Supplementary Material

Supplemental Experimental Procedures

Vector construction and plant and cell transformation

The *HindIII-EcoRI* fragment adjacent to the left border of T-DNA from plasmid pBI121 consists of the *β*-glucuronidase (*gusA*) gene controlled by the *cauliflower mosaic virus* (CaMV) *35S* promoter (P_{35S}) and nopaline synthase terminator (*Tnos*). The neomycin phosphotransferase II (*nptII*) gene controlled by the nopaline synthase promoter and terminator adjoins the right border of the T-DNA. The 54 bp fragment (-49 to +5) of the *35S* promoter was cloned as a mini-promoter. The 834 bp *PNZIP* promoter was produced by PCR amplification of the *Pharbitis nil PNZIP* promoter (AF373414) (Yang et al., 2009). The 807 bp *DREB1* promoter was isolated by adaptor PCR of the *Gossypium hirsutum DREB* promoter (Shan et al., 2007). *HindIII-BamHI* fragments containing the P_{PNZIP}, P_{DREB} and $P_{mini35S}$ were respectively inserted into the P1 construct in pBI121 to produce the P4, P6 and P8 constructs. The TM6 MAR isolated form the tobacco NC89 was inserted into *HindIII* site at the upstream or/and *EcoRI* site at the downstream of the *gusA*-gene cassettes. The construct with RB7 (P2) was used as the control (Halweg et al., 2005). The PCR primers were shown in Supplementary Table S1.

The above 9 vectors were transferred into *Agrobacterium tumefaciens* LBA4404 by electroporation and then transformed into tobacco by the leaf-disc method (Horsch et al., 1985). Transformed plants were selected on MS medium containing 100 μg · ml^{-1} kanamycin and 250 μg · ml^{-1} carbenicillin. After regeneration, shoots were transferred to root-inducing medium for 2 to 3 weeks and then transferred to a greenhouse to generate T0 plants. T1

plants were obtained by *in vitro* sowing surface-sterilized seedlings of the inbred T0 plants on MS medium containing 100 mg · l^{-1} kanamycin to select transformed resistant plants.

Overlap PCRs were used to subclone the TM6II fragments with different deletions of the four motifs in Fig. 4, and the PCR primers were shown in Supplementary Table S1. These different deletions of TM6 were then inserted into the upstream of g*usA* gene under the control of *35S* promoter of binary vector pBI121.

GUS activity assays and histochemical staining

Protein concentrations were measured by ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE 19810, USA). The average GUS activity was obtained from 20 independent transformants and each assay was repeated three times. We calculated the GUS activity using only 16 lines without the two highest lines and two lowest lines.

Hand-cut sections or the whole tissues were incubated in a solution of 1 mM X-gluc in 50 mM sodium phosphate (pH 7.0) for 12h at 37˚C. Green tissues of tobacco seedlings were cleared of chlorophyll by incubation in 70% ethanol. The samples were observed and photographed.

Nuclei extraction and treatment with micrococcal nuclease

Nuclear pellets were suspended in buffer (20 mM Tris-HCl (pH 8.0), 5 mM NaCl, 2.5 mM $CaCl_2$) and 0.3 μl micrococcal nuclease (MNase) (TaKaRa, Japan). After addition of the 0.5U MNase, the digestion mixtures were incubated at 37˚C. Aliquots were taken from the digestion mixture at 0-, 5-, 10-, 15-, 20-, 25- and 30 min and terminated immediately with 20 mM EDTA. After extraction with phenol and phenol:chloroform:isoamyl alcohol (25:24:1), the target DNA fragments were precipitated with 0.3 mM NaAc (pH 5.2) and ethanol, dried

and suspended in 100 μl of TE.

The sepcific primers of P_{35S} and P_{NOS} were shown in Supplementary Table S1. The coding region of *tubulin* was used as an endogenous control. The PCR products were quantified with an Alpha Imager 1200 (Alpha Innotech Corp., San Leandro, CA). Alternately, the fragments were blotted onto GeneScreen Plus membrane (DuPont-New England Nuclear Research Products), probed with ^{32}P-labeled PCR products, and exposed to X-ray film, and the bands were scanned with a Molecular Dynamics 300S laser scanning densitometer. Hybridization and washing were performed as described previously (Zheng et al., 1998), and the radioactive signal was scanned by a phosphorimager FLA-7000 (FUJIFILM, http//www.fujifilm.co.jp).

Electrophoretic mobility shift assay (EMSA)

Reactions were incubated at room temperature for 15 min and the resulting protein-DNA complexes were electrophoresed in 6% native polyacrylamide gel. After electrophoresis, the gel was transferred to a nylon membrane by electro-blotting. Nylon membranes were rinsed briefly in washing buffer, and incubated for 30 min in anti-Digoxigenin-AP (1:10000) for 30 min. Then the membranes were equilibrated and placed on hybridization bag and CSPD working solution was applied. Finally, the membranes were exposed to X-ray film for 40 min.

The EMSA competitions were performed by adding unlabeled specific competitor DNAs (TM6II-1, TM6II-2 and RB7 fragments themselves) or nonspecific competitors (the labeled TM6II-2 or TM6II-1 probe, the labeled TM6II-1 or TM6II-2 probe) to the reactions and incubating the combined mixtures for an additional 10 min prior to electrophoresis.

Supplemental Figures

Fig. S1. Nucleotide sequence of TM6.

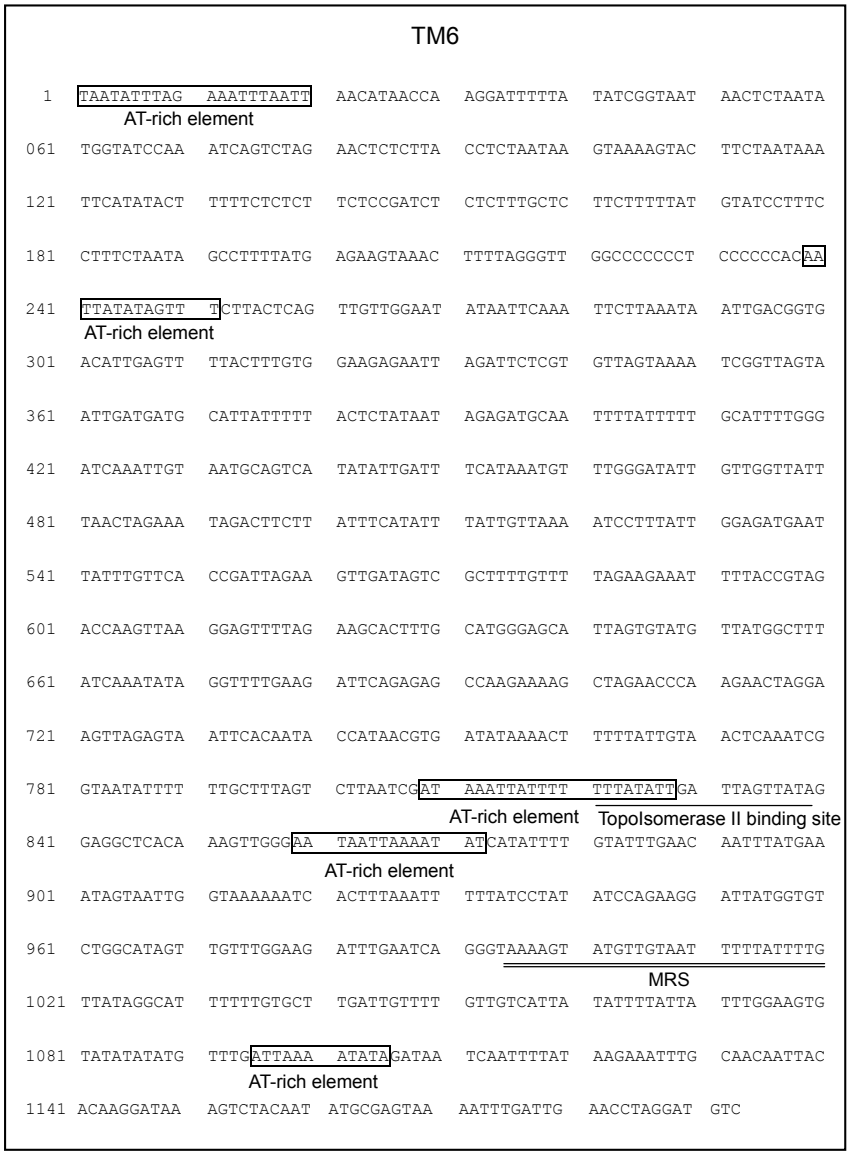
Fig. S2. Comprehensive representations of the methylation status of the sense strand in transgene *35S* promoter regions of transgenic tobaccos.

Bisulfite-PCR products from the full-length CaMV-*35S* promoter were subcloned; then six independent clones were showed for each line. H, transgenic lines with high GUS activity. L, transgenic lines with low GUS activity. "-", transgenic lines without TM6. "+", transgenic lines with TM6. Filled symbols, methylated cytosine residues. Red circles, CG sites. Blue circles, CHG sites. Green squares, CHH sites.

Fig. S3. The methylation status of the full-length *NOS* promoter in tobacco lines with (+TM6) or without TM6 (-TM6).

Fig. S4. Alignment of NtMBP1 with related proteins from other plant species.

The deduced amino acid sequence of NtMBP1 was aligned with PtHMGA (XP_002318524), RcHistone (EEF27887), AtHON4 (AT3G18035) and unknown protein in *Vitis vinifera* (CAO68173) using ClustalW with default parameters through EMBnet (http://www.ch.embnet.org/software/ClustalW.html). Black and gray shadings, done with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html), indicate conserved amino acid residues. NLSs predicted by the Psort algorithm (http://psort.nibb.ac.jp/form.html).


TM6
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AT-rich element
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121 TTCATATACT TTTTCTCTCT TCTCCGATCT CTCTTTGCTC TTCTTTTTAT GTATCCTTTC
181 CTTTCTAATA GCCTTTTATG AGAAGTAAAC TTTTAGGGTT GGCCCCCCCT CCCCCCACAA
241 TTATATAGTT TCTTACTCAG TTGTTGGAAT ATAATTCAAA TTCTTAAATA ATTGACGGTG
AT-rich element
301 ACATTGAGTT TTACTTTGTG GAAGAGAATT AGATTCTCGT GTTAGTAAAA TCGGTTAGTA
361 ATTGATGATG CATTATTTTT ACTCTATAAT AGAGATGCAA TTTTATTTTT GCATTTTGGG
421 ATCAAATTGT AATGCAGTCA TATATTGATT TCATAAATGT TTGGGATATT GTTGGTTATT
481 TAACTAGAAA TAGACTTCTT ATTTCATATT TATTGTTAAA ATCCTTTATT GGAGATGAAT
541 TATTTGTTCA CCGATTAGAA GTTGATAGTC GCTTTTGTTT TAGAAGAAAT TTTACCGTAG
601 ACCAAGTTAA GGAGTTTTAG AAGCACTTTG CATGGGAGCA TTAGTGTATG TTATGGCTTT
661 ATCAAATATA GGTTTTGAAG ATTCAGAGAG CCAAGAAAAG CTAGAACCCA AGAACTAGGA
721 AGTTAGAGTA ATTCACAATA CCATAACGTG ATATAAAACT TTTTATTGTA ACTCAAATCG
781 GTAATATTTT TTGCTTTAGT CTTAATCGAT AAATTATTTT TTTATATTGA TTAGTTATAG
AT-rich element
Topoisomerase II binding site
841 GAGGCTCACA AAGTTGGGAA TAATTAAAAT ATCATATTTT GTATTTGAAC AATTTATGAA
AT-rich element
901 ATAGTAATTG GTAAAAAATC ACTTTAAATT TTTATCCTAT ATCCAGAAGG ATTATGGTGT
961 CTGGCATAGT TGTTTGGAAG ATTTGAATCA GGGTAAAAGT ATGTTGTAAT TTTTATTTTG
MRS
1021 TTATAGGCAT TTTTTGTGCT TGATTGTTTT GTTGTCATTA TATTTTATTA TTTGGAAGTG
1081 TATATATATG TTTGATTAAA ATATAGATAA TCAATTTTAT AAGAAATTTG CAACAATTAC
AT-rich element
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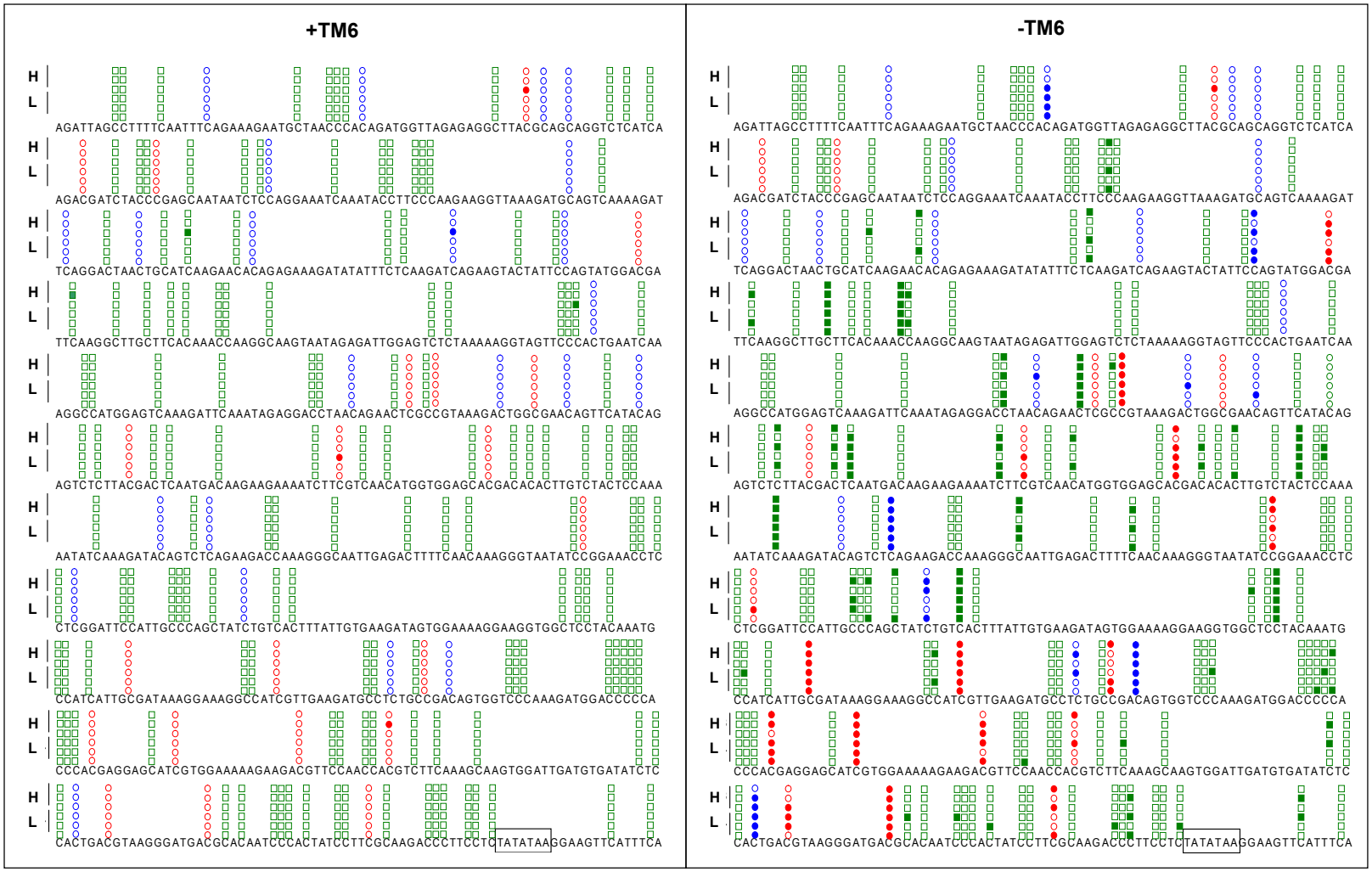
Fig. S1


+TM6
-TM6
H
L
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CTCGGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCCTACAAATG
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CCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGATTGATGTGATATCTC
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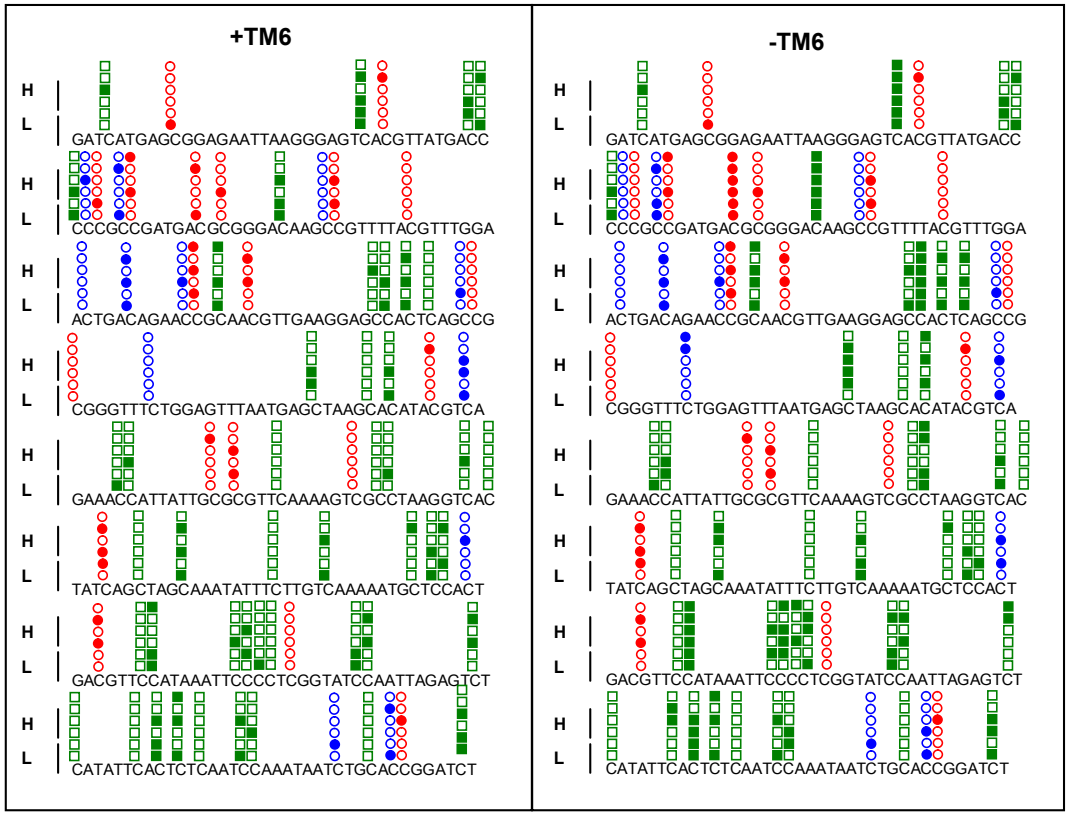
Fig. S2


+TM6
-TM6
H
L
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CCCGCCGATGACGCGGGACAAGCCGTTTTACGTTTGGA
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CGGGTTTCTGGAGTTTAATGAGCTAAGCACATACGTCA
GAAACCATTATTGCGCGTTCAAAAGTCGCCTAAGGTCAC
TATCAGCTAGCAAATATTTCTTGTCAAAAATGCTCCACT
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Fig. S3

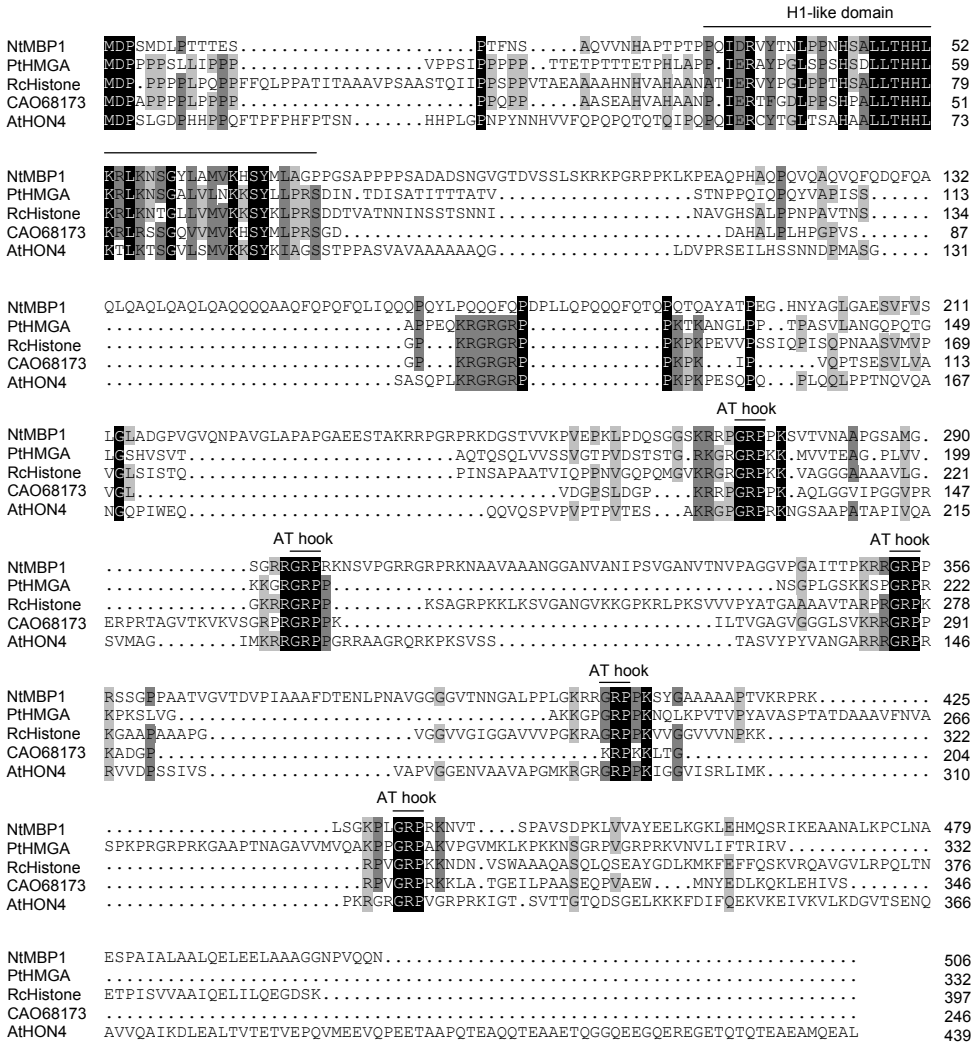
H1-like domain
AT hook
AT hook
AT hook
AT hook
NtMBP1
PtHMGA
RcHistone
CAO68173
AtHON4

Fig. S4

Table S1. Gene names and primer sequences used in experimental analysis in this article.

Sequence Name	Primer Sequence
CaMV 35 (P35S)	**F:** 5′-GGCCATGGAGTCAAAGATTC-3′; **R:** 5′-CCGTGTTCTCTCCAAATG-3′
Promoter of *PNZIP* gene (*PPNZIP*)	**F:** 5′-AAGCTTCAATCAAGCTGGCCTGTC-3′; **R:** 5′-GGATCCGGGTAGAGTGTACTGT-3′
Promoter of *DREB* gene (*PDREB*)	**F:** 5′-GTACATATTGTCGTTAGAACGCGTAATACGACTCA -3′; **R:** 5′-ATAATCTTGAACTACAAAATCCA -3′
mini35S (Pmini35S)	**F:** 5′-TGGAGAGAACACGGGGGACTCT-3′; **R:** 5′-AACATAAGGGACTGACCACCC-3
RB7	**F:** 5′-ATATTTGCGACTCTTCTGGC-3′; **R:** 5′-TCAGAAGAAGTTCCCAATAG-3′
Neomycin phosphotransferase II gene (*nptII*)	**F:** 5′-ATGATTGAACAAGATGGAT-3′; **R:** 5′-TCCCGCTCAGAAGAACTCGTC-3′
β-glucurindase gene	**F:** 5′-GGTGATTACCGACGAAAACG-3′; **R:** 5′-CGGTTTGTGGTTAATCAGGAAC-3′
Promoter of *nptII gene*(P_{NOS})	**F:** 5′-GGAGAATTAAGGGAGTCACG-3′; **R:** 5′-GTCAGTGGAGCATTTTTGAC-3′
TM6I (0 to 650 bp)	**R:** 5′-CATACACTAATGCTCCCATGC-3′
TM6II (651 to 1193 bp)	**F:** 5′-GTTATGGCTTTATCAAATATAGGT-3′
TM6I-1 (760 to 870 bp)	**F:** 5′-TTATTGTAACTCAAATCGGT-3′; **R:** 5′-ATTTTAATTATTCCCAACTTTGTG-3′
TM6I-2 (934 to 1013 bp)	**F:** 5′-CAGGGTAAAAGTATGTTGTAAT-3′; **R:** 5′-AAATAATAAAATATAATGACAAC-3′
TM6 deletion of MRS element (D-MRS)	**F:** 5′-GGAAGATTTGAATCAGGGGAAGTGTATATAT-3′; **R:** 5′-ATATATACACTTCCCCTGATTCAAATCTTCC-3′
TM6 deletion of AT-rich element (D-AT)	**F:** 5′-AGGGTAAAAGTATGTTGTGGCATTTTTTGTGCTT-3′; **R:** 5′-AAGCACAAAAAATGCCACAACATACTTTTACCCT-3′
TM6 deletion of Toposamerase II-binding site (D-Topo)	**F:** 5′-CATAACGTGATATAAAACTCGGTAATATTTTTTG-3′; **R:** 5′-CAAAAAATATTACCGAGTTTTATATCACGTTATG-3′
NtMBP1	**F:** 5′-ATGGACCCATCCATGGATCT-3′; **R:** 5′-ATTTTGCTGCACTGGATTCC-3′
NtHMBP	**F:** 5′-ATGAAAGGAGGTAAATCAAAGGC-3′; **R:** 5′-GTCATCATCTTCCTCCTCCT-3′
Methylation of *P35S* – 1(*MetP35S–1*)	**F:** 5′-AATTTATAGATGGTTAGAGAGGTTTA-3′; **R:** 5′-TCTATTACTTACCTTAATTTATAAAACAAA-3′
Methylation of *P35S* – 2(*MetP35S–2*)	**F:** 5′-ATTTAAATAGAGGATTTAATAGAATT-3′; **R:** 5′-TTAATATTTTTAAAATAAACAAATATATC-3′
Methylation of *P35S* – 3(*MetP35S–3*)	**F:** 5′-GACACACTTGTCTACTCCAAAAATATCAA-3′; **R:** 5′-TCAATAAAAATATCACATCAATCCA-3′
Methylation of *P35S* – 4(*MetP35S–4*)	**F:** 5′-TGGATTGATGTGATATTTTTATTGA-3′; **R:** 5′-AACATAAAAAACTAACCACCC-3′
Methylation of *P35S* – 4(*MetP35S–5*)	**F:** 5′-GAAAAGGAAGGTGGTTTTTATAAATG-3′; **R:** 5′-TCAATAAAAATATCACATCAATCCACT-3′
Methylation of *PNOS* – 1 (*MetPNOS–1*)	**F:** 5′-ATGGCGATGCCTGCTTGCCGAATATCATGGTG-3′; **R:** 5′-TCATAGAAGGCGGCGGTGGAATCGAAATCTCG-3′
Methylation of *PNOS* – 2 (*MetPNOS–2*)	**F:** 5′- TTTGGAGTTTAATGAGTTAAGTATATA-3′; **R:** 5′-ATACAAATTATTTAAATTAAAAATAAATAT-3′

Table S2. Percentage of cytosine methylation of the P_{35S} and P_{NOS} regions in transgenic lines.

	+TM6-H				+TM6-L				-TM6-H				-TM6-L			
	Total	CG	CHG	CHH	Total	CG	CHG	CHH	Total	CG	CHG	CHH	Total	CG	CHG	CHH
P_{35S}	3.3%	2.5%	0.8%	0	5%	4.2%	0.4%	0	26.5%	19.2%	6.7%	0.4%	33.9%	20.8%	9.2%	4.2%
P_{NOS}	6.7%	3.3%	2.5%	0	10%	6.7%	3.3%	0.4%	15%	10%	4.2%	0.4%	23.3%	14.1%	6.7%	3.3%

H, transgenic lines with high GUS activity.

L, transgenic lines with low GUS activity.

"+", transgenic lines with TM6

"-", transgenic lines without TM6.