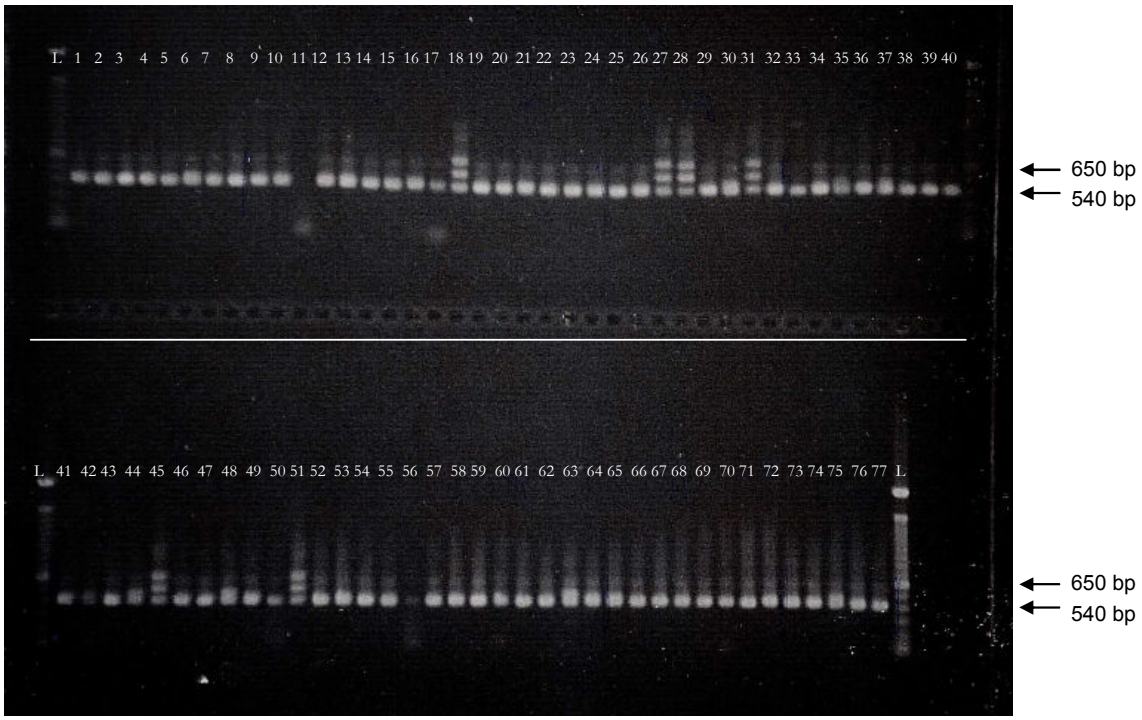
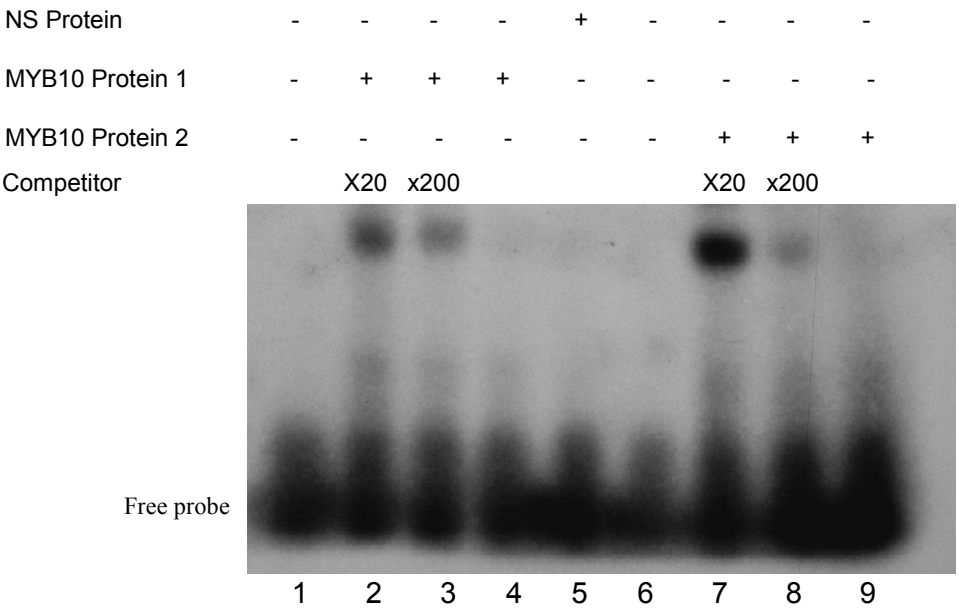
Supplemental Data. Espley et al., (2009). Multiple repeats of a promoter segment causes transcription factor autoregulation in red apples



Supplemental Figure 1. **Gel picture showing products from the apple germplasm PCR assay.**

Apple genomic DNA from 77 individuals was amplified using a pair of PCR primers located in the *MYB10* promoter (forward: 5'-GGAGGGGAATGAAGAAGAGG-3'; reverse: 5'-GCTATTCTTTTGCCTGCTACC-3'). The varieties are listed in supplemental Table 1. Amplicons from the PCR reaction were found at 547bp, corresponding to the R_1 allele, and 647 bp, corresponding to the the R_6 allele. The higher band present in the R_1/R_6 lanes represents a heteroduplex



Supplemental Figure 2. Electrophoretic mobility shift assays comparing full length and truncated MYB10 protein and a non-specific His-tagged control protein.

No difference was observed in the binding and competition assays for the two versions. A non-specific (NS) His-tagged protein (α-farnesene synthase from apple, Green et al., 2007) was also included in the experiment and did not bind with MYB10 protein. Lane 1, r1 DNA probe only; lane 2, r1 DNA probe with MYB10 full length protein; lanes 3 and 4, r1 DNA probe with MYB10 full length protein and 20-fold and 200-fold excess cold r1 competitor; lane 5, r1 DNA probe with non-specific His-tagged protein; Lane 6, r1 DNA probe only; lane 7, r1 DNA probe withMYB10 truncated protein; lanes 8 and 9, r1 DNA probe with MYB10 truncated protein and 20-fold and 200-fold excess cold r1 competitor

Supplemental Table 1. Data for the 77 apple varieties tested for the presence of the R_1 and R_6 alleles.

The genotype and phenotype of the 77 individuals tested for the presence of the minisatellite-like insertion. The individuals tested were Open Pollinated (OP) and the cultivar name refers to the maternal parent. The repeat motif genotype refers to R_1 (no minisatellite-like insertion) and R_6 which contains the insertion. Results from the PCR reaction are shown in Supplemental Figure 1.

Lane	Mother cultivar name	Repeated motif genotype	Foliage colour	Fruit core colour
1	Suiselpas Rozabols	$R_1 : R_1$	Green	White
2	Antonovka kamenichka	$R_1 : R_1$	Green	White
3	King of the Pippins	$R_1 : R_1$	Green	White
4	Grenadier	$R_1 : R_1$	Green	White
5	Red Baron	$R_1 : R_1$	Green	White
6	Korichnoe Polosatoje	$R_1 : R_1$	Green	White
7	Golden Sweet	$R_1 : R_1$	Green	White
8	Krievu Rosmarins	$R_1 : R_1$	Green	White
9	Bessemianka Muchirin	$R_1 : R_1$	Green	White
10	Korichnoe Polosatoje	$R_1 : R_1$	Green	White
11	Campanino	na	Green	White
12	*M. sieversii 45-0$_1$*	$R_1 : R_1$	Green	White
13	*M. sieversii 42-03*	$R_1 : R_1$	Green	White
14	Appio	$R_1 : R_1$	Green	White
15	Grenoble	$R_1 : R_1$	Green	White
16	*M. sieversii 09-0$_1$*	$R_1 : R_1$	Green	White
17	Paides Ziemas-Abols	$R_1 : R_1$	Green	White
18	Pomme Grise	$R_1 : R_6$	Red	Red
19	Octovo	$R_1 : R_1$	Green	White
20	A. Brioallay/Aromatde Vera	$R_1 : R_1$	Green	White
21	Styrmka	$R_1 : R_1$	Green	White
22	Patte de Loup	$R_1 : R_1$	Green	White
23	Jeptiska	$R_1 : R_1$	Green	White
24	De Jaune	$R_1 : R_1$	Green	White
25	Pomme a Cotes	$R_1 : R_1$	Green	White
26	Geelzoet	$R_1 : R_1$	Green	White
27	Grimes Golden	$R_1 : R_6$	Red	Red
28	Zelenovka Sotchnaya	$R_1 : R_6$	Red	Red
29	Wijnappel	$R_1 : R_1$	Green	White
30	Pepinka Litovka	$R_1 : R_1$	Green	White
31	Redfield	$R_1 : R_6$	Red	Red
32	Mother	$R_1 : R_1$	Green	White
33	Gloire de Ponchartrain	$R_1 : R_1$	Green	White
34	Zure Kroon	$R_1 : R_1$	Green	White

Supplemental Table 1 (cont.).

35	Mildew immune seedling	$R_1 : R_1$	Green	White
36	Pepino Jaune	$R_1 : R_1$	Green	White
37	Chadimova	$R_1 : R_1$	Green	White
38	Early almond	$R_1 : R_1$	Green	White
39	*M. sieversii $_1$9-0$_1$*	$R_1 : R_1$	Green	White
40	Hornokrajske Malinove	$R_1 : R_1$	Green	White
41	Mere de Menage	$R_1 : R_1$	Green	White
42	Shaphran Letnij	$R_1 : R_1$	Green	White
43	Isayev's Desertnyi	$R_1 : R_1$	Green	White
44	Lady Williams	$R_1 : R_1$	Green	White
45	Redfield	$R_1 : R_6$	Red	Red
46	Reinette Franche	$R_1 : R_1$	Green	White
47	Belle Fleur Jaune	$R_1 : R_1$	Green	White
48	Frau Margaret Von Stosch	$R_1 : R_1$	Green	White
49	Wyken Pippin	$R_1 : R_1$	Green	White
50	Frequin Rouge	$R_1 : R_1$	Green	White
51	Court Pendu Rose	$R_1 : R_6$	Red	Red
52	Tare de Ghinda	$R_1 : R_1$	Green	White
53	Zelenovka Sotchnaya	$R_1 : R_1$	Green	White
54	Binet Blanc Dore	$R_1 : R_1$	Green	White
55	Cerina	$R_1 : R_1$	Green	White
5_6	Unknown4.99	$R_1 : R_1$	Green	White
57	Calville d'Aout	$R_1 : R_1$	Green	White
58	Blanchard	$R_1 : R_1$	Green	White
59	*M. sieversii 42-05*	$R_1 : R_1$	Green	White
60	Commercio	$R_1 : R_1$	Green	White
61	Pigeonet Rouge	$R_1 : R_1$	Green	White
62	Lemon Pippin	$R_1 : R_1$	Green	White
63	London Pippin	$R_1 : R_1$	Green	White
64	Reinette Franche	$R_1 : R_1$	Green	White
65	Pepino Schafranovij	$R_1 : R_1$	Green	White
66	*M. sieversii_3-02*	$R_1 : R_1$	Green	White
67	Hvezdnata Reneta	$R_1 : R_1$	Green	White
68	Decio	$R_1 : R_1$	Green	White
69	*M. sieversii $_1$2-02*	$R_1 : R_1$	Green	White
70	Gooseberry pippin	$R_1 : R_1$	Green	White
71	*M. sieversii 44-03*	$R_1 : R_1$	Green	White
72	*M. sieversii 24-02*	$R_1 : R_1$	Green	White
73	M. Kirzistan (Kazak)	$R_1 : R_1$	Green	White
74	Winter Queening	$R_1 : R_1$	Green	White
75	Gul Richard	$R_1 : R_1$	Green	White
76	Panenske Ceske	$R_1 : R_1$	Green	White
77	Smiricke	$R_1 : R_1$	Green	White

Supplemental methods

Plasmid Construction

The promoter deletion constructs for R_1 and R_6 were cloned into the vector *pGreen 0800-LUC* (Hellens et al., 2005), containing the luciferase reporter. An inverse PCR approach was used for the R_1+ construct with the inclusion of unique restriction sites (*Bam*HI and *Sac*I) for the cloning of non-specific DNA (from pGEM T Easy, Promega) using the primers 5' – GGATCCTTCTGCACGACAACATTGACAA – 3' and 5' – GAGCTCATGTTAGCTTTTCTATATATCGA – 3'.

Promoter deletions were performed on R_1 and R_6 sequences using the following restriction enzymes; ΔA, *Hind*III (retaining the 5' fragment only), ΔB, *Spe*I, ΔC, *Ssp*I and ΔD, *Hind*III (retaining the 3' fragment only).

Deletion of the first repeat unit and microsatellite (*R_0:LUC*) was based on the R_1:LUC promoter construct and performed using a partial restriction digest with *Dra*I. The linear fragment was further digested with *Bsg*I and treated with T4 DNA polymerase (Promega) prior to re-ligation. For the construction of $R_{1\text{-}R}$ and $R_{1\text{-}MS}$, R_0 was digested with *Bsg*I and annealed oligos were directionally cloned in with an AA (underlined below) overhang: $R_{1\text{-}R}$, 5'-CTATATATCGAGTGTGTGTGTGTGTGTATTTCACAACTGTTGGAATGTTTGAA – 3'; $R_{1\text{-}ms}$, 5'- CTATATATCGAATTTCACAACTGTTGGAATGTTTGAA - 3'. The R_6 versions were based on $R_{1\text{-}R}$ and $R_{1\text{-}MS}$. The minisatellite repeat unit from R_6 was amplified by PCR using the primers 5'-ATGTTGTCGTGCAGAAATGTTAG -3' and 5'-GTAGCTATTAACAAGTTAGACTGTGT -3' and cloned in at the *Bsg*I restriction site to create R_5 and $R_{6\text{-}MS}$. All clones were positively identified by bi-directional sequencing.

Transactivation Analysis using Transformed Tobacco Leaves

Nicotiana benthamiana plants were grown until at least six leaves were available for infiltration with *Agrobacterium*. A 10 µl loop of confluent bacteria was re-suspended in 10 ml of infiltration media (10 mM $MgCl_2$, 0.5 µM acetosyringone), to an OD^{600} of 0.2, and incubated at room temperature without shaking for 2 h before infiltration. Approximately 150 µl of this *Agrobacterium* mixture were infiltrated at six points into a young tobacco leaf. Transient expression was analyzed three days after inoculation. Six technical replicates of 3 mm diameter leaf discs were excised from each plant using a leaf hole-punch and buffered in Phosphate Buffer Saline (PBS). Plate-based assays were conducted using a Berthold Orion Microplate Luminometer (Berthold Detection Systems) according to the manufacturer's specifications for the dual luciferase assay, using the Dual Glow assay reagents (Promega) for firefly luciferase and *Renilla* luciferase. Luminescence was calculated using Simplicity version 4.02 software (Berthold Detection Systems).

Production and Purification of MYB10 Protein

Constructs for the preparation of recombinant histidine-tagged MYB10 protein extracts were built using pET30 expression vectors (Invitrogen). A full length version of *MYB10* cDNA (amino acids 1-243) was cloned into pET-30a using the *Sac*I/*Kpn*I restriction sites. Protein yield following expression in *E. coli* BL21-CodonPlus™-RIL (Stratagene) was found to be low. Previous studies using purified plant MYB transcription factor proteins have encountered problems with toxicity of the expressed protein in host cells. These problems have been successfully resolved in by using truncated versions of the protein using a carboxy-terminal deletion (Yang et al., 2001; Gális et al., 2006). A carboxy-terminal deletion of the *MYB10* cDNA was made (retaining amino acids 1-167) and cloned into pET-30 using *Bgl*II/*Nde*I restriction sites. The truncated version contained the R2R3 DNA-binding domain. Expression levels in *E. coli* of the truncated version were found to be approximately 10-fold higher than for the full length protein. Both versions were used in EMSA analysis and showed similar results for binding and competition assays (Supplemental Figure 2). The truncated version was used for all subsequent binding studies using

EMSA. An unrelated HIS-tagged protein, α-farnesene synthase from apple (Green et al., 2007) was used in EMSAs to test for non-specific binding (Supplemental Figure 1).

Notes on Apple Genealogy

Some of the apple varieties used in this study have a common ancestor, while others are the product of open pollination (OP) and as such have an undefined parent. Below is a summary of the genealogy of apple varieties as described in Figure 2.

Common ancestry through 'Delicious' with three varieties analysed:

1. 'Mildew Immune Seedling' = OP of 'Delicious' ('Delicious' is a seedling of unknown origin) (Dayton, 1977).
8 'Royal Gala' = 'Gala' ('Golden Delicious' x 'Kidd's Orange Red') x ('Delicious' x 'Cox's Orange Pippin') (Brooks and Olmo, 1972).
9. 'Fuji' = 'Ralls Janet' x 'Delicious' (Brooks and Olmo, 1972).

Common ancestry through *M. pumila* var. *Niedzwetzkyana* with three varieties analysed:

3. *M. pumila* var. *Niedzwetzkyana* = seedling of unknown origin (Fialo, 1994).
4. 'Prairifire' = 'Liset' x OC 6-1 (*M. astrosanguinea* x (OC 1-7 (*M. zumi calocarpa* x *M. pumila* var. *Niedzwetzkyana*))) (Dayton, 1982).
5. 'Geneva' = O.P. of *M. pumila* var. *Niedzwetzkyana* (Brooks and Olmo, 1972).

Any further pedigree relationships among remaining genotypes examined are unknown:

2. *M. purperea* 'Aldenhamensis' = seedling of unknown origin (Fialo, 1994).
6. 'Pomme Grise' = seedling of unknown origin (Smith, 1971).
7. 'Granny Smith' = thought to be OP of 'French Crab' (Smith, 1971).
10. 'Braeburn' = seedling of unknown origin (Brooks and Olmo, 1972).

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