Structural Characteristics of Two Wheat Histone H2A Genes Encoding Distinct Types of Variants and Functional Differences in Their Promoter Activity

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To investigate the regulation of plant histone H2A gene expression, we isolated two H2A genes (TH254 and TH274) from wheat, which encode different types of variants. Both genes had an intron in the coding region. In the promoters, some characteristic sequences, such as Oct and Nona motifs, which are conserved among plant histone genes were also found, and they were located in a short region (about 120 bp) upstream from the putative TATA box. Analyses of promoter activity with H2A-GUS fusion genes in the transient system using tobacco protoplasts revealed novel types of positive *cis*-acting sequences in the TH254 promoter: a direct repeat of a 13-bp sequence (AGTTACATTATTG) and a stretch composed of an AT-rich sequence (ATATAGAAAATTAAAA) and a G-box (CACGTG). A quantitative S1 assay of the mRNA amounts from the TH254/GUS and TH274/GUS chimeric genes in stably transformed and cell cycle-synchronized tobacco cell lines showed that the promoters of both genes contained at least one *cis*-acting element responsible for S phase-specific expression. Histochemical analysis of transgenic tobacco plants carrying the chimeric genes showed that the promoters of the two H2A genes were both active in developing seedlings and flower organs but regulated in different manner.

Histones are highly conserved eukaryotic proteins, constituting nucleosomes with the chromosomal DNA. Histone genes are known to form multigene families and are divided into two groups based on their expression patterns. One class of genes encode replication-dependent histone variants, which are predominantly expressed during the S phase along with DNA synthesis and the other encodes replication-independent or tissue-specific variants expressed constitutively throughout the cell cycle or expressed transiently and post-proliferatively after the onset of differentiation (Stein et al., 1992). It has been revealed by studies mostly with yeast and mammals that histone gene expression is regulated at both transcriptional and post-transcriptional levels (Heintz, 1991: Osley, 1991: wolff 1994). However, current knowledge about the regulation of plant histone gene expression is still poor.

Studies of plant histone genes, which were initiated with the wheat histone H3 and H4 genes and subsequently done with other monocot and dicot genes, have revealed both unique and conserved features in their structure and expression, compared with mammalian and yeast histone genes (Nakayama and Iwabuchi, 1993). From the analyses of the H3 and H4 genes of wheat and maize, Hex (CCACGTCA), Oct (CGCGGATC), and nonamer (Nona, CATCCAACG) motifs have been identified as positive cis-acting elements (Lepetit et al, 1993: Nakayama et al., 1992). Among them, Oct is the most conserved motif, and has been found in all plant histone promoters sequenced so far (Chaboute et al., 1993). A reverse-oriented Oct is often paired with Hex by a 2-bp interval to form the type I element (CCACGTCANCGATCCGCG) (Mikami et al., 1993), which has been shown to contribute to the S phase-specific and meristem-specific expression of the wheat H3 gene TH012 (Terada et al., 1995: Ohtsubo et al, submitted). Oct is also present as the type II element with a 3-bp extention of conservation (TCACGCGGATC) or the type III element in which the CCAAT sequence is located

just 15 bp away (Mikami et al., 1993: Yang et al., 1995). However, the importance of the type II and III elements in histone gene regulation is not known. The Hex and Nona motifs are often, but not always, found and have been implicated in the replication-dependent activation of the promoter (Brignon and Chaubet, 1993: Terada et, 1995: Ohtsubo et al., submitted). It has been shown that HBP-1a and -1b-type bZIP transcription factors and the nuclear DNA-binding activity HBP-2 interact specifically with Hex and Nona, respectively (Iwabuchi et al., 1996).

In contrast to the histone H3 and H4 genes, only a few genes for H1, H2A and H2B have been identified, i.e., *Arabidopsis* and tomato H1 genes (Gantt and Lenvik, 1991: Jayawardene and Riggs, 1994), wheat H2B genes (Yang et al., 1995) and a part of the PCR-amplified H2A gene of Norway spruce (Sundas et al., 1993), so not much is known about the gene regulation of these classes of histones. We previously isolated wheat cDNA clones encoding two structurally different types of H2A proteins (Huh et al., 1995). A remarkable feature is that the C-terminal variable region of the type 1 H2As carries one or two copies of the SPKK motif, which is known to interact with the minor groove of DNA (Suzuki, 1991), whereas the type 2 H2A proteins have a shorter C-terminal variable region and no SPKK motif there. A larger amount of mRNAs for the type 1 H2As was detected in portions of wheat plants rich in proliferating cells and their accumulation level was changed, coupling with DNA synthesis during the early stage of germination. In contrast, a considerable amount of the mRNA for a type 2 H2A was also found in tissues with only a few actively dividing cells and the amount did not change along with DNA synthesis.

In the present paper, we have identified two H2A genes, which encode type 1 and type 2 H2A variants, and performed structural and functional analyses of their promoters. Results have shown that both promoters have several characteristic sequences inplicated in the promoter activity and have at least one element responsible for cell cycle-dependent expression. In addition, the histochemical study of transgenic tobacco plants has shown that promoter activity of the two H2A genes is differently regulated in seedlings and flower organs.

MATERIALS AND METHODS

Isolation of H2A Genomic Clones and DNA Sequencing

All the recombinant techniques used were according to (Sambrook et al., 1989) unless otherwise specified. A wheat genomic DNA library in EMBL-3 (Yang et al., 1995) was initially screened with the coding region of a wheat H2A cDNA, wcH2A-2, as a probe as described previously (Huh et al., 1995). In further purification of positive plaques, specific probes derived from the 3'-untranslated sequences of two wheat H2A cDNA clones, wcH2A-4 and wcH2A-9 were used. Two positive clones λTH254 and λTH274 were isolated with wcH2A-4 and wcH2A-9-derived probes, respectively, from approximately 5×10^6 phage. After three rounds of plaque purification, the inserts were cut out with *Sal*I, recloned into the *Sal*I site of pUC118 and sequenced by the dideoxy method.

Construction of Plasmids

Chimeric translational fusion genes, TH254/GUS and TH274/GUS, consisting of an H2A promoter and the coding region of the GUS gene were constructed by inserting the 1.2 kb *Eco*RI-*Pst*I fragment of TH254 (from base -1102 to +73) into the *Pst*I-*Xba*I sites of pBI221 (Jeffeerson, 1987) after both fragments were blunt-ended and by inserting the 1.8 kb *Hin*dIII-blunt-ended *Bst*PI fragment of TH274 (from base -1789 to +41) into the *Hin*dIII-*Sma*I sites of pBI221. The GUS proteins expressed from these chimeric genes were expected to have 24 (for TH254/GUS) and 14 (for

TH274/GUS) amino acids derived from the N-terminal portions of the corresponding H2A proteins. A 5'-deletion series of H2A/GUS constructs was prepared by using exonuclease III and mung bean nuclease and deletion end points were determined by sequencing. The resulting constructs were named 5' Δ-xxTH254G and 5'Δ-xxTH274G, where xx corresponds to the 5' end of the deleted promoters.

The *Sph*I-*Bam*HI fragment of 5'Δ-544TH254G containing the bases between -544 and -247 was purified, further digested with *Afa*I, *Mbo*II and *Taq*I, and blunt-ended to prepare three short fragments, A (from base -544 to -485), B (-484 to -381) and C (-379 to -333), which were inserted into the filled-in *Sph*I site of 5'Δ-141TH254G located just upstream of the truncated promoter to generate A/-141TH254G, B/-141TH254G and C/-141TH254G.

Base substitution mutations were introduced by the oligonucleotide-aided method (Kunkel, 1985). Template ssDNA was derived from plasmids constructed by inserting the *Hin*dIII-*Eco*RI fragment of B/-141TH254G or C/-141TH254G into pUC119. Synthetic oligonucleotides used were:

mDR 5- CCACCAATCATGTACCTTTGCCTTAGAAAACAAGAATGTCACTCCCGAC - 3,

mAT 5- TGGCACGTGTTTTCCTGTTCTATATGGCCCAC - 3,

mG 5- ACGGAGTGGTTACTTGTTTTAATTTTC - 3,

mAT/mG. 5 - ACGGAGTGGTACTTGTTTTCCTGTTCTATATGGCCCA - 3.

For Ti plasmid-mediated transformation, the *Eco*RI-*Hin*dIII fragment of TH254/GUS and the *Eco*RI fragment of 5Δ'-658TH274G, containing the H2A promoter, the GUS coding region and the *nos* terminator, were recloned into the *Eco*RI-*Hin*dIII sites and the *Hin*dIII site of the binary vector BIN19 (Bevan, 1984) to make pB1102TH254G and pB658TH274G.

Transient Gene Expression in Tobacco Protoplasts

Preparation of protoplasts from suspension-cultured tobacco BY2 cells, electroporation of plasmid DNA and fluorometric mesurement of GUS activity were all carried out according to the method described by (Yang et al., 1995).

Preparation of Stably Transformed BY-2 Cells and Synchronization of DNA Synthesis

Transformation of suspension-cultured BY2 cells with *Agrobacterium tumefaciens* LBA4404 carrying pB1102TH254G or pB658TH274G and the culture of transformed cells were carried out essentially according to the method described by (Matsuoka and Nakamura, 1981). GUS-positive and kanamycin-resistant calli were selected as stable transformants and 40 – 60 independent calli were collected and cultured. Synchronization of the cell cycle of suspension-cultured cells with aphidicolin and the measurement of the rate of DNA synthethis by [^{3}H]thymidine pulse-labeling were performed essentially as described by Ohstubo et al. (1993).

Nuclease S1 Assay

Total RNAs were prepared from transformed BY-2 cells using the TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instruction. The transcripts from H2A/GUS chimeric genes and the NPT-II gene were quantified by the S1 nuclease assay as described by Ohstubo et al. (1993). Single-stranded uniformly labeled DNAs covering the region around the transcription intitiation site were synthesized from pUC118-derived ssDNA templates with the Klenow fragment. After S1 digestion, protected DNAs were separated by polyacrylamide gel electrophoresis and their radioactivity was measured with a Fuji Imaging analyzer.

Preparation of Transgenic Tobcco Plants

A tobacco cultivar SR1 was transformed with *Agrobacterium tumefaciens* LBA4404 harboring pB1102TH254G or pB658TH274G by the leaf disc method [9]. Seeds of primary transformants were obtained by self-pollination. Histochemical analysis of the GUS activity in T2 plants was performed as described by Benfey et al. (1989) and Jefferson, (1987).

RESULTS

Characterization of Two H2A Genes Encoding Distinct Types of Variants

To know the structures of wheat H2A genes corresponding to type 1 and type 2 variants, we screened a genomic library with 3'-untranslated sequences of representative cDNAs for the two variants as probes (wcH2A-9 for a type 1 and wcH2A-4 for a type 2 (Huh et al., 1995)). Two genomic clones were identified and the regions containing the H2A genes (TH254 and TH274) were sequenced (Figure 1A). Comparison of the genomic and cDNA sequences revealed that both genes isolated contained an intron in the coding region, which were 266 bp in TH254 and 95bp in TH274 in length and had the canonical 5' and 3' splicing junctions. Interestingly, the location of the intron was the same not only for both genes but also a gymnosperm H2A gene from Norway spruce (Sundas et al., 1993) with respect to the encoded H2A protein sequences. TH254 and TH274 encode 135-amino-acid type 2 and 146-amino-acid type 1 H2As, respectively. That is, the TH274-encoding H2A has high similarity with type 1 H2As (99% for wcH2A-9, 93-95% for other wheat H2As and 71-76% for the other angiosperm H2As) and two SPKK motifs near the C-terminus. On the other hand, the TH254-encoding H2A has a shorter C-terminal variable region and no SPKK motif. Sequence similarity of the TH254-encoding H2A was 89% and 75% for wcH2A-4 and Norway spruce type 2 H2As, respectively, and 60-63% for the type 1 H2As.

Many characteristic motifs common in many plant histone genes were also present in the 5' upstream region of each gene (Fig. 1B). A putative TATA box is located about 120-130 bp upstream of the first Met codon. Three and 4 copies of the Oct motif (CGCGGATC and its complement), which have been identified in all the plant histone gene promoters sequenced so far, are found in the proximal promoter regions of TH254 and TH274, respectively, and two and one of them are present as the type II element (TCACGCGGATC). Neither type I nor type III element was found. As for other cis-acting elements often found in the promoters of plant histone genes, the Nona and CCGTCC motifs and derivatives there of were present, some of which partly overlap with each other or with an Oct motif. These motifs may also be involved in regulation of the TH254 and TH274 genes.

Functional Analysis of Two H2A Gene Promoters

To examine the function of two H2A gene promoters, the H2A/GUS chimeric genes, consisting of the TH254 or TH274 promoter and the GUS-coding region, were prepared (-1102TH254/GUS and -1789TH274/GUS) and the promoter activity was investigated by measuring transiently expressed GUS activity in tobacco protoplasts. As shown in Figure 2, a high level of GUS expression was observed for both chimera, indicating that the activity of the TH254 and TH274 promoters was at a comparable level to that of the strong promoter for the CaMV 35S RNA: 110% for -1102TH254/GUS and 56% for -1789TH274/GUS.

To roughly localize the regulatory regions important for such high levels of promoter activity, a series of 5' deletion mutants was prepared and their promoter activity was examined (Figure 2). The TH254/GUS-derived mutants with a deletion from -1102 to -835, -545 and -355 showed a decreased level of GUS expression, i.e., 90%, 72%, and 38%, respectively, compared with the full-sized construct, -1102TH254/GUS (Figure 2A). A marked reduction was brought about by the deletion from -544 to -355 (compare between the 5'Δ-544 and 5'Δ-354 mutants), suggesting the presence of (a) positive cis-acting sequence(s) in this region of the promoter. Further deletions from -354 to -142 had little affected on the activity, although the deletion from -354 to -248 slightly increased the GUS expression level. The 5'Δ-141 mutant, which had only the putative TATA box as the recognizable motif, still

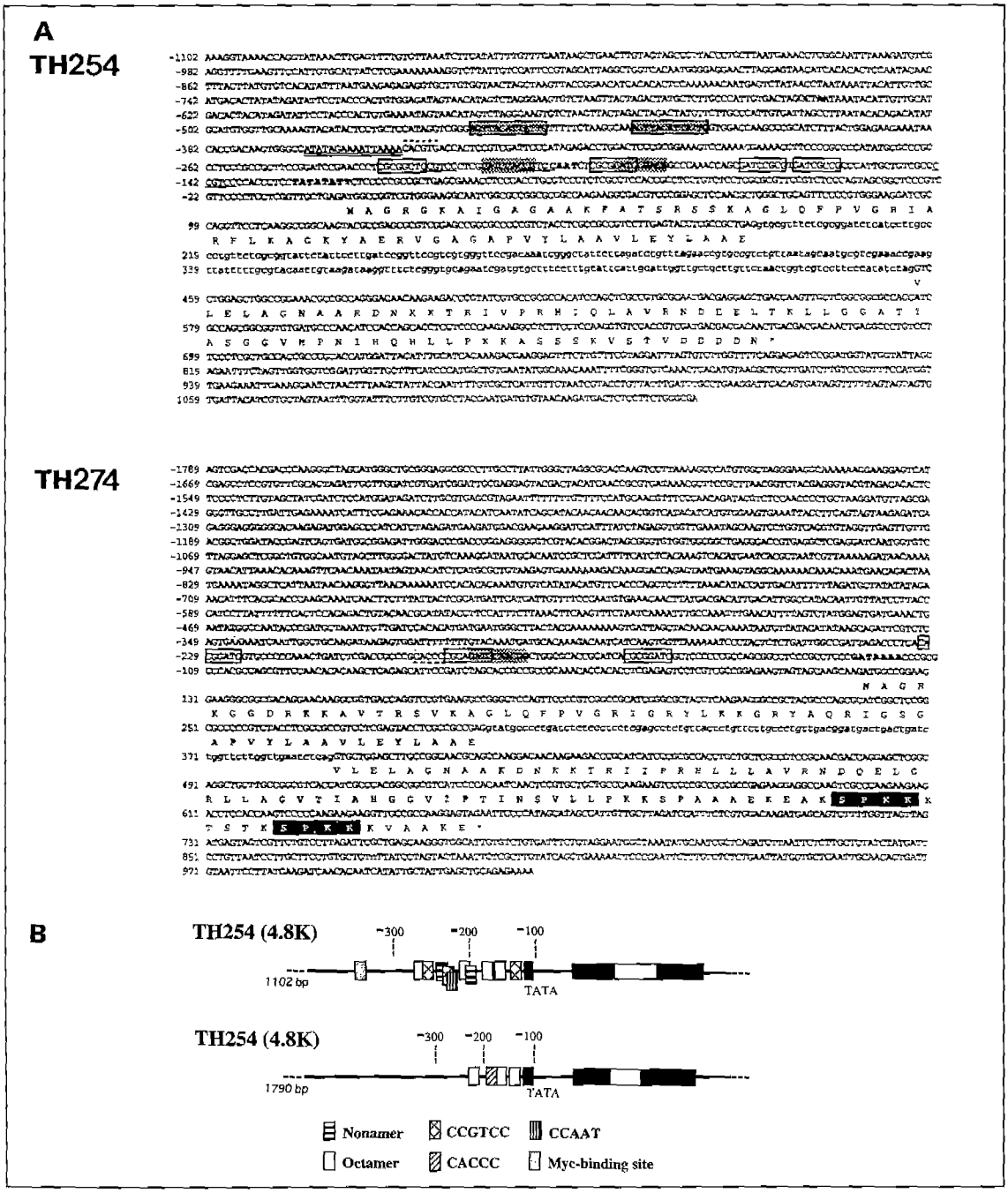


Figure 1. Structures of two wheat histone H2A gene variants (TH254 and TH274). A: Nucleotide and deduced amino acid sequences of TH254 and TH274 are represented. Amino acid sequences are shown with single capital letters and intervening nucleotide sequences are indicated with small letters. The nucleotide numbers refer to the positions relative to A(+) of the start codon ATG(+1). The asterisk denotes the stop codon. The SPKK motifs are black-boxed. The putative TATA sequence is presented with bold letters. Other characteristic sequences in the 5'-flanking region are shown by cross-hatched box for 13-bp direct repeat, double underline for AT-rich box, closed circles above sequence for Myc-binding site, open box for Oct motif, cross-hatched for nonamer motif, single underline for CCGTCC motif, and broken underline for CACCC motif.

B: Schematic representation of the TH254 and TH274 genes. Some characteristic sequences locating in their 5'-flanking regions are indicated by different small boxes. Exons and introns are represented by large open and close boxes, respectively.

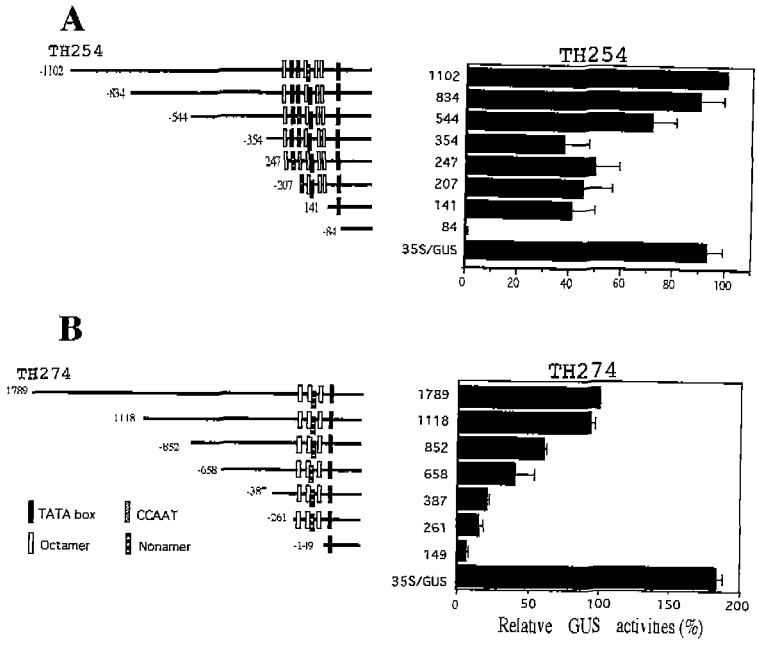
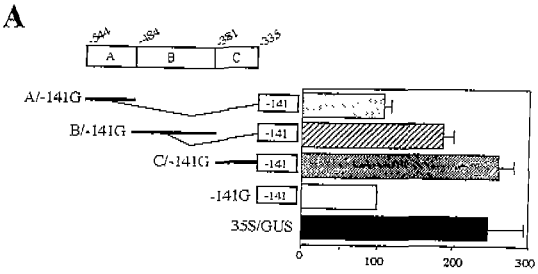


Figure 2. Changes in the activity of the 5'-deleted promoters of the H2A gene variants in tobacco (BY-2) protoplasts.
5'-deletion mutants and their activities in the TH254/GUS (A) and TH274/GUS (B) chimeric genes. The TATA box and conserved sequences are represented by small boxes. The numbers of deleted end-points refer to the position relative to the A site (+1) of the start codon ATG. 10 μg each of the reporter gene constructs was electrophorated into tobacco protoplasts (BY-2) as described by Yang et al. (1995). GUS activities are the average values of four independent assays and they are expressed relative to the value of the wild type promoter. Vertical bars show standard deviations.



B

B domain (-483 ~ -381)	13-bp direct repeat (DR) (AGTTACATTATTG $(N)_{14}$ AGTTACATTATTG)
C domain (-380 ~ -335)	AT rich box (ATATAGAAAATTAAAA)
	Myc-binding site (CACGTG)

Figure 3. Dissection analysis of the positive cis-acting region of the H2A variant (TH254) promoter.
A: Promoter activities of tested chimeric genes were examined by GUS activity as described in the legend to Figure 2. The positive cis-control region from -544 to -335 was dissected into three regions, A, B and C. Each of these dissected region was joined to the 5' end of the 5'D-141G chimeric gene which has the basal promoter of the H2A gene (TH254). 10 μg of report constructs transfected into tobacco protoplasts and meseared GUS activities. The relative GUS activity is the average of four independent assays.
B: Characteristic sequences (13 direct repeat, AT rich and Myc-binding sequences) being contained in the B (-463 to -381) and C (-389 to -335) regions.

yielded a significantly high level of the GUS activity. The mutant 5'Δ-84, without the TATA sequence, showed no GUS activity, suggesting that this sequence is the TATA box.

In the case of the TH274/GUS chimeric gene, the reporter GUS activity was lowered as the deletion became longer (Figure 2B). Significant decrease (about 60%) was observed by the deletion from -261 to -150 (compare between 5'Δ-261 and 5'Δ-149 mutants), where all the known motifs including the Oct and Nona motifs were located. Therefore, these motifs are suggested to be involved in the regulation of the TH274 gene.

Although no conserved motifs among plant histone genes could be found in the region from -544 to -355 of the TH254 promoter, the deletion experiment suggested the presence of (a) positive *cis*-acting element(s) there (Figure 2A). To confirm this and to further localize the responsible *cis*-acting regions, three subfragments, A (from -544 to -485), B (from -484 to -381) and C (from -379 to -333), were placed just upstream of the 5'Δ-141TH254G gene (named A/-141G, B/-141G and C/-141G, respectively). As shown in Figure 3, no significant

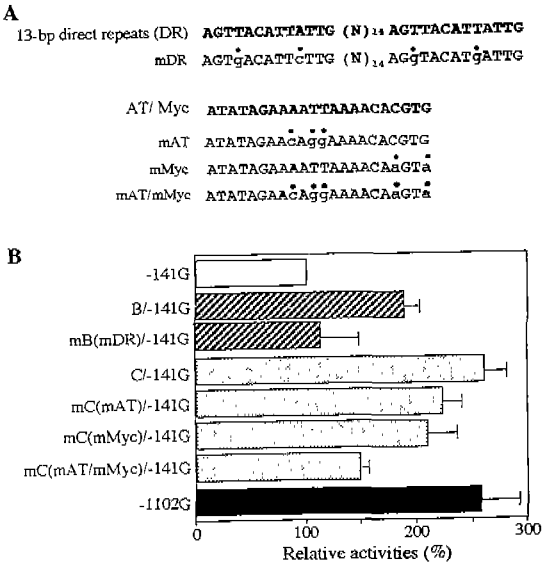


Figure 4. Effect of base-substitutions in 13-bp direct repeat and AT/Myc sequences of the H2A variant (TH254) promoter on its activity.
A: The base-substituted mutants of the 13 direct repeat and AT/Myc sequence. Substituted bases are shown by small letters.
B: GUS activites of the 5'D-141G chimeric genes to which the base-substituted 13-bp repeat and AT/Myc sequences were joined. The relative GUS activities are the average of four independent assays.

difference in GUS activity was observed between the 5'Δ-141TH254G and A/-141G genes. In contrast, the addition of fragment B or C resulted in 1.7- to 2.5-fold enhancement of GUS activity. It is marked that the construct C/-141G with only the 47-bp sequence from -379 to -333 gave almost the same level of GUS activity as the full-sized construct -1102TH254/GUS.

As characteristic sequences, fragment B has a direct repeat consisting of a 13-bp sequence (AGTTACATTATTG) and fragment C has an AT-rich sequence (ATATAGAAAATTAAAA) *flanking* a G-box (CACGTG). To learn whether these three sequences were involved in the enhancing effect of the B and C fragments, base-substitution mutations were introduced into the B/-141G and C/-141G constructs and GUS expression from the resulting mutants was examined (Figure 4). When the direct repeat in the B/-141G construct was point-mutated (the mB (mDR)/-141G construct), relative GUS activity was reduced to the level of the 5'Δ-141TH254G gene (-141G in Figure 4). Therefore, the direct repeat functions as a positive *cis*-acting element. Regarding fragment C, mutation into either the AT-rich sequence (mC (mAT)/-141G) or the G-box (mC (mG)/-141G) reduced GUS activity about 20%. Simultaneous introduction of mutations (mC (mAT/mG)/-141G) resulted in a more reduced level of activity but was still higher than the control (-141G). These observations suggest that both AT-rich and G-box sequences function additively and that there may be another element in fragment C that is able to enhance the promoter activity.

Cell Cycle-Dependent Activation of the H2A Promoters

We have recently shown that the type I element can mediate S phase-specific transcription in rice (Ohtsubo et al., submitted). However, neither TH254 nor TH274 gene contain the type I element, so we did experiments to address the question whether the two H2A gene promoters have the ability to confer S phase-specific expression. Suspension-cultured tobacco BY-2 cells were transformed with TH254/GUS and TH274/GUS chimeras and stably transformed cell lines were established. DNA synthesis of transformed cells was synchronized by the aphidicolin method (Ohstubo et al., 1993): the cell cycle is arrested at the G1/S boundary in the presence of aphidicolin and upon removal of aphidicolin DNA synthesis resumes. At 1-2 h after the removal, the rate of DNA synthesis reaches its maximum and then rapidly decreases.

The amount of transcript from the -1102TH254G gene, which was quantified by the S1 nuclease assay, increased as much as 13-fold at 1 to 2 h after release of aphidicolin and thereafter decreased rapidly (Figures 5A and B). This pattern is in parallel with that of the DNA synthesis examined by pulse-

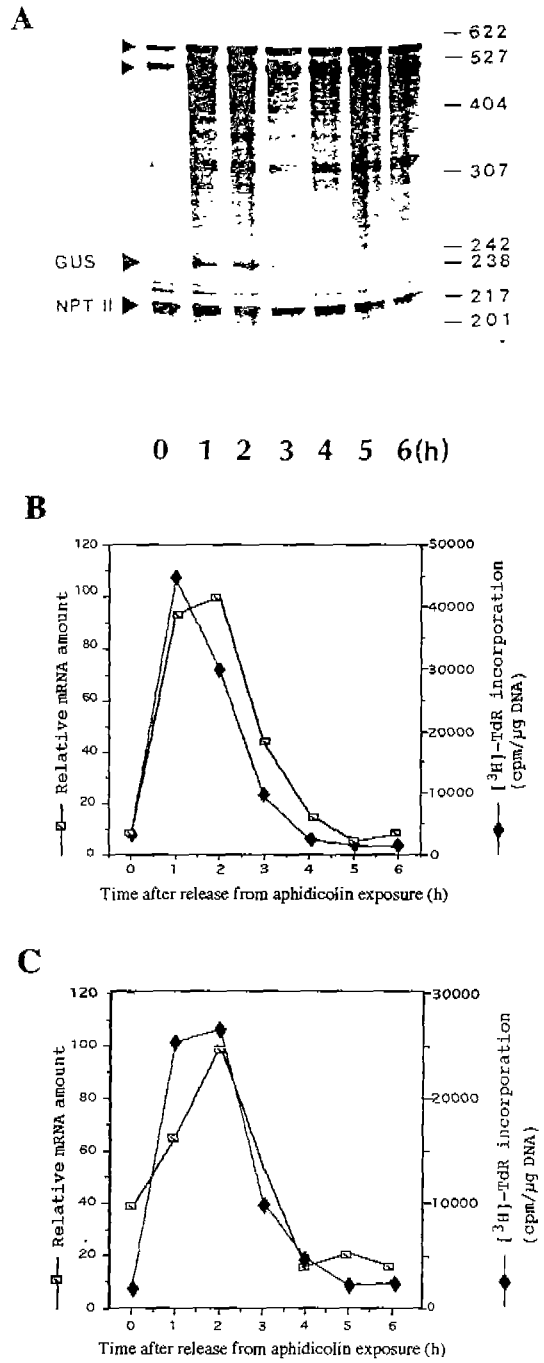


Figure 5. Changes of mRNA levels of integrated H2A/GUS genes and DNA synthesis in stably transformed tobacco cells after the aphidicoline treatment.
A and B: mRNA level and DNA synthesis in transformed cells with -1102TH254G. The autoradiogram shows the S1 pattern of transcripts from H2A/GUS gene and neomycine phoshotransferase (NPT)-Ⅱ gene which was deriven by the NOS promoter as an internal control for cell cycle-independent expression. The graphic representation indicates changes in the relative amount of the H2A/GUS mRNA, which was corrected by the NPT-Ⅱ mRNA, and that of DNA synthesis (H^3-thymidin incorporation into DNA of transformed cells).
C: mRNA level and DNA synthesis in transformed cells with -658TH274G. The relative amounts of the H2A/GUS mRNA and DNA synthesis were examined as described in A.

labeling with [^{3}H]thymidine. The same result was obtained with the transgenic line harboring the 5'Δ-247TH254G gene (not shown data). Therefore, it is highly probably that at least one *cis*-acting element involved in the S phase-specific expression is located downstream of position -247 of the TH254 gene.

Such S phase-specific induction of the promoter activity was also observed with the transformed cells carrying the 5'Δ-658TH274G gene (Figure 5C). Thus, we conclude that both genes bear the *cis*-acting element (s) to confer the cell cycle-dependent expression. Taken together with the previous observation that the accumulation level of the wcH2A-4 mRNA encoding a type 2 H2A was not correlated with DNA synthesis during an early stage of germination (Huh et al., 1995), it is suggested that there are both replication-dependent and -independent type-2 H2A variants.

Histochemical Analysis of the Function of the H2A Promoters in Transgenic Tobacco Plants

To know the regulation of the H2A gene expression during plant development, transgenic tobacco plants carrying the -1102TH254G or -658TH274G genes were produced by a Ti plasmid-mediated method and transgene expression in various tissues and organs was histochemically analyzed.

The same pattern of transgene expression was observed among independent transformants with the same construct, although the intensity of GUS staining was variable from one transformant to another. In younger seedlings of transgenics harboring

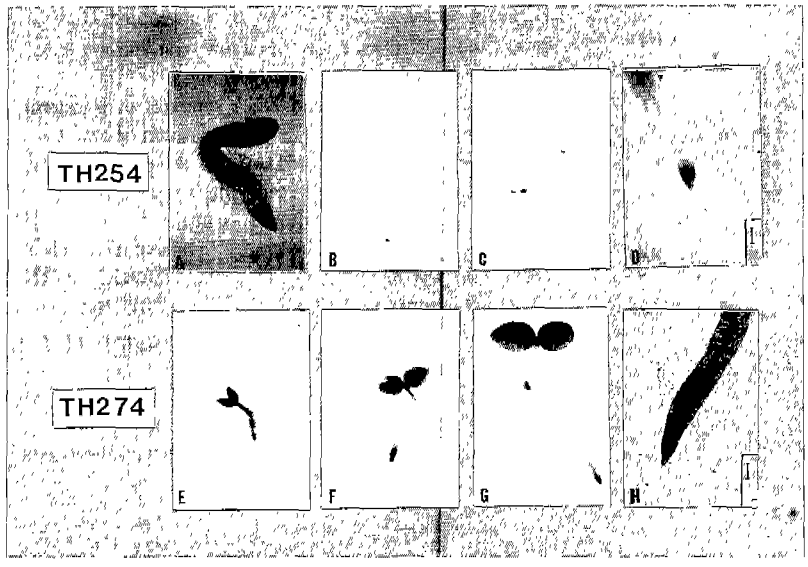


Figure 6. Histochemical localization of GUS activity in seedlings of transgenic tobacco plants with H2A/GUS genes.
A-D: GUS expression in -1102TH254G transformed tobacco seedlings.
E-H: GUS expression in -658TH274G transformed tobacco seedlings. Arrowheads in H indicate the lateral roots.

-1102TH254G, weak GUS staining was first observed in meristematic zones of both primary root tip and epicotyl (Figure 6A) and as plants grew, the level of GUS staining rose in the root meristematic region (Figure 6B and D). Around the time when lateral roots began to develop, GUS staining was no longer detected in the primary root tip but was instead visible in the lateral root primordia (Figure 6C). The TH254 promoter appeared to be developmentally regulated in young seedlings and was quite different from that of other plant histone genes (Atanassova et al., 1992; Terada et al., 1995; Yang et al., 1995).

In contrast, very young seedlings of transgenic plants with the -658TH274G gene showed a high level of GUS activity in the root tip, hypocotyl and cotyledons (Figures 6E and F). As plants grew, the GUS staining became restricted to the primary and lateral root tips, cotyledons and the apical meristem (Figure 6G and H). The GUS activity in the root tip including the meristem and root cap persisted throughout development but in the mature leaves no activity was detected (not shown). Such expression patterns in cotyledons and root tips was similar to that of the wheat H2B/GUS fusion gene in transgenic tobacco plants (Yang et al., 1995) and the wheat H3/GUS gene in transgenic rice plants (Terada et al., 1993).

Since the wheat H3 gene (TH012) promoter has been shown to be active in non-proliferating tissues such as the anther wall and the pistil as well as in proliferating tissues (Terada et al., 1993), GUS activity in the flower organs of transgenic tobacco plants with the H2A/GUS chimeric genes was investigated. In transgenics with the -1102TH254G gene, no GUS activity was detected in the stigma or style (Figure 7A), while the activity was observed weakly in the nectary of the ovary (Figures 7D) and strongly in pollen grains (Figure 7H). In transgenic tabaccos with the -658TH274G gene, strong GUS staining was observed in the secretory zone of the stigma (Figure 7B and C) and various ovarian tissues such as ovules, receptacles, vascular bundle, placenta and nectary (Figure 7F and G) but hardly detected in pollen grains (Figure 7I).

DISCUSSION

In the present study, we have isolated two genomic DNA clones each encoding a type 1 (TH274) or type 2 (TH254) H2A variant of wheat.

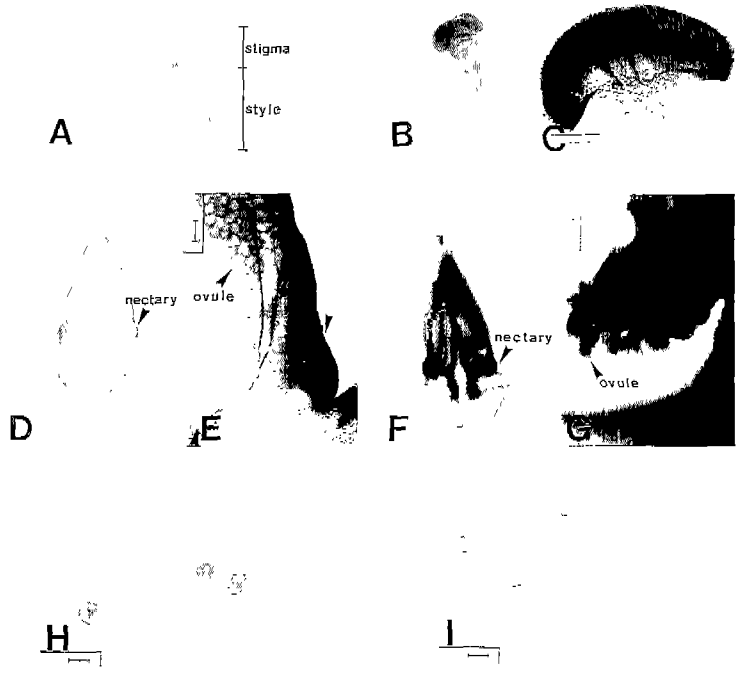


Figure 7. Histochemical localization of GUS activity in various flower organs of transgenic tobacco plants with H2A/GUS genes
A, D, E, and H: GUS expression in stigma (A and B), ovary (D and E), and pollen (H) of transformed plants with -1102TH254G.
B, C, F, G, and I: GUS expression in the stigma (B and C), ovary (F and G), and pollen (I) of transformed plants with -658TH274G. Samples in B, C, F, G and H are longitudinal sections of the organs.

Promoter analysis with the H2A/GUS chimeric genes showed that the promoters of both genes are active in tobacco, and regulated cell cycle-dependently coupling with DNA synthesis. Histochemical GUS staining of transgenic tobaccos with these chimeric genes showed distinct regulation of the two promoters.

Both TH254 and TH274 genes contain an intervening sequence at the same position in the coding region as that found in a Norway spruce H2A gene (Sundas et al., 1993). Introns have been found in some genes for plant histone H1 and H3 (Chaubet et al., 1992: Gantt et al., 1991: Jayawardane and Riggs, 1994: Kanazin et al., 1996). Expression of the intron-containing Arabidopsis H3 genes has been suggested to be replication-independent (Chaubet et al., 1992), while proliferation-dependent expression of the Norway spruce H2A and tomato H1 genes has been demonstrated (Sundas et al., 1995) or strongly suggested (Jayawardene and Riggs, 1994). Our observation that the chimeric genes with the TH254 or TH274 promoter were predominantly expressed during the S phase implies that the presence of an intron is not necessarily related with replication-independent expression. Further, the coincidence of the intron position in the two wheat (monocot) and one Norway spruce (gymnosperm) H2A genes suggests that the intron was already present before the type 1 and type 2 H2A variants were diverged.

The promoter sequences of the TH254 and TH274 genes are not similar to each other and this could be related to the distinct expression patterns between the TH254/GUS and TH274/GUS genes in the floral organs as well as in young seedlings of transgenic tobaccos (Figures 6 and 7). On the other hand, both promoters have the ability to confer S phase-specific expression in transgenic suspension-cultured cells and to direct expression in the root meristems of transgenic plants (Figures 5 and 6). These common features of the promoter function could result from the sequence motifs shared between the two promoters. Of the motifs found in many plant histone genes, some of which has been implicated in the histone gene regulation (Brignon and Chaubet, 1993: Nakayama and Iwabuchi, 1993),

the Oct, Nona, CCGTCC, and CCAAT motifs are present in TH254 and the Oct, Nona, and CACCC motifs are in TH274. It is interesting to note that all of these motifs are clustered within about 100 bp just upstream of the TATA element. It is therefore likely that they specify the basic promoter functions of the two H2A genes.

The type I element has been shown to be involved in the S phase-specific as well as meristematic tissue-specific expression of the wheat H3 gene (TH012) (Terada et al., 1995: Ohtsubo et al., submitted). However, neither the TH254 nor TH274 promoter has the type I element. Therefore, another *cis*-acting element conferring S phase specificity must be located downstream of position -247 in the TH254 promoter and position -658 in the TH274 promoter, where all the above-mentioned conserved motifs are included. Considering the universality of the Oct motif in plant histone gene promoters and its indispensability in type I-mediated S phase-specific expression, Oct is a probable candidate for the element involved in the S phase-specific expression of the TH254 and TH274 genes. Of particular interest is the type II element (TCACGCGGATC), an 11-bp conserved sequence containing an Oct motif, which is found twice in TH274 and once in TH254. A 126-bp promoter region of the *Arabidopsis* H4 gene (H4A748) has been shown to contain elements involved in preferential expression in meristems (Atanassova et al., 1992), where a type II element can be found. The Nona motif has been shown to function as a positive *cis*-acting element 8Lepetit et al., 1993: Nakayama et al., 1992) and suggested to be involved in proliferation-dependent expression (Brignon and Chaubet, 1993). The TH254 and TH274 promoters contain two and one Nona motif, respectively, with an overlapping CCGTC or Oct motif. Involvement of the Nona will have to be considered in future works.

The 5'-deletion and gain-of-function analyses of the TH254 promoter revealed three positively functioning cis-acting elements in the region between -544 and -355: a 13-bp direct repeat, an AT-rich sequence and a G-box. Since a deletion mutant lacking this region still showed S phase-specific expression, these elements may be involved in enhancing promoter activity and/or in tissue-specific expression. In fact, while the GUS expression pattern of the 5'Δ-544TH254G-transformed tobaccos was essentially the same as that of the tobaccos carrying the full-sized construct -1102TH254G, tobaccos with a 5'Δ-544TH254G-derived mutant lacking the sequence between -247 and -142 showed GUS activity only in pollen grains and no other tissues examined (data not shown).

Histochemical analysis of tobacco plants transformed with H2A/GUS chimeric genes indicated that the H2A promoters of the two gene variants (TH254 and TH274) were active not only in meristematic tissues but also in non-proliferating tissues (Figures 6 and 7). However, their promoter activity and expression pattern were apparently different. Activity of the TH274 promoter, exemplified by preferential expression in meristematic tissues of young seedlings, was similar to that of the promoters of an *Arabidopsis* H3 (Atanassova et al., 1992), a wheat H3 (Terada et al., 1995) and two wheat H2B (Yang et al., 1995) genes. On the other hand, activity of the TH254 promoter was low and spaciotemporally restricted: that is, the transition of promoter activity from the primary root to the lateral roots was observed during the root development. In the flower organs, the TH254 promoter was highly active in pollen, whereas the TH274 promoter was active in both stigma and ovary but not in pollen. Thus, it is speculated that the two H2A variants are differently expressed in various cells and tissues.

Important functions of histones in the regulation of gene expression have been reported (Wolff, 1994). Differential expression of H2A variants is thought to cause small changes in nucleosome structure, which could result in a delicate effect on chromatin structures and consequently in altered gene expression. Two histone H2As encoded by the

TH254 and TH274 genes differ in the basic stretch of the C-terminal variable region which lies on the surface of the nucleosome core particle interacting with DNA. The C-terminal region of the TH274-encoding type 1 H2A contains two SPKK motifs conserved in angiosperm H2As, but the type 2 H2As including the TH254-encoding one do not have the SPKK motif. The SPKK motif has been shown to bind to the minor groove of DNA in A/T-rich regions (Suzuki, 1991) and reported that in the case of sea urchin H1, its phosphorylation affects the chromatin condensation/decondensation during spermatocyte maturation (Roth and Allis, 1992). The SPKK motif in plant histone H2As may also play an important role in conformational changes of the chromatin structure. The two types of the wheat H2A variants may be differently involved in gene expression and regulation through their differential expression as well as modification. Our previous work showed that the accumulation of the type 2 H2A-encoding wcH2A-9 mRNA was uncoupled with DNA synthesis (Huh et al., 1995). However, that the TH254 promoter had a potential to confer cell cycle-dependent regulation implies that the type 2 H2As contain both replication-dependent and -independent variants. Further work will be needed to elucidate the functional difference of the two types of H2As.

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