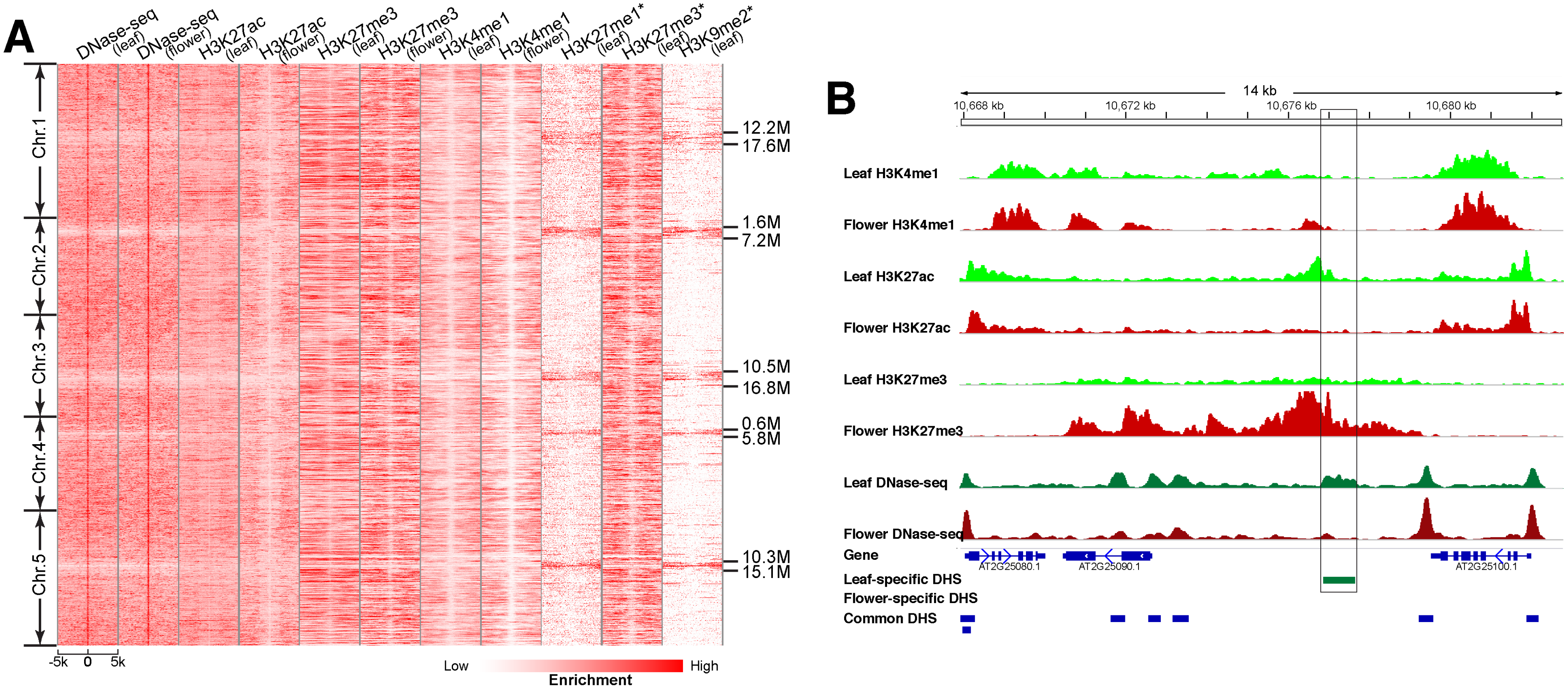
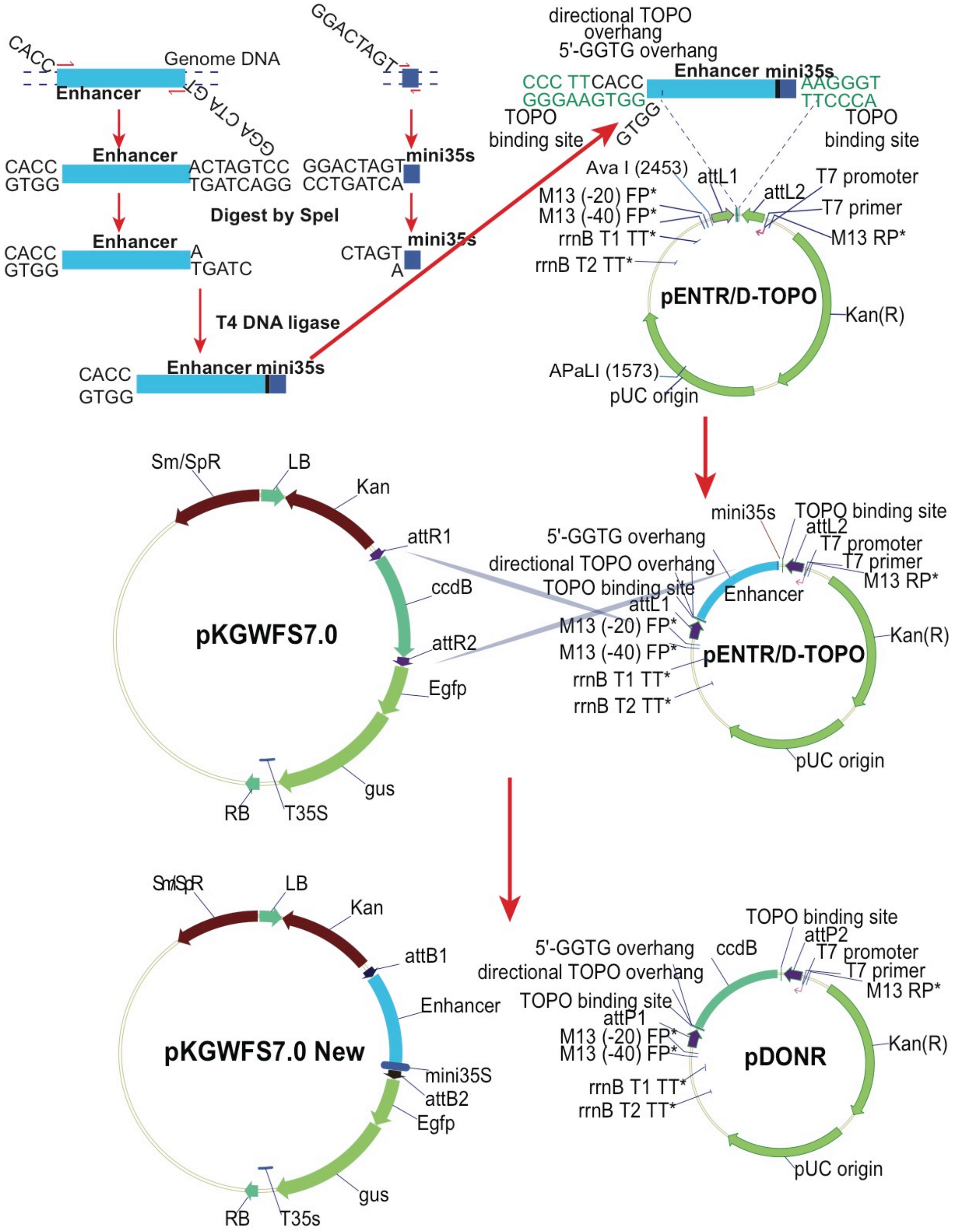
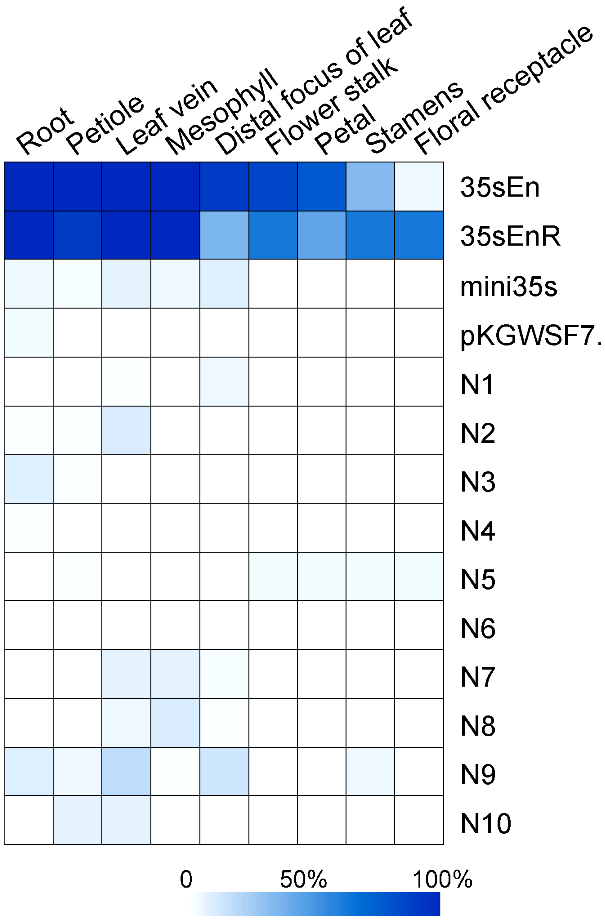


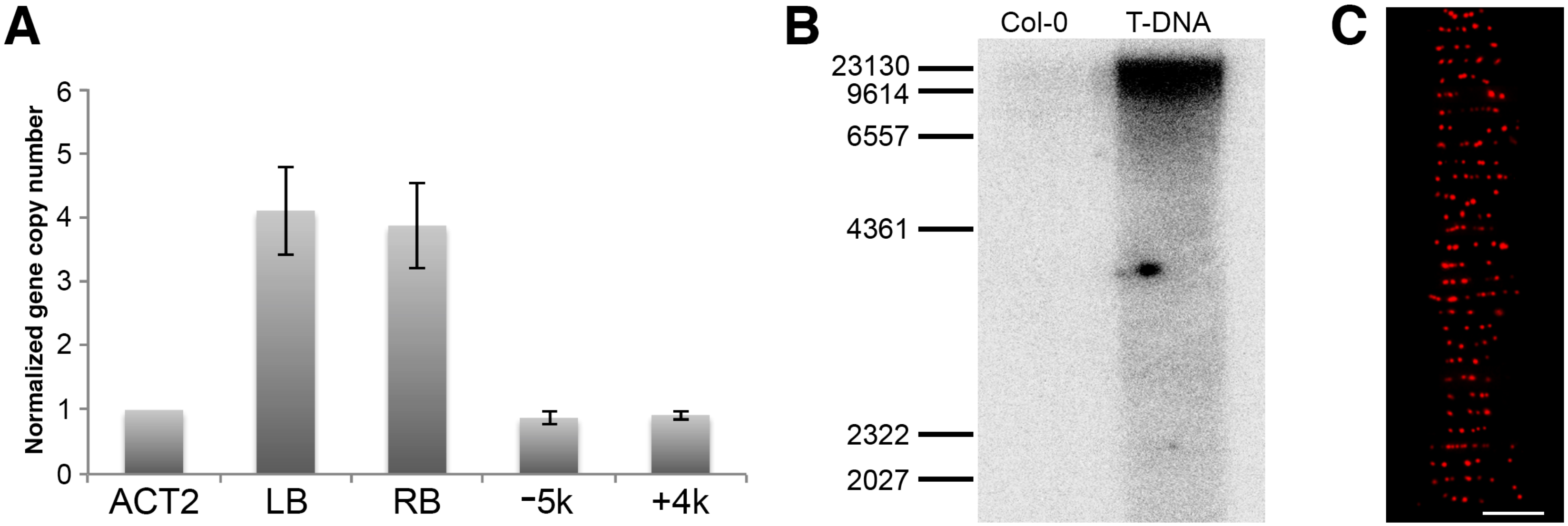
Supplemental Figure 1. Histone modification patterns associated with predicted enhancers in *Arabidopsis*. (**A**) Heatmaps of histone modifications associated with all predicted intergenic enhancers. A total of 10,044 predicted enhancers are arranged according to their positions on the five *Arabidopsis* chromosomes. The flanking regions (± 5 kb) of the predicted enhancers are enriched with H3K27ac and H3K27me3, but not with H3K27me1 and H3K9me2. Enrichment of H3K27me1 and H3K9me2 are only associated with the flanking regions of a small number of predicted enhancers located near the pericentromeric regions of the five chromosomes, which are marked by chromosomal positions (Mb) on the right side of the figure. Three datasets marked by "*" were generated by Luo et al. (2013, Plant J. 73: 77-90). The H3K27me3 data from Luo et al. showed a similar pattern as H3K27me3 data from this study. (**B**) An example of histone modifications associated with a single predicted leaf-specific enhancer.



Supplemental Figure 2. Diagram of the development of enhancer validation constructs. The genomic sequence of each predicted enhancer is isolated from *Arabidopsis thaliana* ecotype Col-0. The predicted enhancer region is amplified using a forward primer specific to the genomic sequence with the bases CACC attached to the 5’ end, and a reverse primer containing the *Spe*I restriction enzyme site GGACTAGT also attached to the 5’ end. The minimal 35S promoter region is simultaneously amplified from any plasmid containing the 35S promoter using a forward primer also including the *Spe*I site. These two amplified fragments are digested with *Spe*I, and then ligated together with T4 DNA ligase. The CACC sequence at the 5’ end of the enhancer fragment facilitates double stand invasion during the TOPO reaction. This directionally clones the enhancer sequence and minimal 35s into the entry vector pENTR/D-TOPO, and allows for downstream Gateway cloning. The entry vector containing the enhancer and mini35s insert is used in the LR recombination reaction along with pKGWFS7.0. The LR recombination replaces the ccDB gene in the destination vector (pKGWFS7.0) with the enhancer and mini35s sequence. The recombination places the enhancer and mini35s sequence upstream of the eGFP and β-glucuronidase reporter genes.



Supplemental Figure 3. Heatmap of GUS expression in different tissues of transgenic plants transformed with 10 non-enhancer constructs and control constructs. White indicates no GUS expression was detected in any plants. Blue indicates that GUS expression was detected in 100% of the transgenic plants.



Supplemental Figure 4. Characterization of the T-DNA inserted in L3 enhancer. (**A**) Quantitative PCR analysis of the copy number of the T-DNA insert. Two single-copy genes, -5k and +4k, which flank the L3 enhancer, and the ACT2 gene were used as controls. The error bars represent standard deviation based on qPCR data from three biological replicates. (**B**) Southern blot hybridization of T-DNA in the T-DNA line and wild type Col-0. A single hybridization band >20 kb is detected in the T-DNA line. (**C**) 30 independent fiber-FISH signals collected from the T-DNA line. Bar represents 10 μm, which are equivalent to approximately 30 kb.

Supplemental Table 1. Genomic locations and primers for all predicted enhancers and non-enhancer controls

Chromosome location	**Construct** (short name)	**Size** (bp)	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
Chr4_14841212_14841779	Common DHS 1 (C1)*	568	caccTGTGACCATCTTTTAAGGCGAATGA***	ggactagtTAGACAGACTTGAGAGCATTTGTGA
	Common DHS 1R (C1R)	568	ggactagtTGTGACCATCTTTTAAGGCGAATGA	caccTAGACAGACTTGAGAGCATTTGTGA
Chr5_23022352_23023035	Common DHS 2 (C2)*	684	caccTGGGTGCGACGACACCATAGTC	ggactagtGCCGGCCACAGTTAACCTCCT
	Common DHS 2R (C2R)	684	ggactagtTGGGTGCGACGACACCATAGTC	caccGCCGGCCACAGTTAACCTCCT
Chr2_11500116_11500529	Common DHS 3 (C3)*	393	caccGCTTGTAAGAACTCATGTATCC	ggactagtGTAATCGATGCTATGTTGTAGG
	Common DHS 3R (C3R)	ND	ND**	ND
Chr3_9043279_9043858	Common DHS 4 (C4)	580	caccTTCCCATTCATCCATTTGCG	ggactagtGTCGTAAAATGAAATTACAGAAGCA
	Common DHS 4R (C4R)	580	ggactagtTTCCCATTCATCCATTTGCG	caccGTCGTAAAATGAAATTACAGAAGCA
Chr4_17183498_17184077	Common DHS 5 (C5)	580	caccTGGTGGCTAAGTTTTCTCTCT	ggactagtGTCTTGGGAACAAGCGAAAG
	Common DHS 5R (C5R)	580	ggactagtTGGTGGCTAAGTTTTCTCTCT	caccGTCTTGGGAACAAGCGAAAG
Chr5_2247896_2248279	Common DHS 6 (C6)	384	caccCTGACGAGCGTATATTGATGC	ggactagtTGCAATGAAATTGAAGAACTGAA
	Common DHS 6R (C6R)	ND	ND	ND
Chr4_13816308_13816710	Common DHS 7 (C7)	403	caccATCCAGTGTTTGGTGTCCTA	ggactagtTCGCTGAATATTCGGATGGG
	Common DHS 7R (C7R)	ND	ND	ND
Chr2_17839521_17840082	Common DHS 8 (C8)	562	caccCTAGGAGGAGTTTGAGATG	ggactagtGAGCACAATGAGAGATTGCTGTTC
	Common DHS 8R (C8R)	562	ggactagtCTAGGAGGAGTTTGAGATG	caccGAGCACAATGAGAGATTGCTGTTC
Chr1_7700553_7701140	Leaf DHS 1 (L1)	588	caccAAACTGTAAACGTTTGGCTGAAAAA	ggactagtCGGACAATTAAACGTCTCCAATGC
	Leaf DHS 1R (L1R)	588	ggactagtAAACTGTAAACGTTTGGCTGAAAAA	caccCGGACAATTAAACGTCTCCAATGC
Chr2_14064942_14065385	Leaf DHS 2 (L2)	444	caccAGAGTCCAACGATTACGCAGC	ggactagtACAGATAAACTGTGGTACGGTTCT
	Leaf DHS 2R (L2R)	444	ggactagtAGAGTCCAACGATTACGCAGC	caccACAGATAAACTGTGGTACGGTTCT
Chr2_18722871_18723495	Leaf DHS 3 (L3)	625	caccAATCGGGATGGTCCGCATAA	ggactagtTGTACCGTTTTGTTTTTCTCAACCA
	Leaf DHS 3R (L3R)	625	ggactagtAATCGGGATGGTCCGCATAA	caccTGTACCGTTTTGTTTTTCTCAACCA
Chr1_17578056_17578677	Flower DHS 1 (F1)	622	caccTCTCGTAAACCAGCGCCGTC	ggactagtACGTCGATAAGTGTTTCGGCCA
	Flower DHS 1R (F1R)	622	ggactagtTCTCGTAAACCAGCGCCGTC	caccACGTCGATAAGTGTTTCGGCCA
Chr1_24662761_24663293	Flower DHS 2 (F2)	533	caccAGGCTGAAAACGAGATGTTATCG	ggactagtAGAGTAGATGTCTGTGCACAATGTT
	Flower DHS 2R (F2R)	533	ggactagtAGGCTGAAAACGAGATGTTATCG	caccAGAGTAGATGTCTGTGCACAATGTT
Chr1_27175205_27176010	Flower DHS 3 (F3)	806	caccTTTACGCTGTCGGGTGACGA	ggactagtTTTGTTACCATGTGTTGACAGTTCT
	Flower DHS 3R (F3R)	ND	ND	ND
Chr1_14153271_14153725	Non-DHS 1 (N1)	455	caccTGCACCATACCAATGTTGAAG	ggactagtCCGACGTTGGTTTGAAATGT
Chr1_21647295_21647699	Non-DHS 2 (N2)	405	caccGAGATGACGAGGATTGAAACG	ggactagtTGAAACCGATTATGTGTCAAAA
Chr2_15963538_15964250	Non-DHS 3 (N3)	713	caccGGATCGTCAGTAAGAGACCC	ggactagtACTCTCCATTTGCTCTACCG
Chr3_11223420_11223838	Non-DHS 4 (N4)	419	caccCAGTCCAACCAATCACCTTG	ggactagtTGCTTTAGCGAAATCTGCTC
Chr3_11903139_11903725	Non-DHS 5 (N5)	587	caccAAGCAAGGATACCAAGGGAG	ggactagtGTGTAATGAGCCCTACTGGG
Chr3_4842741_4843184	Non-DHS 6 (N6)	444	caccCCAACGAACTAAACGACTGC	ggactagtGTACTGCACAAATTCCGTGT
Chr5_10387620_10387978	Non-DHS 7 (N7)	359	caccGGCACACAAGGGATGAGATA	ggactagtACCAGTTGACCAAACCTGAT
Chr5_10388716_10389094	Non-DHS 8 (N8)	379	caccGTCCATTTTGGGTGCTTGAA	ggactagtCCAAGATGTGTTTGGTGCTT
Chr5_10423499_10423791	Non-DHS 9 (N9)	293	caccATGTTTGTTTGGTGTCCGTC	ggactagtTGACAGGATTTGGCAAGGTA
Chr2_15964858_15965334	Non-DHS 10 (N10)	477	caccGAAGCCGAAAAGTGAAGGTG	ggactagtAGTCAAAACCATCCAACAACC

* These three putative enhancers were identified previously by the traditional enhancer trapping method (Michael and McClung, 2003, Plant Physiology 132: 629-639).

** ND: not developed.

*** Low case letters represent added bases for cloning purposes, capital letters represent Arabidopsis genomic DNA sequences.

Supplemental Table 2. GUS expression in transgenic plants derived from all enhancer constructs and non-enhancer control constructs

Construct	Tissue with GUS expression in transformed T1 plants								
	Root (%)	**Petiole** (%)	**Leaf vein** (%)	**Mesophyll** (%)	**Distal focus of leaf** (%)	**Flower stalk** (%)	**Petal** (%)	**Stamens** (%)	**Floral receptacle** (%)
35sEn	100 (23/23)	100 (23/23)	100 (23/23)	100 (23/23)	91 (21/23)	86 (12/14)	79 (11/14)	36 (5/14)	7 (1/14)
35sEnR	100 (13/13)	92 (12/13)	100 (13/13)	100 (13/13)	38 (5/13)	64 (7/11)	45 (5/11)	64 (7/11)	64 (7/11)
mini35s	7 (4/61)	5 (3/61)	10 (6/61)	7 (4/61)	11% (7/61)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
pKGWSF7.0	6 (1/18)	0 (0/18)	0 (0/18)	0 (0/18)	0 (0/18)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
Common DHS 1	8 (1/12)	83 (10/12)	75 (9/12)	75 (9/12)	8 (1/12)	88 (14/16)	19 (3/16)	19 (3/16)	6 (1/16)
Common DHS 1R	67 (8/12)	92 (11/12)	50 (6/12)	100 (12/12)	67 (8/12)	36 (4/11)	18 (2/11)	9 (1/11)	9 (1/11)
Common DHS 2	4 (1/24)	13 (3/24)	8 (2/24)	21 (5/24)	17 (4/24)	67 (4/6)	0 (0/6)	0 (0/6)	0 (0/6)
Common DHS 2R	0 (0/24)	4 (1/24)	13 (3/24)	21 (5/24)	0 (0/24)	0 (0/19)	0 (0/19)	0 (0/19)	0 (0/19)
Common DHS 3	0 (0/28)	0 (0/28)	4 (1/28)	0 (0/28)	0 (0/28)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
Common DHS 4	8 (1/12)	100 (12/12)	33 (4/12)	42 (5/12)	25 (3/12)	83 (10/12)	33 (4/12)	33 (4/12)	42 (5/12)
Common DHS 4R	0(0/11)	82 (9/11)	9 (1/11)	9 (1/11)	0 (0/12)	0 (0/10)	0 (0/10)	0 (0/10)	10 (1/10)
Common DHS 5	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/11)	36 (4/11)	73 (8/11)	0 (0/11)
Common DHS 5R	56 (5/9)	22 (2/9)	11 (1/9)	11 (1/9)	11 (1/9)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/11)
Common DHS 6	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/25)	0 (0/13)	0 (0/13)	0 (0/13)	0 (0/13)
Common DHS 7	0 (0/23)	4 (1/23)	0 (0/23)	0 (0/23)	4 (1/23)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
Common DHS 8	4 (1/24)	4 (1/24)	4 (1/24)	4 (1/24)	42 (10/24)	0 (0/18)	0 (0/18)	6 (1/18)	0 (0/18)
Common DHS 8R	0 (0/18)	6 (1/18)	17(3/18)	11 (2/18)	78 (14/18)	0 (0/15)	0 (0/15)	0 (0/15)	0 (0/15)
Leaf DHS 1	7 (1/15)	13 (2/15)	27 (4/15)	0 (0/15)	47 (7/15)	0 (0/13)	0 (0/13)	0 (0/13)	0 (0/13)
Leaf DHS 1R	0 (0/7)	29 (2/7)	14 (1/7)	0 (0/7)	57 (4/7)	-	-	-	-
Leaf DHS 2	36 (5/14)	7 (1/14)	21 (3/14)	21 (3/14)	21 (3/14)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)
Leaf DHS 2R	11 (2/18)	0 (0/18)	0 (0/18)	0 (0/18)	17 (3/18)	-	-	-	-
Leaf DHS 3	89 (16/18)	11 (2/18)	44 (8/18)	11 (2/18)	11 (2/18)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
Leaf DHS 3R	100 (11/11)	36 (4/11)	36 (4/11)	55 (6/11)	18 (2/11)	-	-	-	-
Flower DHS 1	10 (2/21)	71 (15/21)	52 (11/21)	57 (12/21)	29 (6/21)	0 (0/14)	64 (9/14)	14 (2/14)	14 (2/14)
Flower DHS1R	17 (2/12)	100 (12/12)	83 (10/12)	58 (7/12)	42 (5/12)	0 (0/8)	75 (6/8)	0 (0/8)	0 (0/8)
Flower DHS 2	15 (2/13)	15 (2/13)	8 (1/13)	0 (0/13)	8 (1/13)	0 (0/13)	0 (0/13)	46 (6/13)	0 (0/13)
Flower DHS 2R	8 (1/12)	0 (0/12)	0 (0/12)	0 (0/12)	8 (1/12)	0 (0/16)	6 (1/16)	69 (11/16)	0 (0/16)
Flower DHS 3	0 (0/21)	0 (0/21)	0 (0/21)	0 (0/21)	0 (0/21)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)
N1	0 (0/24)	0 (0/24)	4 (1/24)	0 (0/24)	8 (2/24)	0 (0/18)	0 (0/18)	0 (0/18)	0 (0/18)
N2	4 (1/23)	4 (1/23)	13 (3/23)	0 (0/23)	0 (0/23)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)
N3	11 (3/27)	4 (1/27)	0 (0/27)	0 (0/27)	0 (0/27)	0 (0/18)	0 (0/18)	0 (0/18)	0 (0/18)
N4	4 (1/26)	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/17)	0 (0/17)	0 (0/17)	0 (0/17)
N5	0 (0/23)	4 (1/23)	0 (0/23)	0 (0/23)	0 (0/23)	6 (1/16)	6 (1/16)	6 (1/16)	6 (1/16)
N6	0 (0/28)	0 (0/28)	0 (0/28)	0 (0/28)	0 (0/28)	0 (0/11)	0 (0/11)	0 (0/11)	0 (0/11)
N7	0 (0/20)	0 (0/20)	10 (2/20)	10 (2/20)	5 (1/20)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)
N8	0 (0/24)	0 (0/24)	8 (2/24)	13 (3/24)	4 (1/24)	0 (0/12)	0 (0/12)	0 (0/12)	0 (0/12)
N9	12 (3/25)	8 (2/25)	20 (5/25)	4 (1/25)	16 (4/25)	0 (0/13)	0 (0/13)	8 (1/13)	0 (0/13)
N10	0 (0/30)	10 (3/30)	10 (3/30)	3 (1/30)	3 (1/30)	0 (0/14)	0 (0/14)	0 (0/14)	0 (0/14)

Supplemental Table 3. Expression of six genes flanking enhancer L3 in wild-type Col-0 and T-DNA line GABI-Kat 909A07

Gene Name	**-10k**	**-9k**	**-5k**	**+4k**	**+7k**	**+11k**	**ACT2**
Gene ID	AT2G45410	AT2G45412	AT2G45420	AT2G45430	AT2G45440	AT2G45450	AT3G18780
Location	Chr2:18712287-18713116	Chr2:18714574-18714729	Chr2:18718348-18720729	Chr2:18727561-18728935	Chr2:18730840-18733015	Chr2:18733275-18734127	Chr3:6474842-6477204
Annotation	LOB domain-containing protein 19	Unknown protein	LOB domain-containing protein 18	AT hook motif nuclear-localized protein 22	Dihydrodipicolinate synthase	Protein binding	Actin gene
Forward primer	5’CGAAATAAGGAGCGAAAACG3’	5’GCCAACAGAATCGACGGTTG3’	5’AACCGCAACCGCTCTTTT3’	5’GGGCTAAAACGTGACCGAGA3’	5’CTGGTCGAACAGGGCAAGAT3’	5’CTGAAGCAGAGAGAAGAGAGTT3’	5’CTTGCACCAAGCAGCATGAA3’
Reverse primer	5’GGTGGAAGAGGAGGAGAAGG3’	5’CGACGTGGCTTGTTTTGTGT3’	5’TGAGGGCAAGGGTGAGACT3’	5’GTGGCCTTCTCGTGATGTGA3’	5’TCTTCGACTCGCTTGTTCCC3’	5’CGTGGAGAATCAAAACATCA3’	5’CCGATCCAGACACTGTACTTCCTT3’
Expression in Col-0 (root) (1)	28.48 ± 0.98	28.34 ± 0.85	27.76 ± 0.89	24.68 ± 0.44	25.92 ± 0.65	26.72 ± 0.84	21.31 ± 0.47
Expression in Col-0 (leaf) (1)	32.77 ± 0.14	34.75 ± 0.16	31.79 ± 0.20	29.25 ± 0.13	25.90 ± 0.37	27.81 ± 0.85	20.39 ± 0.34
Expression in T-DNA line (root) (1)	28.09 ± 0.71	28.11 ± 0.76	27.49 ± 0.64	25.03 ± 0.15	25.77 ± 0.23	26.28 ± 0.32	20.94 ± 0.27
Expression in T-DNA line (leaf) (1)	32.69 ± 0.23	34.22 ± 0.34	31.77 ± 0.23	28.07 ± 0.24	26.05 ± 0.21	26.93 ± 0.23	20.66 ± 0.23
Col-0 root vs. Col-0 leaf (t-test) (2)	0.067	0.033	0.034	0.011	0.026	0.025	
Col-0 root vs. T-DNA line root (t-test) (2)	0.927	0.925	0.925	0.022	0.271	0.926	
Col-0 leaf vs. T-DNA line leaf (t-test) (2)	0.351	0.056	0.354	0.001	0.016	0.01	

(1) Real time qRT-PCR of gene expression is presented as the average number (from three biological replicates) of PCR cycles ± standard deviation.

(2) t-test of expression of the same gene in different tissues or different lines.