levels of enhanced GUS expression from A748-based promoters, for instance in roots (Figure 4A), were 10-fold lower than from the other promoters tested, irrespective of the promoter strength in the absence of introns (Figure 6A). The factors that enhance GUS expression, which may include the contribution of post-transcriptional processes like IME, also appear to exist at different levels in distinct plant tissues. The 10–20-fold higher GUS expression levels, driven by 35S or replacement H3 promoters with introns (Figure 6), suggest that enhancement factors are more abundant in roots.

The observation that the replacement H3 gene introns enhance the activity of cell cycle-regulated, weak and strong promoters can be exploited to enhance expression of transgenes, especially for root tissues where apparent promoter strength is highest (Figures 4A, 6A). This conclusion is useful even at a time when the mechanism of promoter activation has not been defined. We are currently pursuing the possibility that the presence of intron sequences i1 or i2, immediately following a promoter sequence, activate that promoter by excluding nucleosomes and other repressive factors. This possibility was suggested by the unusual high density of GAGA-like polypyrimidine sequence elements in both introns (Waterborg and Robertson, 1996) and the capability of GAGA factor in *Drosophila* to displace nucleosomes allowing transcription factor access to transcription factor binding sites and basal promoter alike (O'Donnell and Wensink, 1994; Tsukiyama *et al.*, 1994; Wall *et al.*, 1995). The functional importance of polypyrimidine sequences was suggested by the repeated polypyrimidine sequences in the intron-less 5′-UTRs of the three highly and constitutively expressed replacement H3 genes of alfalfa (Robertson *et al.*, 1996). Polypyrimidine-binding factors have been shown to exist in nuclear extracts (T. Kapros, unpublished) but whether these are responsible for the intron-based enhancement of reporter gene expression reported here awaits further study. The contributions of promoter derepression and transcript stabilization to the observed enhancement of GUS expression by introns i1 and i2 will be quantified.

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References

Atanassova, R., Chaubet, N. and Gigot, C. 1992. A 126 bp fragment of a plant histone gene promoter confers preferential expression in meristems of transgenic *Arabidopsis*. Plant J. 2: 291–300.

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

Bhattacharyya, N. and Banerjee, D. 1999. Transcriptional regulatory sequences within the first intron of the chicken apolipoprotein AI (apoAI) gene. Gene 234: 371–380.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Brignon, P. and Chaubet, N. 1993. Constitutive and cell-division-inducible protein-DNA interactions in two maize histone gene promoters. Plant J. 4: 445–457.

Callis, J., Fromm, M. and Walbot, V. 1987. Introns increase gene expression in cultured maize cells. Genes Dev. 1: 1183–1200.

Chaboute, M.E., Chaubet, N., Philipps, G., Ehling, M. and Gigot, C. 1987. Genomic organization and nucleotide sequences of two histone H3 and two histone H4 genes of *Arabidopsis thaliana*. Plant Mol. Biol. 8: 179–191.

Chaubet, N., Clement, B. and Gigot, C. 1992. Genes encoding a histone H3.3-like variant in *Arabidopsis* contain intervening sequences. J. Mol. Biol. 225: 569–574.

Chaubet, N., Flenet, M., Clement, B., Brignon, P. and Gigot, C. 1996. Identification of *cis*-elements regulating the expression of an *Arabidopsis* histone H4 gene. Plant J. 10: 425–435.

Gallie, D.R. and Young, T.E. 1994. The regulation of gene expression in transformed maize aleurone and endosperm protoplasts. Plant Physiol. 106: 929–939.

Gardner, R.C., Howarth, A.J., Hahn, P., Brown-Luedi, M., Shepherd, R.J. and Messing, J. 1981. The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13mp7 shotgun sequencing. Nucl. Acids Res. 9: 2871–2888.

Gidekel, M., Jimenez, B. and Herrera-Estrella, L. 1996. The first intron of the *Arabidopsis thaliana* gene coding for elongation factor 1β contains an enhancer-like element. Gene 170: 201–206.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusion: 1β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901–3907.

Kanazin, V., Blake, T. and Shoemaker, R.C. 1996. Organization of the histone H3 genes in soybean, barley and wheat. Mol. Gen. Genet. 250: 137–147.

Kapros, T., Robertson, A.J. and Waterborg, J.H. 1995. Histone H3 transcript stability in alfalfa. Plant Mol. Biol. 28: 901–914.

Kapros, T., Stefanov, I. Magyar, Z., Ocsovszky, I. and Dudits, D. 1998. A short histone H3 promoter from alfalfa specifies expression in S phase cells and meristems. In Vitro Cell Dev. Biol. 29: 27–32.

Lepetit, M., Ehling, M., Chaubet, N. and Gigot, C. 1992. A plant histone gene promoter can direct both replication-dependent and -independent gene expression in transgenic plants. Mol. Gen. Genet. 231: 276–285.

Luehrsen, K.R. and Walbot, V. 1991. Intron enhancement of gene expression and the splicing efficiency of introns in maize cells. Mol. Gen. Genet. 225: 81–93.

Mascarenhas, D., Mettler, I.J., Pierce, D.A. and Lowe, H.W. 1990. Intron-mediated enhancement of heterologous gene expression in maize. Plant Mol. Biol. 15: 913–920.

O'Donnell, K.H. and Wensink, P.C. 1994. GAGA factor and TBF1 bind DNA elements that direct ubiquitous transcription of the α1-tubulin gene. Nucl. Acids Res. 22: 4712–4718.

Ohtsubo, N., Nakayama, T., Kaya, H., Terada, R., Shimamoto, K., Meshi, T. and Iwabuchi, M. 1997. Cooperation of two distinct cis-acting elements is necessary for the S phase-specific activation of the wheat histone H3 promoter. Plant J. 11: 1219–1226.

Rethmeier, N., Seurinck, J., Van Montagu, M. and Cornelissen, M. 1997. Intron- mediated enhancement of transgene expression in maize is a nuclear, gene-dependent process. Plant J. 12: 895–899.

Robertson, A.J., Kapros, T., Dudits, D. and Waterborg, J.H. 1996. Identification of three highly expressed replacement histone H3 genes of alfalfa. DNA Seq. 6: 137–146.

Robertson, A.J., Kapros, T. and Waterborg, J.H. 1997. A cell cycle-regulated histone H3 gene of alfalfa with an atypical promoter structure. DNA Seq. 7: 209–216.

Rose, A.B. and Beliakoff, J.A. 2000. Intron-mediated enhancement of gene expression independent of unique intron sequences and splicing. Plant Physiol. 122: 535–542.

Shen, W.H. and Gigot, C. 1997. Protein complexes binding to *cis* elements of the plant histone gene promoters: multiplicity phosphorylation and cell cycle alteration. Plant Mol. Biol. 33: 367–380.

Snowden, K.C., Buchholz, W.G. and Hall, T.C. 1996. Intron position affects expression from the tpi promoter in rice. Plant Mol. Biol. 31: 689–692.

Tanaka, A., Mita, S., Ohta, S., Kyozuka, J., Shimamoto, K. and Nakamura, K. 1990. Enhancement of foreign gene expression by a dicot intron in rice but not in tobacco is correlated with an increased level of mRNA and an efficient splicing of the intron. Nucl. Acids Res. 18: 6767–6770.

Taoka, K., Ohtsubo, N., Fujimoto, Y., Mikami, K., Meshi, T. and Iwabuchi, M. 1998. The modular structure and function of the wheat H1 promoter with S phase-specific activity. Plant Cell Physiol. 39: 294–306.

Terada, R., Nakayama, T., Iwabuchi, M. and Shimamoto, K. 1995. A Type I element composed of hexamer wheat histone (ACGTCA) and octamer (CGCGGATC) motifs plays a role(s) in meristematic expression of a wheat histone H3 gene in transgenic rice plants. Plant Mol. Biol. 27: 17–26.

Topfer, R., Matzeit, V., Gronenborn, B., Schell, J. and Steinbiss, H.H. 1987. A set of plant expression vectors for transcriptional and translational fusions. Nucl. Acids Res. 15: 5890.

Tsukiyama, T., Becker, P.B. and Wu, C. 1994. ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. Nature 367: 525–532.

Valvekens, D., Van Montagu, M. and Van Lijsebettens, M. 1988. *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc. Natl. Acad. Sci. USA 85: 5536–5540.

Wall, G., Varga-Weisz, P.D., Sandaltzopoulos, R. and Becker, P.B. 1995. Chromatin remodeling by GAGA factor and heat shock factor at the hypersensitive *Drosophila* hsp26 promoter in vitro. EMBO J. 14: 1727–1736.

Warnecke, C., Willich, T., Holzmeister, J., Bottari, S.P., Fleck, E. and Regitz-Zagrosek, V. 1999. Efficient transcription of the human angiotensin II type 2 receptor gene requires intronic sequence elements. Biochem. J. 340: 17–24.

Waterborg, J.H. 1992. Existence of two histone H3 variants in dicotyledonous plants and correlation between their acetylation and plant genome size. Plant Mol. Biol. 18: 181–187.

Waterborg, J.H. 1993. Histone synthesis and turnover in alfalfa. Fast loss of highly acetylated replacement histone H3.2. J. Biol. Chem. 268: 4912–4917.

Waterborg, J.H. and Robertson, A.J. 1993. Efficient large-scale purification of restriction fragments by solute-displacement ion-exchange HPLC. Nucl. Acids Res. 21: 2913–2915.

Waterborg, J.H. and Robertson, A.J. 1996. Common features of analogous replacement histone H3 genes in animals and plants. J. Mol. Evol. 43: 194–206.