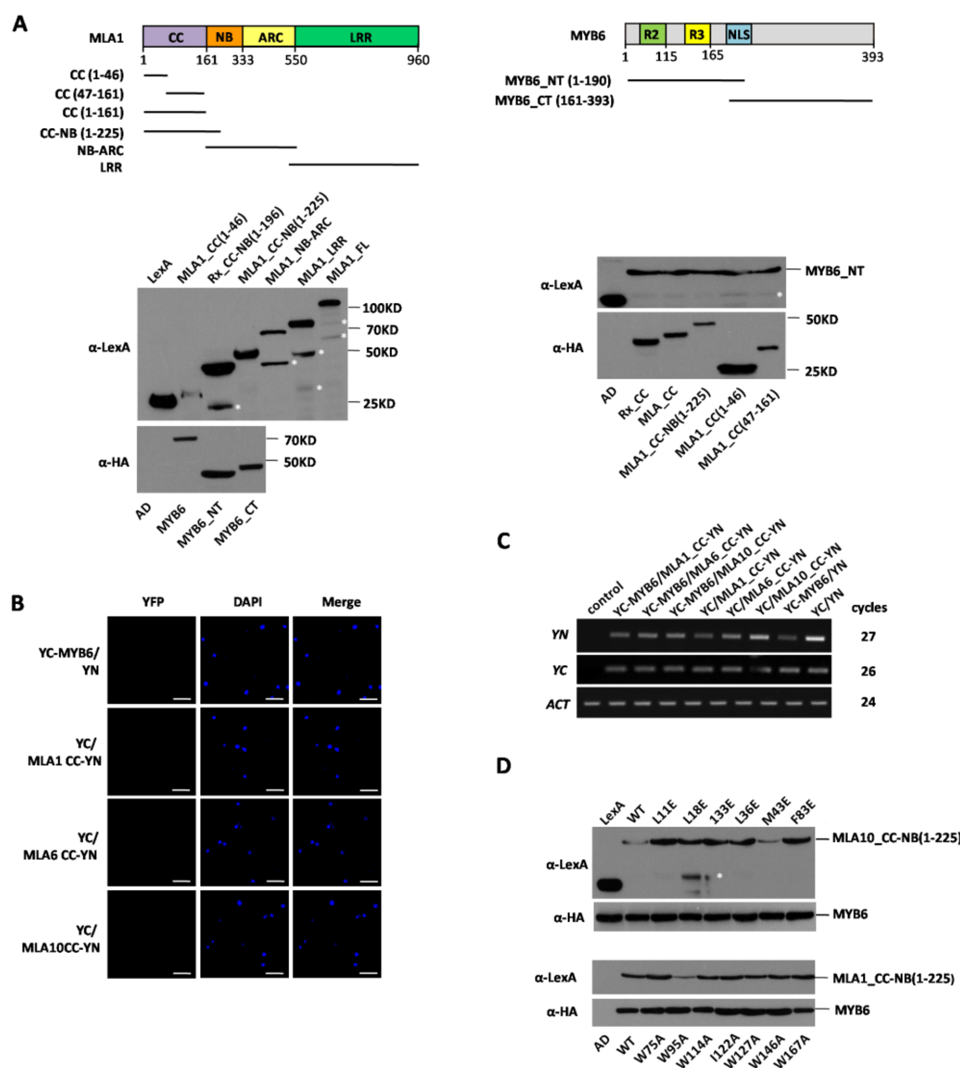
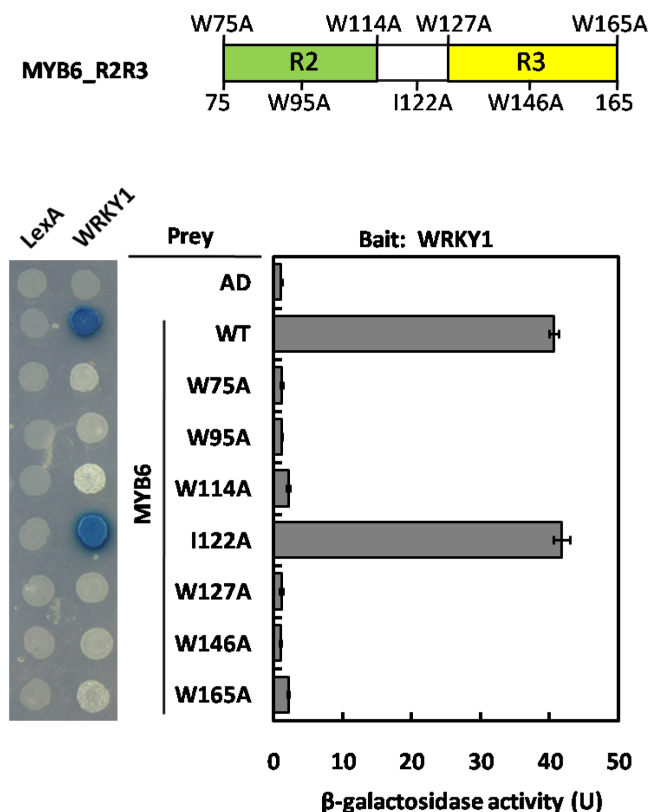


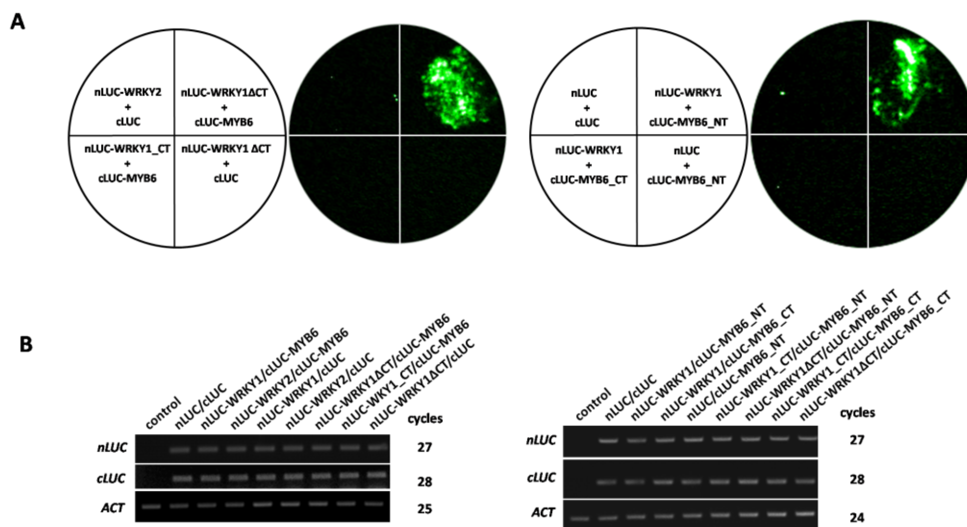
Supplemental Figure 1. Protein Accumulation of Baits and Preys and BiFC Controls for MLA_CC and MYB6 Interaction Analysis.

(A) Schematic depictions of MLA1 and MYB6 domain structures. Conserved domains are represented in colour boxes. Solid lines represent fragments tested for interaction in yeast (upper panels). Anti-LexA or anti-HA antibodies detected the accumulation of all bait or prey fusions used in Fig. 1A or Fig. 1D for Y2H assays. Asterisk indicates degraded fragments (lower panels). **(B)** Fusion pairs used in BiFC assays in Figure 1C did not interact with the nYFP/cYFP vector. The experiments settings were same as in Fig. 1C. Scale bar is 50 µm. **(C)** Transcript accumulation of all fusions used in **(A)** and **(B)**. RT-PCR analysis using RNA samples derived from Agroinfiltrated *N. benthamiana* leaves in the BiFC assays. The *Actin* (*ACT*) gene was used as an internal control. **(D)** Anti-LexA or anti-HA antibodies detected the accumulation of all bait or prey fusions used in Fig. 1D for Y2H assays.



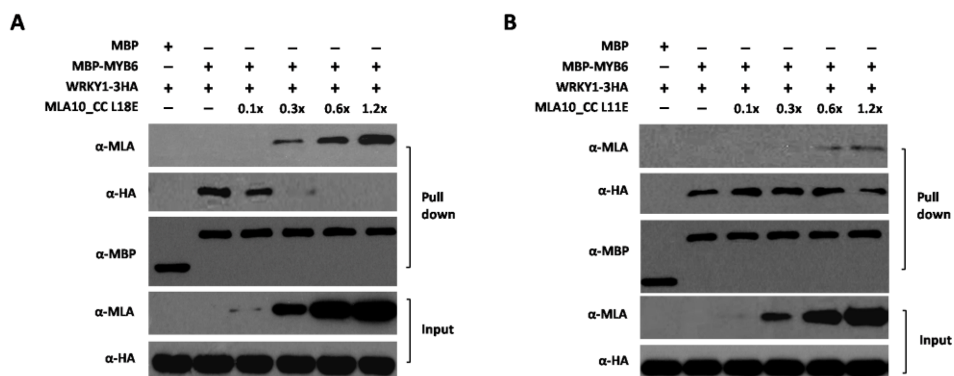
Supplemental Figure 2. Y2H Analysis between WRKY1 and the Mutant Variants of MYB6.

Diagram depicts the relative positions of the conserved Trp in the R2R3 domain and the Ile122 residue in the junction region between R2 and R3 repeat of MYB6 (Top panel), and these residues were each mutated into Ala in MYB6 prey for testing interactions with the WRKY1 bait (bottom panel), same as in Fig. 1E. The β-galactosidase activity was quantified and data are represented as mean +/- SEM (n=8).

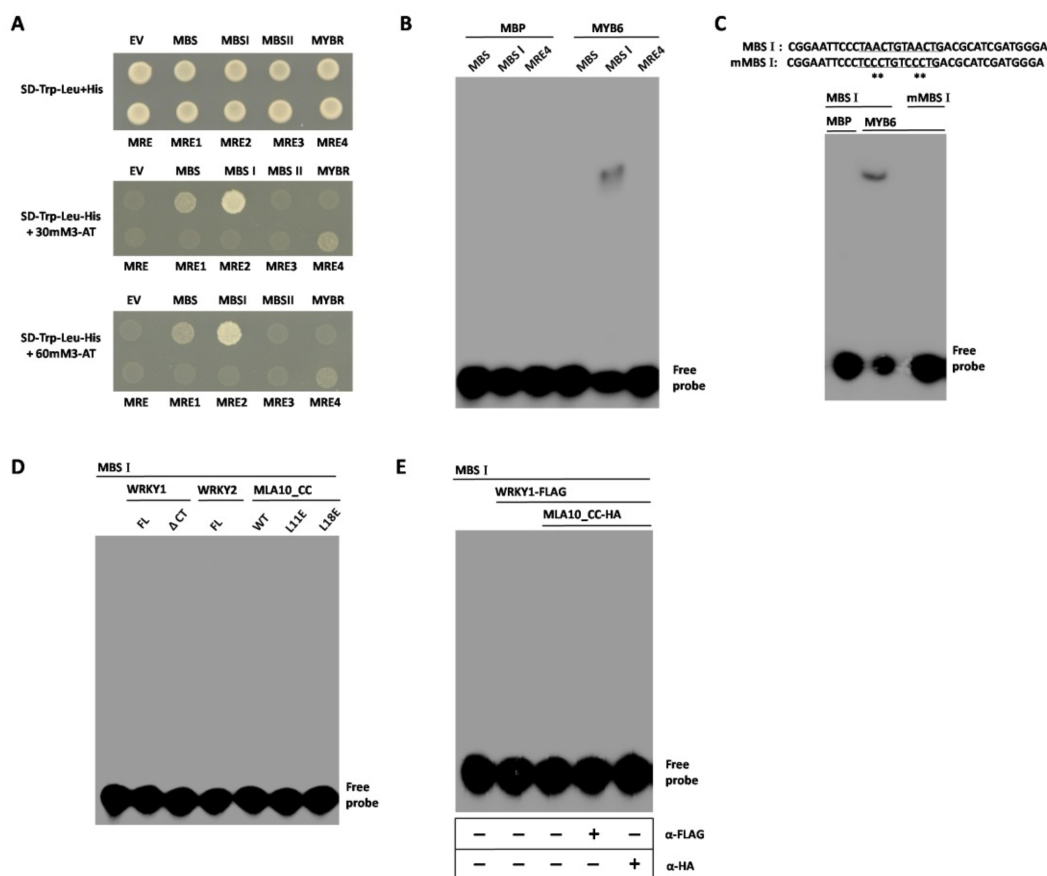


Supplemental Figure 3. Firefly LCI Assays for WRKY1-MYB6 Interaction.

(A) LCI assays showing that C-terminus of WRKY1 and MYB6 are not required for their interactions. Same experimental settings as in Fig. 2C. The luminescent signal was collected at 60 hrs post infiltration. **(B)** Transcript accumulation of all fusions used in LCI assays in Fig. 2C and Fig. S3A.

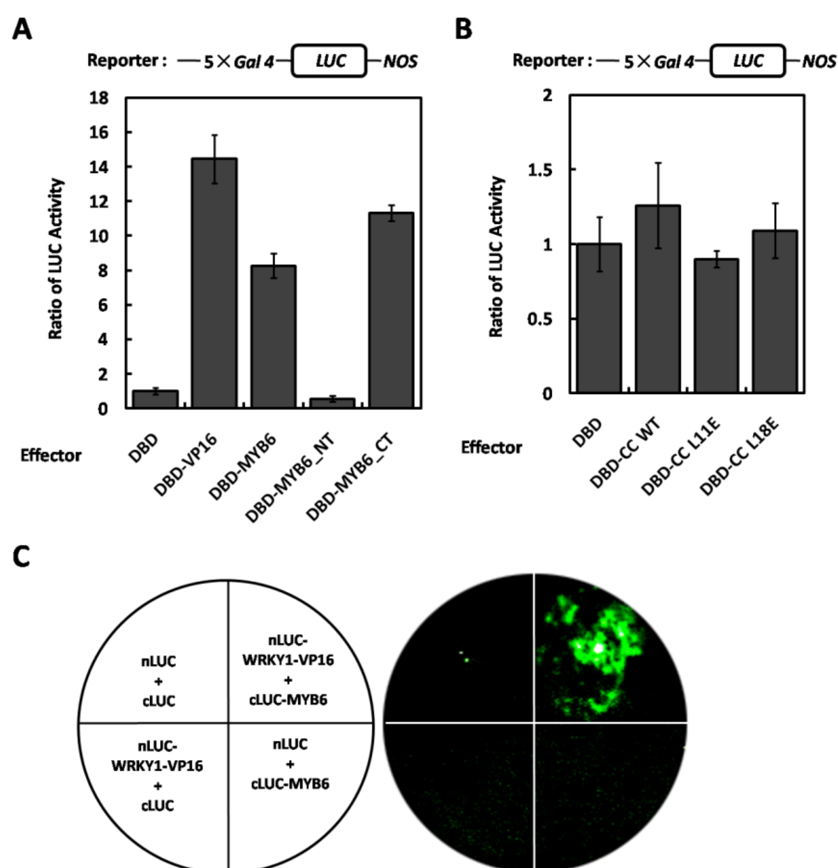


Supplemental Figure 4. MBP pull-down Assays Testing MLA10_CC Variants Competing with WRKY1 to Bind MYB6.
(A) MLA10_CC(L18E) compete out WRKY1 in binding MYB6. Same MBP pull-down assays were conducted as in Figure 3C & 3D, except that MLA10_CC(L18E) was used to replace MLA10_CC. Similar results were obtained from more than five repeats. **(B)** Failure of MLA10_CC(L11E) to compete out WRKY1 in binding MYB6. Same pull-down assays as above except that the MLA10_CC(L11E) was used here.



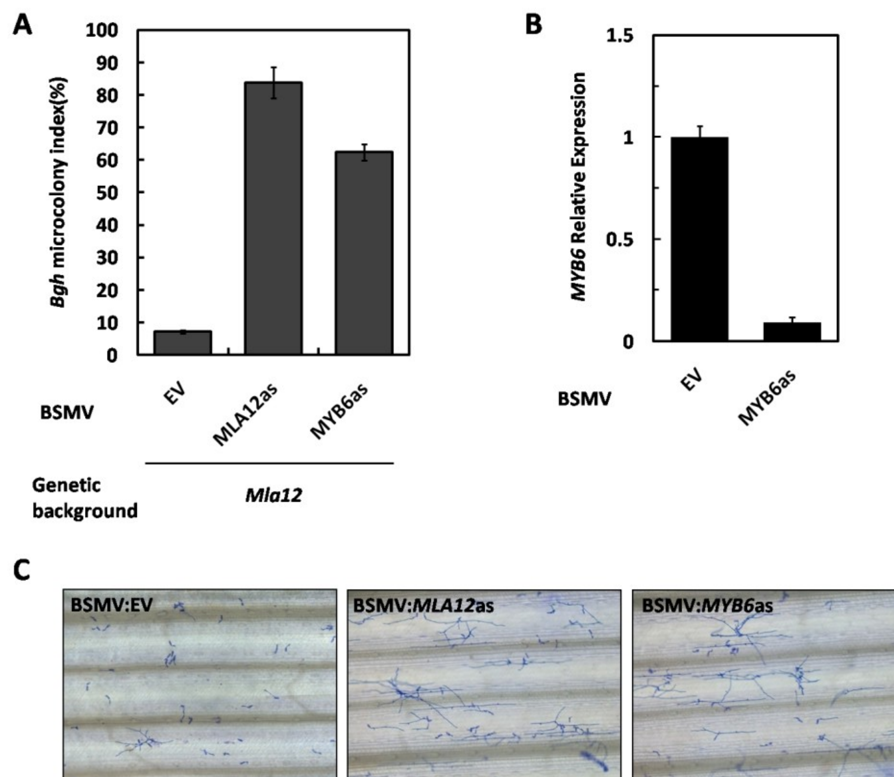
Supplemental Figure 5. MYB6 Specifically Binds the MBS I *cis*-element whereas WRKY1, WRKY2 or MLA10_CC do not.

(A) Screening of cis-elements that MYB6 binds by yeast one-hybrid. Three copies of each of the DNA *cis*-elements *MBS*, *MBS I*, *MBS II*, *MYBR*, *MRE*, *MRE1*, *MRE2*, *MRE3* and *MRE4* were used in the assays. **(B)** MYB6 specifically binds the *MBS I cis*-element but not *MBS* or *MRE 4* in EMSA assay. Indicated *cis*-elements were all ^{32}P-labeled and incubated with recombinant MYB6 or MBP (as a control) as in Figure 4A. **(C)** MYB6 specifically binds the *MBS I cis*-element but not m*MBS I* in EMSA assay. Indicated cis-elements were all ^{32}P-labeled and incubated with recombinant MYB6 or MBP (as a control) as in Figure 4A. **(D)** Failure of WRKY1 or WRKY2 or MLA10_CC to bind *MBS I* probe. EMSA assay was conducted with freshly prepared recombinant proteins, similar to that in Figure 4 but without adding MYB6 in the incubation with *MBS I* probe. **(E)** Failure of WRKY1-FLAG and MLA10_CC-HA to bind the MBS I probe. EMSA assays were similarly done as in Figure 4E but without adding MYB6 in the incubation with *MBS I* probe. All EMSA experiments were repeated three times with similar results.



Supplemental Figure 6. MYB6 is a Transcription Activator; MLA10_CC has no Transcriptional Activity; WRKY1-VP16 Fusion Interacts with MYB6 in LCI Assays.

(A) MYB6 is a transcription activator. Arabidopsis protoplasts were transfected with a mixture of effector, a luciferase (LUC) reporter and an internal control in a ratio of 6:6:1. The LUC reporter contains 5x *Gal4 UAS*. Basal reporter activity was determined by expressing Gal4 DBD alone, which was normalized to 1.0 unit. DBD-VP16 is a transcriptional activator control. This experiments were repeated three times and data presented as average +/- SEM (n=3). **(B)** MLA10_CC, (L11E) or (L18E) variant has no transcriptional activity. Arabidopsis protoplast reporter gene activation assays were done as in **(A)**. The experiments were repeated three times, and data are presented as average +/- SEM (n=3). **(C)** Firefly LCI assays were done as in Figure 2C, showing interaction between WRKY1-VP16 and MYB6.



Supplemental Figure 7. BSMV-VIGS Silencing of *MYB*6 Compromises *MLA12*-mediated Disease Resistance.

(A) BSMV-VIGS silencing of *MYB*6 compromises *MLA12*-mediated disease resistance. *Bgh* microcolony index was scored upon silencing of indicated gene in *MLA12*-containing barley line and inoculated with avirulent isolate A6. The experiments were repeated three times, and data are presented as average +/- SEM. At least 2000 interaction sites were microscopically analyzed in one experiment. **(B)** qRT-PCR analysis of *MYB6* expression after BSMV-VIGS using BSMV empty vector (EV) or vector to silence *MYB6* (*MYB6*as) in barley cultivar I10 that containing the *Mla12* resistance gene. Error bars represent standard error of technical triplicates and the experiments were repeated for three times with similar results. **(C)** *Bgh* microcolony formation on barley leaf surface in BMSV-VIGS assays. Shown are images of representative leaves of barley cultivar I10 upon BMSV-VIGS using indicated BSMV vector, and fixed at 5 days after inoculations of an avirulent *Bgh* isolate.

```
                                                                 50                                                100
HvMyb6     : ATGTCACGGATCGGAGATTCGATGCCGAGGGAGCGGTCTGTCAATTCAACAAGTCAT-TCAAGGAAGGCTGATGCGAA-TGTGAGTGGTACACCACATGA :   98
MLOC_14401 : ATGGCGTCGATGGGGGAGTTGCTTTTGAACCAGGAG-CTGGCGGCGGTGGCGGCCATGTCGACGGACGCGTACGGGAGGTTCGAAAGGTACACCCTGAGC :   99

                                                                150                                                200
HvMyb6     : CACTGCCAAACATTGCCACGGTGAAGAGTTGGGAAACATAGGAG-GACAACTTTGCAAGAATGATGGCCACTCCTTCAAGGAGAGCTGCAGTATCACAAA :  197
MLOC_14401 : C-CTGCCCATGGTTTTCCCACTTCCAGCAAGCTGGCAGCGCTCCGACGGTATTCCCCGACACGGGATTGCTCGTTTCTGG----CTTCGGCATGCCTCC :  194

                                                                250                                                300
HvMyb6     : TTCTGAAGTCCTCACCAGAGTGAAATGGTCAGAAGAGGAAGA----TAAGATGCTCACTCAAA--TGATCAACAAACATGGGCTGAAAAACTGGCAAACT :  291
MLOC_14401 : CTCCACCTTCATCGTCATGCCGGAGGGGACCCACGCGGCCGACGCTTATGGCGCTGAGGCCGTCCCGGTGGAGATCCCCGTGGTCCGGCGACAGAGACCT :  294

                                                                350                                                400
HvMyb6     : GTCGCACATGCTATACCTGGCCGAAGTGCACCACAATGCCGACAAAGGTGGAGATACAAAATAGACTCTGCAATAAACAAGGAGGCATGGTCGGAACAAG :  391
MLOC_14401 : GCCGC-CAGGA-ACGCTGGCCGACGCCGTTCAGGGGACCGT---GGACGGAGGAAGAGGACGAACTTCTCAAGAGATTGGTGGATCAGCATGGCG-AAC :  388

                                                                450                                                500
HvMyb6     : AGGAGTTGAGACTGATTCGTGCTCATCAAATCTATGGAACTAAATGGCGTGAGATG--GTCAAACATTTTCCTGGGAGGACGAATGGTGCAATCAAAGAA :  489
MLOC_14401 : ATAAGTGGGCGACCATTTCAGATCACC----TCCCGGGACGGATCGGCAAGCAGTGCCGTGAGCGATGGACAAATCACGTCCGCCCAGGCATCAAGAAGG :  484

                                                                550                                                600
HvMyb6     : TATTGGAGAGGTCCTATGAAAAGAAAATTAAATTCATATTTAT-CGTCAGGTTTGCTTGAACAATTT-CCAGACATTCTGGAAAACCTGTCAGGCACACA :  587
MLOC_14401 : AGCACATCTGGACTGAGGCGGACGACATACTGCTGATCGACGCGCACAAGATCCACGGAAACCGCTGGTCGTCCATCGCGAGGTGCCTCCCCGGCCGGTC :  584

                                                                650                                                700
HvMyb6     : GAATAATA--GTTTGGATATTCTGAAGGGGACTAAAGTTTCTGATATTCTGAATGATACTGAAGGTTTGTCCGAGAGAAATGAAATGCCGTCAGCTGTAC :  685
MLOC_14401 : GGAGAACGCCGTCAAGAATCACTGGAACGCGACAAAAC----GGAGCCTCAAGT----CGAAGCGCCGGTTCAAGAAGAAGACGAGCCAACAGGCCGCCC :  676

                                                                750                                                800
HvMyb6     : CAAGAATTTCCATATCTGAACTAGGCTTCACAGAATAGGTGAAAATGCTGATGTACCAGGGGAGAAAGTGCAGATTTCAT-GTATGCTAGAGT--GGC :  782
MLOC_14401 : CGGCCAGTTCAC-CCTCCTTGAGGAGT---ACATCCGCGACAAAATGATGCTGACGAGAACGCGGCGCCACCGTGTCCATCGTCCGGCGGCGTCGGT :  772

                                                                850                                                900
HvMyb6     : ATCA-GCCAAAGCGCCTGAAAACATAATCGCAAGATCTGAGGAGGATGCGAAACCAGGAGGAAGTTGGATTTATTGTCAAGCCCAGTGGAACTGAAGTC :  881
MLOC_14401 : ATGACGGCCAGGTGGTGCCAGGTGCCGCTGCAATG-CTGGCCGTCTCCGGCCCACCGCGGATGGGTCAGTACCTCCACCCAGCCGACGCGG--CGGGTC :  869

                                                                950                                               1000
HvMyb6     : ATCCAGTCATGAAGTAAATTCTCAAAGGCCTCTACAGCAAAGGGAACAAATGAGTCCAGCTGTTGGTGGTGTCTCTCCATCAAATGGATGCCATGATGTT :  981
MLOC_14401 : ATCGTCCCAAGCAGGGG----TGATGAACCTGAGCGC-----GCCGTTGCCGGACCTGAACGCCTACGGTGGCGAGATGCTGGAGGGATACTAC--TACC :  958

                                                               1050                                               1100
HvMyb6     : TTACCAAAAATCCCATCAAAATGTGCAA-AGCCGGTTTTATCACCAACTGGTAGCTACCAACCCAGTGATGTTCATTCTGCGGAAACCTCAGATCCATGT : 1080
MLOC_14401 : CAGCCACCTTCCCGACCTACAGCGACAACAACCTGCTGCACCATGGACCAGAGACTGCATTTCCGCAGATGTTTAGTGCCCAGGGAAGC---ATGCATAT : 1055

                                                               1150                                               1200
HvMyb6     : TCATTAGAGCTAGACATATCAGATCTTATGGAAATGTCATACTTTGATAACTTGTTGATCTTTCCTCCTGGTTCTCCCCCTGATGGTAACTCAATGTAAA : 1180
MLOC_14401 : GCATGCAGCATGCACGAACCTGAACTTGTTCCCAC----TCCCCCAGCACCTCGGTGGCGGCTACTAC-GGCAGCGAGACCGGCCGCAGC---------A : 1141

                                                               1250                                               1300
HvMyb6     : ACGAGGAAAGTGACAATGACCAAAATTACAACCTCAGTTATATT-CACTTGCACACTTTTGGCAAAAAAGGTGATGAGCTAAACTCACTGTTACTGCCAT : 1279
MLOC_14401 : GCGCCGGTGGCAGCAGTGATCAGGACGAAGACGTGGTGCAGATGGCGTCCAGGGAGTTCCAGACGTCTGAGGACGAGGCCACACTTGACCTCACTGGCTT : 1241

                                                               1350
HvMyb6     : CGTCCTGTAAACTGACAGTTCCTGTATCATTTAGATGAAAATCTTTAGCCGAACAAAAGGTACAATGAT : 1348
MLOC_14401 : CAGC----------------------------------------------------------------- : 1245
```

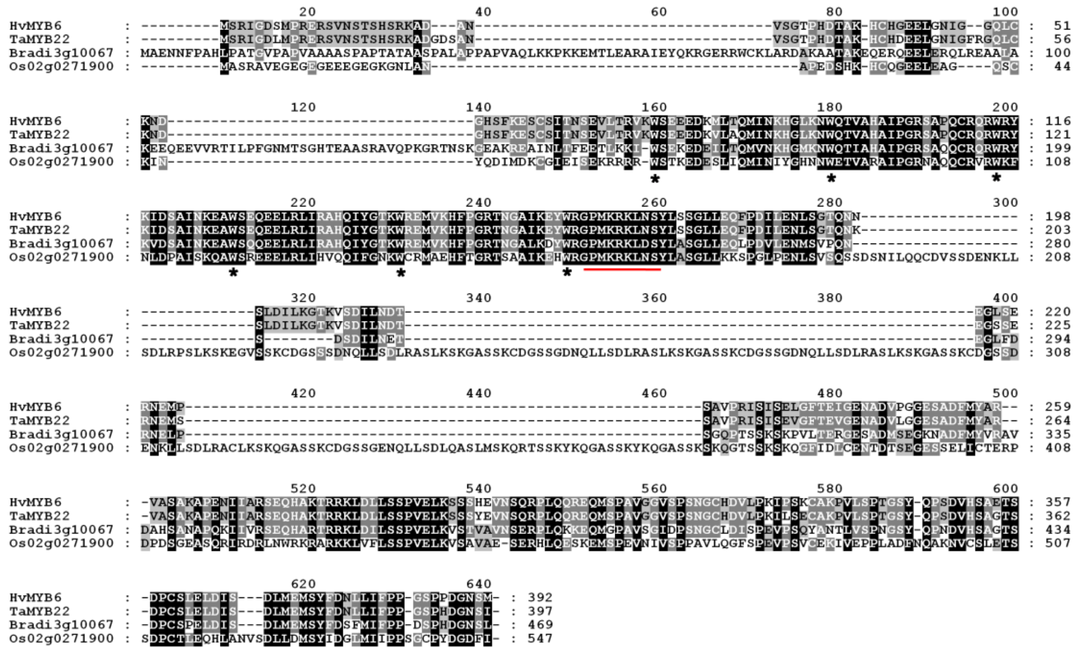
Supplemental Figure 8A. Nucleotide sequence alignment of Hv-*Myb6* and the barley MLOC_14401.

The nucleotide sequence of MLOC_14401 shared 42% identity with Hv-*Myb6*, however, there is no contiguous identical nucleotide stretches between. Identical nucleotides in both DNA sequences are shaded in dark. The Hv-*Myb6* fragment employed in the BSMV and TIGS-mediated silencing experiments is underlined.

```
                              20                  40                  60                  80                  100
HvMYB6     : MSRIGDSMPRERSVNSTSHSRKADANVS--------GTPHDTAKHCHGEELGNIGGQLC---------------KNDGHSFKESCSITNSEVLTR----- :  72
MLOC_14401 : MASMGELLLNQELAAVAAMSTDAYGRFERYTLSPAHGFPHFQQAGSAPTVFPDTGLLVSGFGMPPSTFIVMPEGTHAADAYGAEAVPVEIPVVRRQRPAA : 100

                             120                 140                 160                 180                 200
HvMYB6     : VK---------WSEEEDKMLTQMINKHGLKNWQTVAHAIPGRSAPQCRQRWRYKIDSAIN-KEAWSEQEELRLIRAHQIYGTKWREMVKHFPGRTNGAIK : 162
MLOC_14401 : RNAGPTPFRGPWTEEEDELLKRLVDQHGEHKWATISDHLPGRIGKQCRERWTNHVRPGIKKEHIWTEADDILLIDAHKIHGNRWSSIARCLPGRSENAVK : 200
                       *                   *                  *             *                  *

                             220                 240                 260                 280                 300
HvMYB6     : EYWRGPMKRKLNSYLSSGLLEQFPDILENLSGTQNNSLDILKGTKVSDILNDTEGLS--ERNEMPSAVPRISIS-ELGFTEIGENADVPGGESADFMYAR : 259
MLOC_14401 : NHWN-ATKRSLKSKRRFKKKTSQQAAPGQFTLLEEYIRDKMMADENAAPPCPSSGGVGYDGQVVPGAAAMLAVSGPPGMGQYLHPADAAGSSSQ----AG : 295
               *  ............

                             320                 340                 360                 380                 400
HvMYB6     : VASAKAPENIIARSEQHAKTRRKLDLLSSPVELKSSS-HEVNSQRPLQQREQMSPAVGGVSPSNGCHDVLPKIPSKCAKPVLSPTGSYQPSDVHSAETSD : 358
MLOC_14401 : VMNLSAPL-----PDLNAYGGEMLEGYYYPATFPTYSDNNLLHHGPETAFPQMFSAQGSMHMHAACTNLNLFPLPQHLG------GGYYGSETGRSSAGG : 384

                             420
HvMYB6     : PCSLELDISDLMEMSYFDNLLIFPPGSPPDGNSM-  : 392
MLOC_14401 : SSDQDEDVVQMASREFQT----SEDEATLDLTGFS  : 415
```

Supplemental Figure 8B. Protein sequence alignment of Hv-MYB6 and the barley MLOC_14401.

MLOC_14401 is the most closely related MYB to Hv-MYB6 among the 80 identified R2R3-type MYB TFs from barley with only 18% amino acid sequence identity. Identical residues in both protein sequences are shaded in dark. The highly conserved Trp (W) residues in the MYB repeats are marked with star and the putative nuclear localization signals (NLSs) are underlined.

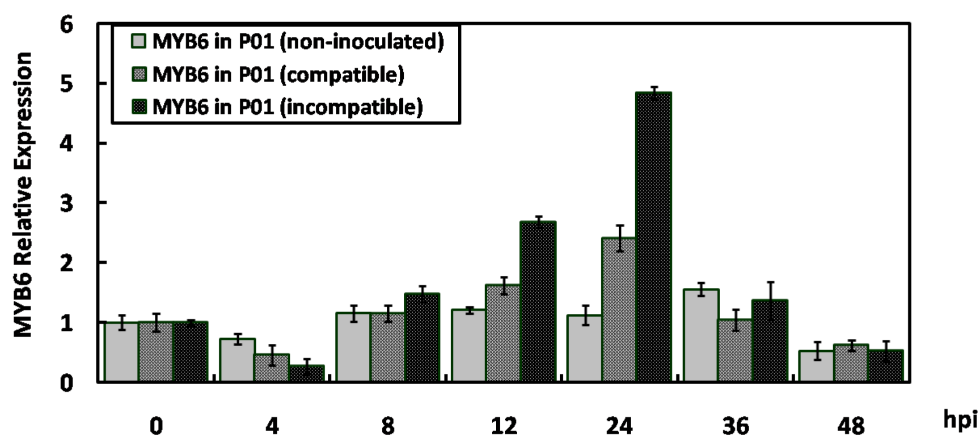


Supplemental Figure 8C. Sequence comparison among Hv-MYB6 and its most closely related R2R3-type MYB TFs from wheat, brachypodium and rice.

Hv-MYB6 is homologous to wheat Ta-MYB22, brachypodium MYB Bradi3g10067 and rice MYB Os02g02719 (same as NM_001053059) with 95%, 51% and 32% sequence identity, respectively. Residues shaded in dark are highly conserved among four proteins and gray among three proteins. The highly conserved Trp (W) residues in the MYB repeats are marked with star and the putative nuclear localization signals (NLSs) are underlined.

Supplemental Figure 8D. Relationship of barley *Hv*-MYB6 with the R2R3-type MYB proteins from *Arabidopsis thaliana*.

The tree was constructed using the neighbor-joining method and 1000 bootstraps with the 125 *Arabidopsis* R2R3-type MYB TFs (Stracke et al., 2001). Similar same subgroups were obtained as before (Stracke et al., 2001). The 125 *Arabidopsis* R2R3-type MYB sequences were gained from http://www.arabidopsis.org/ using HMMER3.0 with seed alignment PF00249 download from Pfam database, and then motifs were detected using MEME4.9.0. The bar indicates 20% dissimilarity.



Supplemental Figure 9. *MYB6* Expression is Induced by *Bgh* Infection, with the Maximum Increases by the Avirulent Isolate.
qRT-PCR analysis of *MYB6* expression in barley first leaves upon inoculation of *Bgh*. RNA samples were derived from the first leaf of 7-day-old seedling without or with virulent or avirulent *Bgh* isolate inoculations. Error bar indicates SE of technical triplicates. Experiments were repeated at least four times with similar results.

Supplemental Table 1. Plasmids constructed in this study.

Plasmids	**Descriptions**
pLexA-MLA1_CC-NB(1-225)	pLexA carrying MLA1_CC-NB(1-225)
pLexA-*Hv*MYB6_NT	pLexA carrying *Hv*MYB6_NT(1-190)
pLexA-*Hv*WRKY1ΔCT	pLexA carrying *Hv*WRKY1ΔCT(1-260)
pLexA-*Hv*WRKY1_CT	pLexA carrying *Hv*WRKY1_CT(260-355)
pB42AD-*Hv*MYB6	pB42AD carrying *Hv*MYB6 FL (1-392)
pB42AD-*Hv*MYB6_NT	pB42AD carrying *Hv*MYB6_NT (1-190)
pB42AD-*Hv*MYB6_CT	pB42AD carrying *Hv*MYB6_CT (166-392)
pB42AD-MLA1_CC(47-161)	pB42AD carrying MLA1_CC(47-161)
pB42AD-MLA1_CC-NB(1-225)	pB42AD carrying MLA1_CC-NB(1-225)
pB42AD-*Hv*MYB6 (W75A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W75A
pB42AD-*Hv*MYB6 (W95A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W95A
pB42AD-*Hv*MYB6 (W114A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W114A
pB42AD-*Hv*MYB6 (I122A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution I122A
pB42AD-*Hv*MYB6 (W127A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W127A
pB42AD-*Hv*MYB6 (W146A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W146A
pB42AD-*Hv*MYB6 (W165A)	pB42AD carrying *Hv*MYB6 FL (1-392) with the substitution W165A
pGBKT7-*Hv*WRKY1	pGBKT7 carrying *Hv*WRKY1 FL
pGBKT7-*Hv*WRKY1ΔCT	pGBKT7 carrying *Hv*WRKY1ΔCT(1-260)
pGADT7-*Hv*MYB6	pGADT7 carrying *Hv*MYB6 FL
pBridge-MLA10_CC	pBridge carrying wild type MLA10_CC
pBridge-MLA10_CC (L11E)	pBridge carrying MLA10_CC with the substitution L11E
pBridge-MLA10_CC (L18E)	pBridge carrying MLA10_CC with the substitution L18E
pMALc2x-MLA1_CC	pMALc2x carrying MLA1_CC
pMALc2x-MLA6_CC	pMALc2x carrying MLA6_CC
pMALc2x-MLA10_CC	pMALc2x carrying MLA10_CC
pMALc2x-*Hv*MYB6	pMALc2x carrying wild type *Hv*MYB6
pET32a-MLA1_CC	pET32a carrying MLA1_CC
pET32a-MLA6_CC	pET32a carrying MLA6_CC
pET32a-MLA10_CC	pET32a carrying MLA10_CC

pET32a-*Hv*WRKY1	pET32a carrying *Hv*WRKY1
pET32a-*Hv*WRKY2	pET32a carrying *Hv*WRKY2
pET32a-*Hv*WRKY1ΔCT	pET32a carrying *Hv*WRKY1ΔCT(1-260)
CTAP-YC-*Hv*MYB6	CTAP carrying YC-*Hv*MYB6
CTAP-MLA1_CC-YN	CTAP carrying MLA1_CC-YN
CTAP-MLA6_CC-YN	CTAP carrying MLA6_CC-YN
CTAP-MLA10_CC-YN	CTAP carrying MLA10_CC-YN
CTAP-YN-*Hv*WRKY1	CTAP carrying YC-*Hv*WRKY1
CTAP-YN-*Hv*WRKY2	CTAP carrying YC-*Hv*WRKY2
CTAP-YC-*Hv*WRKY1ΔCT	CTAP carrying YC-*Hv*WRKY1ΔCT
CTAP-MLA10_CC	CTAP carrying wild type MLA10_CC
CTAP-MLA10_CC (L11E)	CTAP carrying MLA10_CC with the substitution L11E
CTAP-MLA10_CC (L18E)	CTAP carrying MLA10_CC with the substitution L18E
CTAP-GFP-3HA	CTAP-GW-3HA carrying GFP
CTAP-*Hv*MYB6-3HA	CTAP-GW-3HA carrying *Hv*MYB6
CTAP-*Hv*WRKY1_FL-3HA	CTAP-GW-3HA carrying full length *Hv*WRKY1
CTAP-*Hv*WRKY1ΔCT-3HA	CTAP-GW-3HA carrying *Hv*WRKY1ΔCT(1-260)
CTAP-*Hv*WRKY2_FL-3HA	CTAP-GW-3HA carrying full length *Hv*WRKY2
CTAP-*Hv*MYB6-mYFP	CTAP-GW-mYFP carrying *Hv*MYB6
pCAMBIA-nLuc-*Hv*WRKY1	pCAMBIA-nLuc carrying full length *Hv*WRKY1
pCAMBIA-nLuc-*Hv*WRKY2	pCAMBIA-nLuc carrying full length *Hv*WRKY2
pCAMBIA-nLuc-*Hv*WRKY1ΔCT	pCAMBIA -nLuc carrying *Hv*WRKY1ΔCT(1-260)
pCAMBIA-nLuc-*Hv*WRKY1_CT	pCAMBIA-nLuc carrying *Hv*WRKY1_CT(260-355)
pCAMBIA-nLuc-*Hv*WRKY1-VP16	pCAMBIA-nLuc carrying *Hv*WRKY1-VP16
pCAMBIA-cLuc-*Hv*MYB6	pCAMBIA-cLuc carrying full length *Hv*MYB6
pCAMBIA-cLuc-*Hv*MYB6_NT	pCAMBIA-cLuc carrying *Hv*MYB6_NT(1-190)
pCAMBIA-cLuc-*Hv*MYB6_CT	pCAMBIA-cLuc carrying *Hv*MYB6_CT(166-392)
pAD-*Hv*MYB6	pAD-GAL4-2.1 carrying *Hv*MYB6
pHIS2-*MBS*	pHIS2 carrying three copies of *MBS* cis-elements
pHIS2-*MBS I*	pHIS2 carrying three copies of *MBS I* cis-elements
pHIS2-*MBS II*	pHIS2 carrying three copies of *MBS II* cis-elements
pHIS2-*MYBR*	pHIS2 carrying three copies of *MYBR* cis-elements
pHIS2-*MRE*	pHIS2 carrying three copies of *MRE* cis-elements
pHIS2-*MRE1*	pHIS2 carrying three copies of *MRE1* cis-elements
pHIS2-*MRE2*	pHIS2 carrying three copies of *MRE2* cis-elements
pHIS2-*MRE3*	pHIS2 carrying three copies of *MRE3* cis-elements
pHIS2-*MRE4*	pHIS2 carrying three copies of *MRE4* cis-elements
4x*MBSI*-5x*GAL4*-LUC	An *Arabidopsis* protoplast reporter vector with 4x*MBSI* and 5x*GAL4* cis-element

pRT-BD-*Hv*MYB6	pRT-BD carrying full length *Hv*MYB6
pRT-BD-*Hv*MYB6_NT	pRT-BD carrying *Hv*MYB6_NT(1-190)
pRT-BD-*Hv*MYB6_CT	pRT-BD carrying *Hv*MYB6_CT(166-392)
pRT-BD-*Hv*WRKY1	pRT-BD carrying *Hv*WRKY1
pRT-BD-*Hv*WRKY1-VP16	pRT-BD carrying *Hv*WRKY1-VP16
pRT-BD-MLA10_CC	pRT-BD carrying wild type MLA10_CC
pRT-BD-MLA10_CC (L11E)	pRT-BD carrying MLA10_CC with the substitution L11E
pRT-BD-MLA10_CC (L18E)	pRT-BD carrying MLA10_CC with the substitution L18E
pRT107-*Hv*MYB6	pRT107 carrying full length *Hv*MYB6
pRT107-*Hv*WRKY1	pRT 107 carrying *Hv*WRKY1
pRT107-*Hv*WRKY1-VP16	pRT 107 carrying *Hv*WRKY1-VP16
pRT107-MLA10_CC	pRT 107carrying wild type MLA10_CC
pRT107-MLA10_CC (L11E)	pRT 107 carrying MLA10_CC with the substitution L11E
pRT107-MLA10_CC (L18E)	pRT 107 carrying MLA10_CC with the substitution L18E
pRT107-MLA10	pRT 107carrying wild type MLA10
pRT107-MLA10 (K207R)	pRT 107 carrying MLA10 with the substitution K207R
pRT107-MLA10 (D502V)	pRT 107 carrying MLA10 with the substitution D502V
IPKb007-*Hv*MYB6	IPKb007 carrying an DNA fragment from *Hv*MYB6
pUbi-*Hv*MYB6-mYFP	pUbi-GW-mYFP carrying *Hv*MYB6
pCa-γb-*MLA12*as	pCa-γbLIC carrying an antisense DNA fragment from *MLA12*
pCa-γb-*HvMYB6*as	pCa-γbLIC carrying an antisense DNA fragment from *HvMYB6*

Supplemental Table 2. Plasmids from other sources used in this study.

Plasmids	Description	Source/reference
pLexA	A yeast bait vector with LexA DNA binding domain	Clontech
pLexA-MLA1_NB-ARC	pLexA carrying MLA1_NB-ARC(154-555)	(Shen et al., 2007)
pLexA-MLA1_LRR	pLexA carrying MLA1_LRR(545-960)	(Shen et al., 2007)
pLexA-MLA1 FL	pLexA carrying MLA1 FL (1-960)	(Shen et al., 2007)
pLexA-MLA10_ CC-NB(1-225)	pLexA carrying wild type MLA10 CC-NB(1-225)	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (L11E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution L11E	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (L18E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution L18E	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (I33E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution I33E	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (L36E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution L36E	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (M43E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution M43E	(Maekawa et al., 2011)
pLexA-MLA10_ CC-NB(1-225) (F83E)	pLexA carrying MLA10 CC-NB(1-225) with the substitution F83E	(Maekawa et al., 2011)
pLexA-*Hv*WRKY1	pLexA carrying *Hv*WRKY1 FL	(Shen et al., 2007)
pLexA-*Hv*WRKY2	pLexA carrying *Hv*WRKY2 FL	(Shen et al., 2007)
pB42AD	A yeast prey vector with transcription activation domain	Clontech
pB42AD-MLA1_CC(1-46)	pB42AD carrying MLA1_CC(1-46)	(Shen et al., 2007)
pB42AD-MLA1_CC(1-161)	pB42AD carrying MLA1_CC(1-161)	(Shen et al., 2007)
pGBKT7	A yeast bait vector with GAL4 DNA binding domain	Clontech
pGADT7	A yeast prey vector with transcription activation domain	Clontech
pBridge	A yeast expression vector under the control of the MET25 promoter	Clontech
pMALc2x	An *E.coli* expression vector to produce the MBP fusion protein	NEB
pET32a	An *E.coli* expression vector produce the His fusion protein	Novagen
CTAPi	A *N. benthamiana* expression vector	(Bai et al., 2012)
CTAP-GW-3HA	A *N. benthamiana* expression vector to produce 3HA fusion	(Bai et al., 2012)

CTAP-GW-mYFP	A *N. benthamiana* expression vector to produce mYFP fusion	(Bai et al., 2012)
pCAMBIA-nLuc	A *N. benthamiana* expression vector to produce nLUC fusion	(Chen et al., 2008)
pCAMBIA-cLuc	A *N. benthamiana* expression vector to produce nLUC fusion	(Chen et al., 2008)
pAD-GAL4-2.1	A yeast expression vector with transcription activation domain	Clontech
pHIS2	A yeast reporter vector that contains the nutritional marker gene *HIS3*.	Clontech
5XGAL4-LUC	An *Arabidopsis* protoplast reporter vector with 5 copies of GAL4 cis-element	(Wei et al., 2009)
pRT-BD	An *Arabidopsis* protoplast expression vector to produce GAL4 DBD fusion	(Wei et al., 2009)
pRT-BD-VP16	An *Arabidopsis* protoplast expression vector to produce DBD-VP16	(Wei et al., 2009)
pRT 107	An *Arabidopsis* protoplast expression vector	(Wei et al., 2009)
pUbi-GW	An expression vector for transient overexpression in barley epidermal cell	(Shen et al., 2007)
pUbi-*Hv*WRKY1	pUbi-GW carrying *Hv*WRKY1	(Shen et al., 2007)
IPKb007	An expression vector for transient silencing (TIGS) in barley leaf cell	(Himmelbach et al., 2007)
pUbi-GW-mYFP	An expression vector for transient overexpression to produce mYFP tag	(Shen et al., 2007)
pCa-γbLIC	An expression vector for BMSV mediated gene silencing	(Yuan et al., 2011)

Supplemental Table 3. BLASTN Search Results of the Barley Genome Database with the Fragment Used for *HvMYB6*-Silencing.

Group	Sequence identity with the fragment used for *HvMYB6*-silencing	Sequence ID	Gene
1	100%	AK361679.1	*HvMYB6*
		AK249997.1	*HvMYB6*
2	80%	AK353689.1	*HvMYB6*
3	4%-5%	JF495489.1	*CKX2.2*
		AK376220.1	Unknown
		AK361410.1	Unknown
		AK355396.1	Unknown
		AK371627.1	Unknown
		AY943294.1	Unknown

Supplemental Methods

1. Plasmid Construction

Plasmids constructed in the present study are listed in Supplemental Table 1.

Method for bait and prey plasmid construction for yeast two-hybrid assays has been previously described (Shen et al., 2007; Maekawa et al., 2011). Preys containing single amino acid substitution (W75A, W95A, W114A, I122A, W127A, W146A and W165A) in the R2R3 repeats of MYB6 (in Supplemental Table 1) were constructed using splice overlap extension technique. Baits containing single amino acid substitution (L11E, L18E, I33E, L36E, M43E and F83E) in the CC domain of MLA10_CC-NB(1-225) were directly PCR-amplified from the template constructs previously described (Maekawa et al., 2011) and subcloned.

For BiFC assays, N- or C-terminal half of YFP (YN/YC, 154 or 87 amino acid residues, respectively) was fused to the protein of interests by two different approaches, first one is using the vector pSY736 or pSY735, for example, the coding region of WRKY1 was fused to the YN fragment through ligation into the vector pSY736 while the coding region of MYB6 was fused to YC fragment through ligation into the vector pSY735 (Wei et al., 2009); second one is through PCR amplification using splice overlap extension technique, for example, for MLA1_CC, MLA6_CC or MLA10_CC YN fusions. The above YN/YC fusion protein was further sub-cloned into the CTAPi vector (Bai et al., 2012) through Gateway technology (Invitrogen) for expression in *N. benthamiana*.

For LCI assays, C-terminal or N-terminal half of Luciferase protein (cLUC/nLUC) was fused to the protein of interest for further analysis. The coding region of MYB6, MYB6_NT(1-190) and MYB6_CT(161-392) was fused to cLUC by ligation into the vector of pCAMBIA-cLUC to create the cLUC-*Hv*MYB6/ *Hv*MYB6_NT/*Hv*MYB6_CT constructs (Chen et al., 2008).
The WRKY1, WRKY1_CT, WRKY1ΔCT and WRKY2 were fused to nLUC by ligation into the vector of pCAMBIA-nLUC to create the nLUC-*Hv*WRKY1/*Hv*WRKY1_CT/*Hv*WRKY1 ΔCT/*Hv*WRKY2 constructs.

For yeast three-hybrid assay, the coding region of MYB6 was cloned into the vector pGADT7 to generate the AD-*Hv*MYB6 prey construct. The coding

region of WRKY1 or WRKY1ΔCT was cloned into the pGBKT7 vector to generate the BD-WRKY1 or BD-WRKY1ΔCT bait construct. The coding region of wild type MLA10_CC WT or L11E or L18E was ligated into the pBridge vector to generate the competitor constructs (Li et al., 2011).

To construct the plasmids for in vitro protein-protein interaction assay, the MLA CC domains (including MLA1_CC, MLA6_CC and MLA10_CC) or MYB6 was fused to the MBP domain by ligation of the respective coding region into the pMALc2x vector (New England Biolab). The coding region of the MYB6, WRKY1, WRKY1ΔCT and WRKY2 was recombined into CTAPi-GW-3HA (Bai et al., 2012) through Gateway technology to generate the constructs for protein expression in *N. benthiamiana.*

For electrophoretic mobility-shift assays, the coding region of MYB6 was ligated into the pMALc2x to generate the construct for obtaining MBP-*Hv*MYB6 recombinant. The coding region of WRKY1, WRKY1ΔCT, WRKY2, MLA10_CC WT, L11E or L18E variant was individually ligated into pET32a for protein expression and purification in *E. coli*. The FLAG tag (Asp Tyr Lys Asp Asp Asp Asp Lys) in WRKY1-FLAG and the HA tag (Tyr Pro Tyr Asp Val Pro Asp Tyr Ala) in MLA10_CC-HA are introduced by PCR using nested primer pairs: 5’-AGCCCCTCGAGTCACTTGTCATCGTCGTCCTTGTAGTCATTGATG /5’-ATCGTCGTCCTTGTAGTCATTGATGTCCCTGGTCGGCGAGA and 5’-AGCCCCTCGAGTCAGCCCGCATAGTCAGGAACATCGTATGGGTAAAAG ATGCCGG/5’-AACATCGTATGGGTAAAAGATGCCGGAGCCCAAAGCTCGA AGACAAGGATC, respectively.

For the Arabidopsis protoplast transactivation assay, all effector plasmids containing the GAL4 DNA binding domain (DBD) were constructed by cloning the coding region of gene/fragment of interests into the vector pRT-BD (Wei et al., 2009), for example, for expressing DBD-*Hv*MYB6, DBD-*Hv*MYB6_NT, DBD-*Hv*MYB6_CT, or DBD-VP16. Other effector plasmids without fused to the DBD were constructed by cloning into the vector pRT107, including MYB6, WRKY1, WRKY1-VP16, MLA10_CC domain (WT, or L11E and L18E variant), and MLA10 FL variants (WT, K207R or D502V variant), all driven by the 35S promoter.

For single-cell transient gene overexpression assay, the CDS region of

MYB6 was cloned into a vector driven by the maize polyubiquitin promoter to create the construct pUbi-*Hv*MYB6.

For single-cell transient-induced gene silencing assay, a fragment of the 3′untranslated region of MYB6 was amplified using the primer pairs 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTCCAGCTGTTGGTGGT GTCTC/5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCAGGAACTGTCAG TTTACAGGAC, and cloned into the pIPKb007 vector Gateway technology to create the TIGS:*Hv*MYB6 construct (Himmelbach et al., 2007).

For BSMV-mediated VIGS, we used pCaBS-α, pCaBS-β and pCa-γbLIC vector for transcription of respective BSMV RNAs in *N. benthiamiana* (Yuan et al., 2011). By means of ligation independent cloning technique, an antisense fragment of 368 base pairs of the 3' untranslated region of MYB6 was cloned into the pCa-γbLIC vector to create the $BSMV$:$HvMYB6_{as}$ construct using the primer pairs 5'-AAGGAAGTTTAAGTCCAGCTGTTGGTGGTG TCTC/5'-AACCACCACCACCGACAGGAACTGTCAGTTTACAGGAC.

For yeast one hybrid assay, three copies of each of the DNA cis-elements *MBS* (CACCAT), *MBS I* (TAACTG), *MBS II* (CTTTGGT), *MYBR* (TTGTTAG), *MRE* (AACCAAA), *MRE1* (CCGGCAGTTAGGAT), *MRE2* (TATAACGGTTTTTT), *MRE3* (TCTAACCTACCA) and *MRE4* (CACCTAC) were synthesized (Invitrogen, China), annealed, and cloned into the vector pHIS2. The CDS region of MYB6 was cloned into the vector pAD-GAL4-2.1 to generate the construct pAD-MYB6.

2. Single-Cell Transient Gene Expression, Transient-Induced Gene Silencing Assay and BSMV-Mediated Virus Induced Gene Silencing

The single-cell transient gene expression was conducted essentially as described (Shen et al., 2007; Maekawa et al., 2011). The β-glucuronidase (GUS) reporter gene was codelivered into the barley epidermal cell to mark the transformed cells and better visualization of fungal haustorium in these cells. The pUbi or TIGS constructs were mixed with the GUS reporter vector at the 1:1 molar ratio before coating the DNA microcarrier. The exogenous DNA on the microcarrier were delivered into the barley epidermal cell through the particle inflow gun (Bio-Rad, Model PDS-1000/He). The inoculation of

B. graminis conidia spores was performed 14 hrs post bombardment in transient overexpression assay and at least 60 hrs post bombardment in transient–induced gene silencing assay. The leaf segments were stained for GUS activity 48 hrs post *Bgh* spore inoculation and kept in the destaining solution. Before mounting for microscopy, the leaves were stained with coomassie blue to visualize the fungal epiphytic structure.

For BSMV-mediated VIGS, construct DNA of pCaBS-α, pCaBS-β, and pCa-γbLIC derivatives (BSMV:EV/BSMV:*MLA12as* / BSMV:*HvMYB6as*) were transformed into the *A. tumefaciens* strain GV3101, individually. The Agrobacteria culture was harvested and resuspended in the infiltration buffer (10 mM $MgCl_2$, 100 μM acetosyringone and 10 mM MES). The cell suspension containing pCaBS-α, pCaBS-β and pCa-γbLIC derivatives were mixed at the 1:1:1 ratio and infiltrated the *N. benthamiana* leaves*.* The *N. benthamiana* sap from leaves with *BSMV* infection symptom about 12 days post Agroinfiltration was used to inoculate the first two emerging leaves of barley I10 plants. The upper leaves with virus symptom about 14 days post BSMV-infection was collected and challenged with powdery mildew conidiospores (*Bgh* isolate A6). Leaf segments were fixed and stained with coomassie blue to visualize the fungal structure at 48 hrs post conidia spore inoculation.

3. Phylogenetic Analysis

The 125 Arabidopsis R2R3-type MYB sequences were gained from http://www.arabidopsis.org/ using HMMER3.0 (Finn et al., 2011) with seed alignment PF00249 download from Pfam database (Finn et al., 2010) with E-value threshold 0.01, then motifs were detected using MEME4.9.0 (Bailey et al., 2009) with default options. Sequences were aligned using CLUSTALW (Thompson et al., 1994) with default options, and the alignment was corrected manually using GeneDoc (Nicholas et al., 1997). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011) with default options and internal branch support was estimated with 1000 bootstrap replicates. The bar indicates 20% dissimilarity.

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