bHLH05 Is an Interaction Partner of MYB51 and a Novel Regulator of Glucosinolate Biosynthesis in Arabidopsis[1][W][OPEN]

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By means of yeast (*Saccharomyces cerevisiae*) two-hybrid screening, we identified basic helix-loop-helix transcription factor05 (bHLH05) as an interacting partner of MYB51, the key regulator of indolic glucosinolates (GSLs) in Arabidopsis (*Arabidopsis thaliana*). Furthermore, we show that bHLH04, bHLH05, and bHLH06/MYC2 also interact with other R2R3-MYBs regulating GSL biosynthesis. Analysis of *bhlh* loss-of-function mutants revealed that the single *bhlh* mutants retained GSL levels that were similar to those in wild-type plants, whereas the triple *bhlh04/05/06* mutant was depleted in the production of GSL. Unlike *bhlh04/06* and *bhlh05/06* mutants, the double *bhlh04/05* mutant was strongly affected in the production of GSL, pointing to a special role of bHLH04 and bHLH05 in the control of GSL levels in the absence of jasmonic acid. The combination of two specific gain-of-function alleles of MYB and bHLH proteins had an additive effect on GSL levels, as demonstrated by the analysis of the double *MYB34-1D bHLH05D94N* mutant, which produces 20-fold more indolic GSLs than *bHLH05D94N* and ecotype Columbia-0 of Arabidopsis. The amino acid substitution D94N in *bHLH05D94N* negatively affects the interaction with JASMONATE-ZIM DOMAIN protein, thereby resulting in constitutive activation of bHLH05 and mimicking jasmonic acid treatment. Our study revealed the bHLH04, bHLH05, and bHLH06/MYC2 factors as novel regulators of GSL biosynthesis in Arabidopsis.

Plants produce an enormous variety of secondary metabolites that are known as defense products against microbes, fungi, or herbivores, but also serve as signaling molecules in plant-plant, plant-animal, and plant-microbe interactions. Approximately 200,000 to 1,000,000 metabolites have been predicted to exist in plants (Dixon and Strack, 2003; Wink, 2010; Mohanta, 2013), and tens of thousands have already been described, including 200 glucosinolates (GSLs; Clarke, 2010). The high natural diversity of gene products provides a high variability in secondary metabolites and their functions. However, the metabolic richness derives not only from the number of genes present (about 25,500 in Arabidopsis [*Arabidopsis thaliana*]), but also from multiple substrate specificities of enzymes (Schwab, 2003) and importantly, from the plethora of possibilities to specifically regulate these compounds in particular tissues under certain conditions. However, only a limited number of genes involved in the regulation of plant secondary metabolites (Broun, 2004; Grotewold, 2008; Wu and Chappell, 2008) and GSLs (Sønderby et al., 2010a) have been identified to date.

GSLs are known for their role in plant-herbivore interactions and for their cancer-preventive properties, which has intensified research on GSLs. Together with flavonoids (Lepiniec et al., 2006; Gonzalez et al., 2008; Li, 2014; Xu et al., 2014), GSLs have become model secondary metabolites (Sønderby et al., 2010b) in the field of natural plant products. The availability of genetic sequence information, large mutant collections, quantitative trait loci, and expression profiling tools have facilitated the identification of biosynthetic enzymes of GSLs (Supplemental Fig. S1) and led to the intensive characterization of a large number of genes for the production of GSL in Arabidopsis. However, knowledge concerning the factors that regulate GSL levels is scarce. For example, this is the case for the proteins controlling the levels of different GSLs in different plant tissues, which modulate their biosynthesis, transport, or breakdown in response to different biotic and abiotic factors. Therefore, understanding the regulatory mechanisms that modulate GSL concentrations in plants remains of great interest in plant science.

The network of proteins that controls the production of GSLs was recently characterized and comprises six R2R3 MYB transcription factors (TFs) belonging to subgroup 12 of the R2R3 MYB family, having a conserved [L/F]LN[K/R]VA motif (Stracke et al., 2001; Dubos et al., 2010). The MYB34, MYB51, and MYB122 proteins are involved in the transcriptional regulation of the Trp-derived (indole) GSL pathway (Celenza

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et al., 2005; Gigolashvili et al., 2007a; Malitsky et al., 2008; Frerigmann and Gigolashvili, 2014), and MYB28, MYB29, and MYB76 regulate the Met-derived (aliphatic) GSLs in Arabidopsis (Hirai et al., 2007; Sønderby et al., 2007, 2010a; Gigolashvili et al., 2007b, 2008; Beekwilder et al., 2008; Malitsky et al., 2008; Li et al., 2013). Only a few other nuclear-localized components have been reported, which affect the biosynthesis of GSL in Arabidopsis, but have additionally broader functions. These include IQ-DOMAIN1, DNA-BINDING-WITH-ONE-FINGER1.1, SULFUR LIMITATION1, and TERMINAL FLOWER2. The IQ-DOMAIN1 protein is a nuclear-localized calmodulin-binding protein and a positive regulator of GSL that was identified in a screen for mutants with increased GSL accumulation (Levy et al., 2005; Abel et al., 2013). The DNA-BINDING-WITH-ONE-FINGER1.1 protein (also known as OBP2) is a component of the regulatory network controlling GSL biosynthesis, which induces the transcription of at least *cytochrome P450 CYP83B1* (Skirycz et al., 2006). The SULFUR LIMITATION1 protein represses the biosynthesis of GSLs and activates enzymes catabolizing GSL in response to sulfate deficiency. Finally, TERMINAL FLOWER2 (also known as TU8), which is involved in controlling heterochromatin structure (Kim et al., 2004; Bennett et al., 2005), affects the accumulation of GSL, but is also important in flowering, meristem formation, and leaf morphology. However, both qualitative and quantitative fluctuations in the accumulation of GSLs in different tissues during plant development (Petersen et al., 2002; Brown et al., 2003) or in response to environmental stimuli (Bodnaryk, 1992, 1994; Kiddle et al., 1994; Doughty et al., 1995; Brader et al., 2001; León et al., 2001; Pontoppidan, 2001; Kliebenstein et al., 2002; Mikkelsen et al., 2003; Farnham et al., 2004; Lorenzo et al., 2004; Mewis et al., 2005; Dombrecht et al., 2007; Yan and Chen, 2007; Erb et al., 2011; Verhage et al., 2011; Guo et al., 2013a) cannot be fully explained by activities of MYB or other nuclear-localized factors and more components of the GSL regulatory network are anticipated to exist.

Combinatorial interactions among TFs are central to the gene regulation of any given cellular process (Feller et al., 2011). Combinatorial regulation of transcription has several advantages, including the control of gene expression in response to a variety of signals from the environment and the use of a relatively limited number of TFs to create many combinations of regulators whose activities are modulated by diverse sets of conditions (Pilpel et al., 2001). Examining the expansion of TF families in plants, animals, and fungi revealed that TF families shared among these organisms have undergone a much more dramatic expansion in plants than in other eukaryotes (Shiu et al., 2005; Hanada et al., 2008). This elevated expansion of plant TFs is caused by not only higher duplication rates of plant genomes but also a higher degree of expansion of TFs compared with other plant genes. This diversity of possibilities to fine-tune plant responses is especially important for plants as sessile organisms, which require more sophisticated mechanisms of response to the environment, which will benefit the plant without being too costly.

Despite great advances made in GSL research, no combinatorial control of gene expression has been reported thus far. This study aimed to increase understanding of the molecular control of GSL synthesis by identifying further components of GSL regulation, acting together with MYBs in combinatorial manner.

Yeast (*Saccharomyces cerevisiae*) two-hybrid analysis with MYB51 as a bait revealed several interacting proteins. After thorough selection and verification of these interactions, about 54 proteins belonging to the MYB51 interactome were identified. Within this group, one candidate basic helix-loop-helix transcription factor05 (bHLH05) and its homologous proteins (bHLH04, bHLH06, and bHLH28) were validated as bona fide interaction partners both in vitro and in planta and further analysis of these proteins was pursued in this study. Gene expression analysis in combination with chemotyping single and multiple *bhlh* loss-of-function mutations revealed that the bHLH proteins of the subgroup IIIe are novel regulators of GSL biosynthesis in Arabidopsis. Further analysis of double bHLH and MYB gain-of-function alleles revealed the specific role of combinatorial MYB-bHLH interactions in the transcriptional regulation of GSL biosynthesis.

RESULTS

Interactome of MYB51 Identified by Yeast Two-Hybrid Screening

To identify proteins that function in complex with MYB51, a yeast two-hybrid assay with MYB51 as a bait and an Arabidopsis complementary DNA (cDNA) library as a prey was performed. The full-length coding sequence of MYB51 and three different truncated forms of MYB51 containing 151, 251, and 351 amino acids, all containing the R2R3-MYB domain and a signature typical for the proteins regulating GSL biosynthesis, were initially tested for autoactivation. The full-length MYB51 coding sequence activated reporter gene expression without the need to interact with a prey and therefore was eliminated from the screen. Three other nonautoactivating clones of MYB51 fused to the GAL4 DNA-binding domain were assayed for their ability to bind other proteins fused to the GAL4 activation domain.

In total, 510 putative interacting candidates were obtained, which were further verified by retransformation into the prey vector via gap repair (Kelly and Hoffman, 2002) and by a second mating with yeast containing MYB51. This procedure eliminated false-positive interactors and reduced the number of putative MYB51-interacting candidates to 99. The final step of the assay, including the sequencing of these colonies, resulted in 47 positive candidate proteins presented in Table I, due to multiple copies of sequences of frequently interacting candidates.

Table I. *List of proteins interacting with MYB51 in the yeast two-hybrid screen*

AGI Code	Clones	Name	Description
	n		
At4g19700	17	BOI IAP-LIKE PROTEIN	Has E3 ubiquitin ligase activity
At1g47128	2	RESPONSIVE TO DEHYDRATION21	Has been shown to have peptide ligase activity and protease activity in vitro
At3g24800	2	PROTEOLYSIS1 (PRT1)	Contains two ring finger domains and one ZZ-type zinc finger domain binding two zinc ions
At3g12920	2	BRG3	Encodes one of the BRGs involved in resistance to *Botrytis cinerea*
At5g46760	2	ALTERED TRP REGULATION2 MYC3, bHLH05	MYC3 is a JAZ-interacting TF
At5g25480	1	DNA METHYLTRANSFERASE2	Encodes a DNA methyltransferase homolog
At1g52150	1	CORONA and INCURVATA4	Member of the class III homeodomain-leucine zipper protein family
At4g26930	1	MYB DOMAIN PROTEIN97 (MYB97)	Encodes a putative TF
At1g08370	2	DECAPPING1 (DCP1)	Encodes DCP1 involved in mRNA decapping
At3g62100	4	INDOLE-3-ACETIC ACID INDUCIBLE30 (IAA30)	Encodes a member of the Aux/IAA family of proteins implicated in auxin signaling
At1g06620	1		Encodes a protein whose sequence is similar to a 2-oxoglutarate-dependent dioxygenase
At3g09700	6		Chaperone DnaJ-domain superfamily protein
At1g28210	1	Homologous to *Escherichia coli* DnaJ (AtJ1)	DnaJ homolog
At5g17920	1	MET SYNTHESIS1	Encodes a cytosolic cobalamin-independent Met synthase
At5g05170	1	CELLULOSE SYNTHASE3 CONSTITUTIVE EXPRESSION OF VSP1	Encodes a cellulose synthase isomer
At3g08530	1	CLATHRIN H CHAIN2	Clathrin, H chain
At5g41770	1		Crooked neck protein (putative) and cell cycle protein (putative)
At4g00180	1	YABBY3 (YAB3)	YABBY gene family member, likely has TF activity
At5g11980	1		Conserved oligomeric Golgi complex component related /conserved oligomeric Golgi complex component related
At1g75940	1	BETA GLUCOSIDASE20	Encodes a protein similar to the BGL4 β-glucosidase from *Brassica napus*
At5g33290	1	XYLOGALACTURONAN DEFICIENT1	Acts as a xylogalacturonan xylosyltransferase within the xylogalacturonan biosynthesis pathway
At3g57550	1	AGK2	Guanylate kinase
At3g53620	1	PYROPHOSPHORYLASE4	Encodes a soluble protein with inorganic pyrophosphatase activity
At5g53120	2	SPERMIDINE SYNTHASE3	Encodes a novel spermine synthase
At2g24270	1	ALDEHYDE DEHYDROGENASE11A3	Encodes a protein with glyceraldehyde-3-phosphate dehydrogenase activity
At1g07960	2	PROTEIN DISULFIDE ISOMERASE-LIKE 5-1 (PDIL5-1)	Encodes a PDIL protein
At1g21270	2	WAK2	Cytoplasmic Ser/Thr protein kinase induced by salicylic acid
At5g60340	1	ADENYLATE KINASE6	Encodes a nuclear adenylate kinase that interacts with a putative homolog of Rps14
At5g48545	1	HIS TRIAD NUCLEOTIDE-BINDING3	Encodes a protein that has adenylylsulfate sulfohydrolase activity (E.C. 3.6.2.1) in vitro
At5g26190	1		Cys/His-rich C1 domain family protein
At5g22640	1	EMBRYO DEFECTIVE1211 (EMB1211)	EMB1211 is involved in embryo development and chloroplast biogenesis
At4g32190	2		Myosin H chain-related protein
At2g39795	1		Mitochondrial glycoprotein family protein
At1g79280	2	NUCLEAR PORE ANCHOR and TRANSLOCATED PROMOTER REGION	Encodes a 237-kDA protein with similarity to vertebrate translocated promoter region
At4g17330	1	G2484-1	Gene of unknown function expressed in seedlings, flower buds, and stems
At5g63200	1		Tetratricopeptide repeat-containing protein
At5g62090	1	SEUSS-LIKE2 (SLK2)	Best Arabidopsis thaliana protein match is SLK1 (AT4G25520.1)
At5g51200	1	EMB3142	Protein of unknown function (DUF3414)
At5g48610	3		Unknown protein
At5g25757	1		RNA polymerase I-associated factor PAF67
At4g17240	1		Unknown protein
At3g49570	1	RESPONSE TO LOW SULFUR3	Best Arabidopsis protein match is response to low sulfur2 (AT5G24660.1)
At3g45900	1		RNase P protein subunit P38 related
At3g11690	1		Unknown protein
At3g07780	2	OBE1	Encodes a nuclear Plant Homeo Domain finger protein that is functionally redundant with OBE2
At3g02910	1		AIG2-like (avirulence induced gene) family protein
At1g21170	1	SEC5B	Best Arabidopsis protein match is exocyst complex component sec5 (AT1G76850.1)

AGI, Arabidopsis Genome Initiative.

Among the proteins interacting with MYB51 were TFs (bHLH05, MYB97, and YAB3), regulatory proteins (GUANYLATE KINASE2 [AGK2],PYROPHOSPHORYLASE4 [PPA4], and OBERON1 [OBE1]), proteins involved in pathogen or wounding responses (BOTRYTIS SUSCEPTIBLE 1 INTERACTOR (BOI), BOI-RELATED GENE 3 (BRG3) and WALL-ASSOCIATED KINASE2 (WAK2)), and several with unassigned functions (At5g62090, At5g51200, At5g48610, At4g17240, and At3g11690). In this study, we thoroughly analyzed the interaction between MYB51 and bHLH05, because protein complexes consisting of MYBs and bHLHs have been shown to be important for many essential processes in plants (Baudry et al., 2004; Zimmermann et al., 2004; Feller et al., 2006; Butelli et al., 2008; Gonzalez et al., 2008).

The Spatiotemporal Activity of bHLH05 and MYB51 Overlaps and Meets the Requirement for Their Interactions in Vivo

The interaction between R2R3-MYB and bHLH05 was confirmed via bimolecular fluorescence complementation (BiFC) in tobacco (*Nicotiana benthamiana*). We confirmed the interaction between MYB51 and bHLH05 in tobacco in three independent experiments (Fig. 1). Furthermore, we have uncovered the interactions of MYB34, MYB122, MYB28, and MYB29 with both bHLH05 and bHLH04 using BiFC (Supplemental Fig. S2).

To gain further information on the importance of MYB51-bHLH05 complexes in the regulation of indolic glucosinolates (IGs), the expression patterns of bHLH05 were analyzed via fusions of its promoter to the uidA (GUS) reporter in stable transgenic plants (for details, see the "Materials and Methods"). A relevant interaction of both proteins and thus their combined effect on GSL biosynthesis in planta is only possible if two TFs are coexpressed in the same organs or tissues and at the sites of GSL production. As presented in Figure 2, bHLH05 showed strong expression in both roots and shoots of 7-d-old seedlings and mainly in the vasculature. In adult plants (Fig. 2, C, D, and H), GUS staining was observed in most organs of the plant, including stems, siliques, flowers, and roots. The reproductive organs, including flowers and siliques, displayed weak promoter-driven GUS activity. Taken together, the promoter of *bHLH05* showed a similar expression pattern to that previously found for its interacting partner *MYB51* (Gigolashvili et al., 2007a) and GSL biosynthesis genes (Mikkelsen et al., 2000; Grubb et al., 2004; Schuster et al., 2006; Gigolashvili et al., 2009). Thus, *bHLH05* and *MYB51* are coexpressed under normal growth conditions and the interaction of their gene products is therefore possible in planta. Notably, the expression pattern of *bHLH04* has also been reported to overlap with the expression of *bHLH05* and sites of GSL biosynthesis and regulation (Fernández-Calvo et al., 2011).

Multiple Interactions of R2R3-MYB GSL Regulators with bHLHs Are Revealed by the Luminescence-Based Mammalian Interactome Assay

To extend the yeast two-hybrid and BiFC assays and to systematically analyze possible interactions between R2R3-MYB and bHLHs proteins, protein-protein interaction studies by the luminescence-based mammalian interactome (LUMIER) assay (Barrios-Rodiles et al., 2005) in human HEC293TN cells were conducted. For the LUMIER assay, protein fusions to protein A or to luciferase were expressed in human HEC293TN cells and protein A-tagged protein was immunoprecipitated with IgG beads and quantified by luciferase assay.

Using this approach, the interaction between all known R2R3-MYB regulators of GSL biosynthesis (MYB34, MYB51, MYB122, MYB28, MYB29, and MYB76 with bHLH04, bHLH05, bHLH06, and bHLH28) was addressed. The LUMIER experiments confirmed an interaction of MYB51 with bHLH05 that was observed by BiFC and additionally uncovered the complex interactions of all GSL-regulating MYBs with bHLHs belonging to the subgroup IIIe. As summarized in Figure 3, all analyzed R2R3-MYBs interacted with bHLH04, bHLH05, bHLH06, and bHLH28, suggesting that these bHLH proteins represent an important functional component in the regulation of GSL biosynthesis. Notably, the interaction of MYBs was stronger with bHLH04 and bHLH05 and

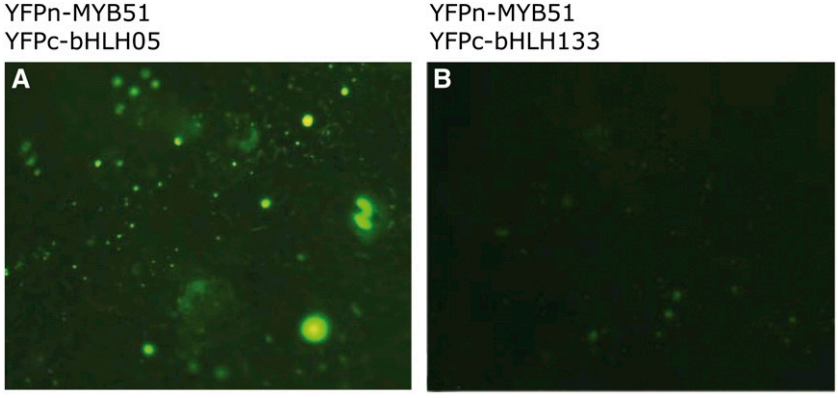

Figure 1. BiFC detection of the protein-protein interaction between MYB51 and bHLH05 in tobacco. BiFC assay in tobacco leaves infiltrated with *A. tumefaciens* containing constructs of interest. A, Interaction between N-terminal yellow fluorescent protein tagged (YFPn)-tagged MYB51 and C-terminal yellow fluorescent protein-tagged (YFPc)-tagged bHLH05 showing the interaction of both proteins. B, Interaction between YFPn-tagged MYB51 and YFPc-tagged bHLH133 (negative control) showing no interaction with MYB51. Supplemental Figure S2 shows the BiFC interaction of MYB51, MYB34, MYB122, MYB28, and MYB29 with bHLH05 and bHLH04.

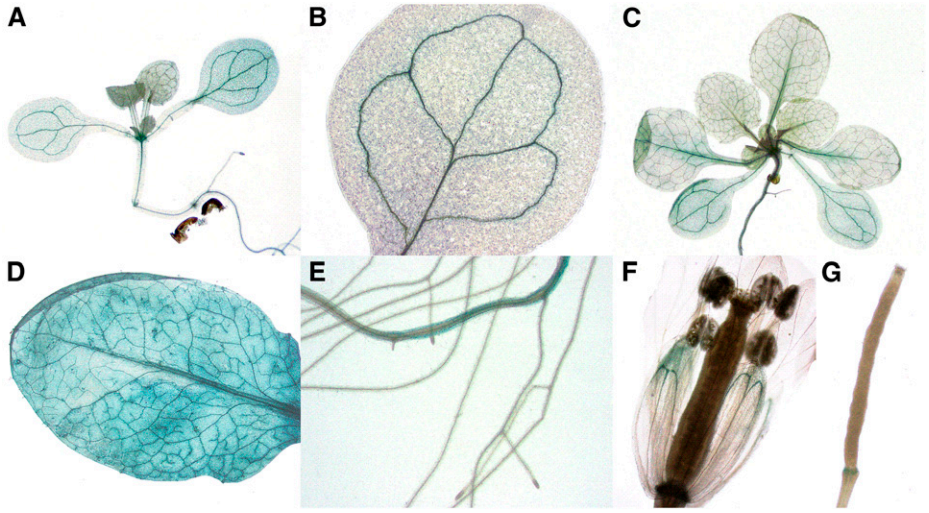

Figure 2. The *bHLH05* promoter is active at sites of GSL production. Histochemical GUS staining of *ProbHLH05-uidA* plants: 7-d-old seedling (A), cotyledon (B), 3-week-old plant (C), mature rosette leaf (D), main and lateral roots (E), flower (F), and young silique (G).

less strong with bHLH06 (Fig. 3), implying that these strong interactions may play a special role in the regulation of GSL. Furthermore, a strong interaction between bHLH28 and either MYB51 or MYB34 was observed, despite the inability of bHLH28 to interact with JASMONATE-ZIM DOMAIN (JAZ) proteins (Fernández-Calvo et al., 2011; Niu et al., 2011). JAZ proteins are negative regulators of jasmonate (JA) signaling that also interact with bHLH proteins, thus inhibiting the activation of target genes of bHLH proteins (Chini et al., 2009). Thus, if bHLH28 is able to influence the production of GSL in plants, its effect on GSL biosynthesis should be independent of JA

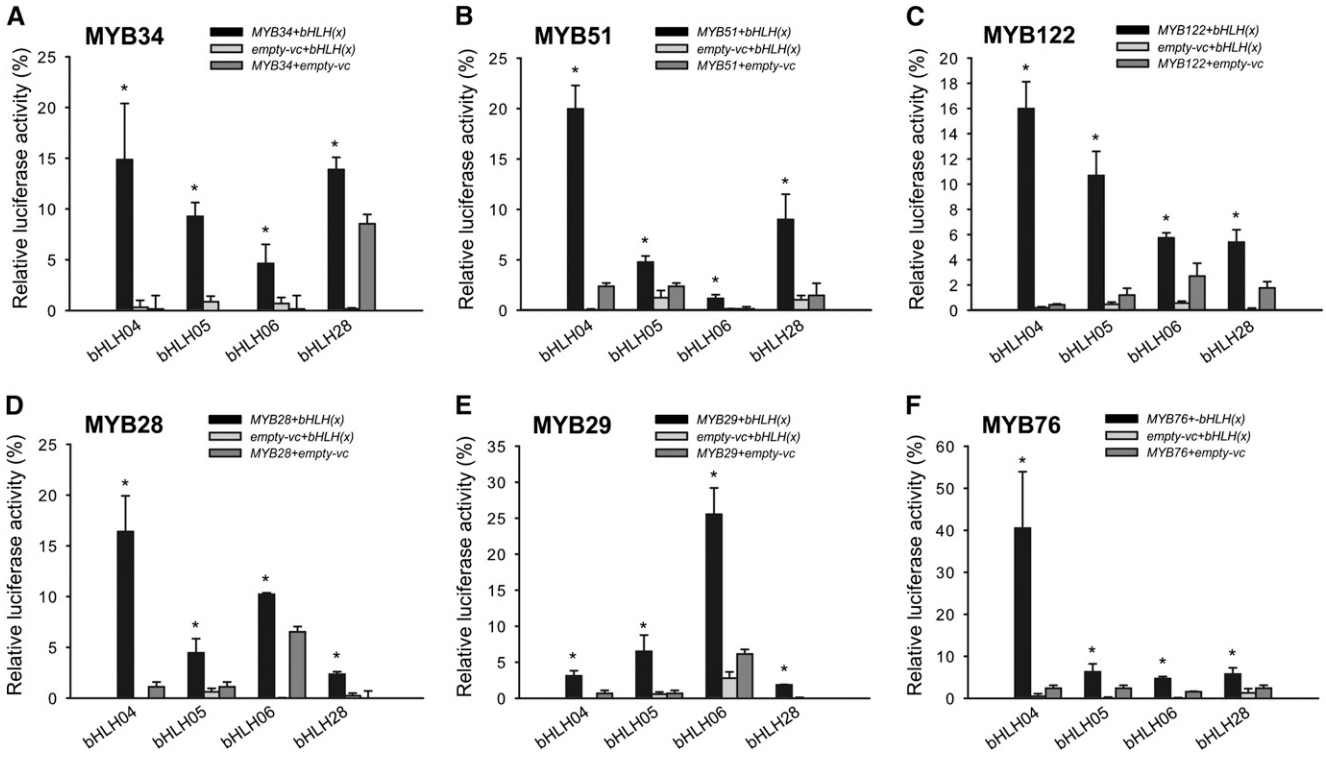

Figure 3. Pull-down experiments revealed an interaction of MYB TFs regulating GSL biosynthesis with bHLH04, bHLH05, bHLH06, and bHLH28. LUMIER assays to analyze the interaction of bHLH05, bHLH04, bHLH06, and bHLH28 with the six MYB TFs regulating IGs (MYB34, MYB51, and MYB122 in A–C) and AGs (MYB28, MYB29, and MYB76 in D–F). Interaction was assessed using fusion proteins of the MYBs with *R. reniformis* luciferase and of bHLHs with protein A. The proteins were coexpressed in human cells (HEK293TN) and coimmunoprecipitated with IgG Dynabeads. Values marked with asterisks are significantly different from both empty vector controls (empty-vc; Student's *t* test; $P < 0.05$).

signaling. However, a strong interaction of bHLH28 with MYBs could be also independent from its importance in GSL biosynthesis and related to structural properties of bHLH proteins representing a negative correlation in the interaction strength of bHLH-JAZ proteins pairs versus bHLH-MYB. Notably, the interaction strength of bHLH06 with MYBs was low, with the exception of the combination bHLH06 and MYB29, which supports the important role of MYB29 in JA signaling (Hirai et al., 2007; Gigolashvili et al., 2008). Moreover, less interaction of bHLH06 with MYBs could be an additional mechanism to fine-tune the GSL production in cells having a relatively high abundance of *bHLH06* mRNA compared with that of *bHLH04* and *bHLH05* (Supplemental Fig. S3).

Simultaneous Loss of Function of *BHLH05* and *MYB51* Causes a Reduction in IG Content in Arabidopsis Seedlings

To evaluate the function of bHLH05 in IG biosynthesis, the publicly available insertional mutants *bhlh05* (GK445B11), *bhlh04* (GK491E10), and *bhlh06* (*jin1.9*) were isolated and double mutants with the main indolic GSL regulators *myb51* and *myb34* (Frerigmann and Gigolashvili, 2014) were generated (for details, see the "Materials and Methods"). These mutants were examined for the accumulation of IG (Fig. 4; Supplemental Fig. S4) and aliphatic glucosinolates (AGs; Supplemental Fig. S4) in Arabidopsis seedlings. As shown in Figure 4, IG biosynthesis was not affected in the *bhlh04* and *bhlh06* mutants compared with the wild type, and was similar in double *myb51 bhlh04* and *myb51 bhlh06* compared with the *myb51* mutant (Fig. 4, A and C).

By contrast, seedlings of the *bhlh05* transfer DNA (T-DNA) insertion line were moderately but significantly affected in IG accumulation, highlighting the importance of bHLH05 in IG regulation in Arabidopsis. Moreover, the concentration of IGs in the double *myb51 bhlh05* mutant was substantially lower than that of single *myb51* mutants (Fig. 4B), suggesting the importance of bHLH05 in the combinatorial regulation of IGs together with MYB51.

In contrast with *myb51 bhlh05,* the IG level in *myb34 bhlh05* seedlings was not significantly lower than that of the single *myb34* mutant. Because MYB34 is known to be the main regulator and rate-limiting step of IG biosynthesis in young Arabidopsis seedlings, loss of *bHLH05* function in double *myb34 bhlh05* seedlings did not further reduce IG production in this double mutant. If we would have analyzed the IG content at a developmental stage when MYB51 instead of MYB34 was rate limiting (e.g. in leaves of adult plants), a different IG level (decreased levels of IG in *myb34 bhlh05* versus *myb34*) would be anticipated.

The Combination of Gain-of-Function Alleles of *bHLH05* and *MYB34* Leads to a Dramatic Increase in GSL Levels in Arabidopsis

In the following experiment, we focused the analysis of GSL biosynthesis on plants either overexpressing both R2R3-MYB and bHLH proteins or containing gain-of-function alleles for both TFs. The *MYB51-1D* (*HIG1-1D*) activation-tagged line and *35S:MYB51* overexpression plants were available from a previous study (Gigolashvili et al., 2007a) and *bHLH05* overexpressors were generated in this study. Whereas the overexpression of *MYB51*

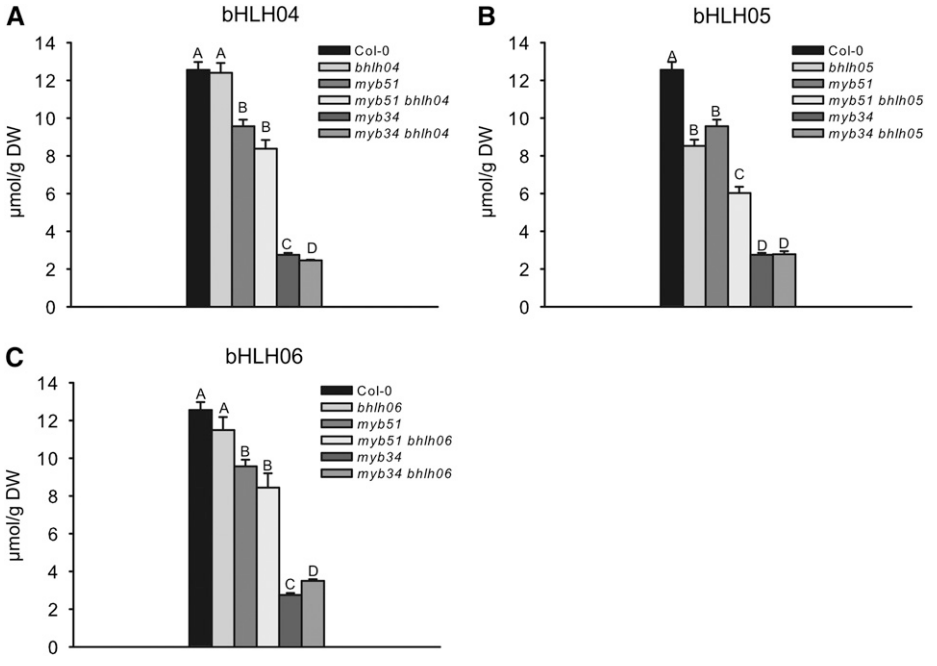

Figure 4. Levels of IGs in seedlings of double *myb bhlh* mutants. Double loss-of-function mutant combinations of *MYB51* and *MYB34* with *bHLH04* (A), *MYB51* and *MYB34* with *bHLH05* (B), and *MYB51* and *MYB34* with *bHLH06* (C). For GSL analysis, 3-week-old seedlings were harvested and the sum of the three major IGs (I3M, 1MO-I3M, and 4MO-I3M) is shown. Results are means ± SE from three independent experiments with four biological replicates each ($n = 12$). Values marked with letters are significantly different from other letters (Student's *t* test; $P < 0.05$). DW, Dry weight.

leads to the increased production of IG, the overexpression of *bHLH05* did not cause an increased accumulation of GSL in plants (Supplemental Fig. S5). The fact that *bHLH05* overexpression did not induce the production of GSL indicates that bHLH05 additionally requires other regulatory proteins to activate the transcription of GSL biosynthesis genes. These findings are in agreement with transactivation analysis of bHLH05 in cultured Arabidopsis cells (see below), which revealed no ability of bHLH05 to activate GSL biosynthetic genes in trans when produced in wild-type cells.

To generate transgenic plants that stably overexpressed both TFs, we transformed the homozygous activation-tagged line *MYB51-1D* (also known as *HIG1-1D*) with *35S::bHLH05* (Supplemental Fig. S5) using *Agrobacterium tumefaciens*-mediated plant transformation and also crossed *MYB51-1D* to homozygous *35S:bHLH05* overexpression plants. Several hundred independent transgenic lines that should contain both *MYB51* and *bHLH05* transgenes were analyzed. However, simultaneous overexpression of *bHLH05* and *MYB51* always led to silencing of at least one of the transgenes in the subsequent generation, most probably because of counter selection of double overexpression transgenes with potentially very high levels of GSLs.

To overcome this problem, we continued to analyze *MYB* and *bHLH* gain-of-function alleles of *MYB34-1D* (*atr1D*) and *bHLH05D94N* (*atr2D*; Bender and Fink, 1998; Smolen et al., 2002) The *MYB34-1D* line harbors a mutation upstream of the *MYB34* open reading frame, which leads to a constitutively high expression level of *MYB34* (Bender and Fink, 1998) and to an accumulation of IGs (Celenza et al., 2005; Fig. 5A). The dominant allele *bHLH05D94N* of bHLH05 is caused by an amino acid exchange in the conserved N-terminal domain, which leads to the up-regulation of some stress-responsive genes (Smolen et al., 2002), but no significant changes in IG levels compared with wild-type plants were recorded (Fig. 5A). Thus, the gain of *bHLH05* function alone as well as constitutive overexpression of *bHLH05* is not sufficient to activate the production of IGs in plants (Fig. 5A; Supplemental Fig. S5). Analysis of IG in the double *MYB34-1D bHLH05D94N* mutant compared with single *MYB34-1D* and *bHLH05D94N* mutants revealed that IG levels in the double mutant were 2-fold higher than in the single *MYB34-1D* mutant and up to 20-fold higher than in ecotype Columbia-0 of Arabidopsis (Col-0) and *bHLH05D94N* (Fig. 5A). In agreement with this finding, the expression level of IG biosynthesis genes was significantly higher than that in the corresponding single *MYB34-1D* or *bHLH05D94N* mutants or Col-0 (Fig. 5B). Together, the analysis of IG biosynthesis in gain-of-function mutants and overexpression lines revealed that increased activity of bHLH05 alone is not sufficient to modulate IG production in plants, but increasing the activity of both MYB34 (in *MYB34-1D* mutants) and bHLH05 (in *bHLH05D94N* mutants) had a strong additive effect on the accumulation of IG. The levels of AGs in double bHLH-MYB gain-of-function mutants were negatively affected by simultaneous overexpression of *MYB34* and *bHLH05*, which is comparable with the GSL profile of MYB34ox and MYB51ox plants (Gigolashvili et al., 2007a) and agrees with the reciprocal negative regulation of AG and IG biosynthetic branches (Supplemental Fig. S6).

The Mutation in the MYB Interaction Region/JAZ Interaction Domain of bHLH05 Impairs the Protein-Protein Interaction with JAZ1 and Stimulates Interaction with MYB34

The previous experiment revealed that gain of function of *bHLH05D94N* causes a doubling in the levels of IGs in *MYB34-1D*. The additive effect of *bHLH05D94N* originates from the D94N mutation, which is localized in the N-terminal conserved amino acid region of the protein. This conserved region was predicted to be important

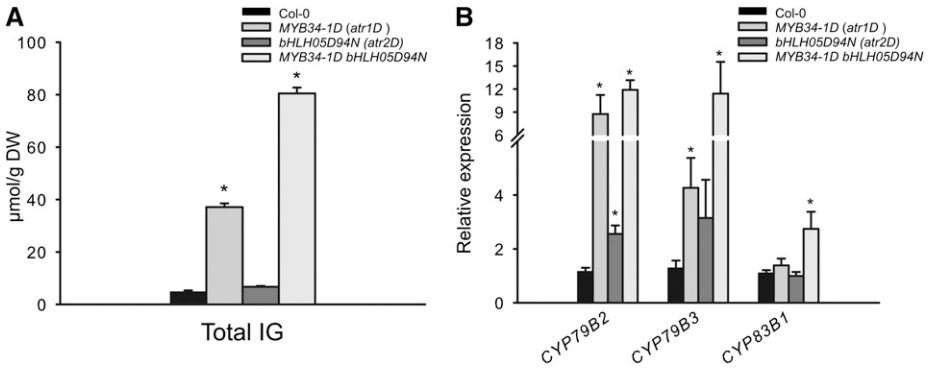

Figure 5. Combined gain of function of *MYB34* and *bHLH05* leads to doubling of GSL levels in Arabidopsis plants. A, Total IG content of the dominant alleles of *MYB34-1D* (*atr1D*) and *bHLH05D94N (atr2D*) are shown. For GSL analysis (A) and expression analysis (Col-0 = 1) of *CYP79B2*, *CYP79B3*, and *CYP83B1* (B), leaves of 6-week-old plants were harvested. Values are means ± SE from four independent experiments with four biological replicates each for A and three independent experiments with four biological replicates each for B ($n = 16$ and 12, respectively). Values marked with asterisks are significantly different (Student's t test; $P < 0.05$) from control plants (Col-0). DW, Dry weight.

for the interaction of subgroup IIIe bHLH proteins with JAZ and MYB (Fernández-Calvo et al., 2011; Schweizer et al., 2013a). In the following experiment, we analyzed the protein-protein interactions of both gain-of-function D94N bHLH05 and native bHLH05 proteins with JAZ1 and MYB34 via the LUMIER assay.

Figure 6A shows that the physical interaction between bHLH05D94N and JAZ1 is strongly attenuated compared with the interaction of native bHLH05 and JAZ1, substantiating the role of D94N, which is predicted to be localized in the MYB interaction region (MIR)/JAZ interaction domain (JID) interaction region, to alter the interaction ability of the bHLH05 protein. In agreement with this finding, bHLH28, which is also unable to interact with JAZ proteins, contains a nonconservative amino acid exchange (Gly to Lys) in the conserved protein region, also predicted to be a JID/MIR interaction region (Fernández-Calvo et al., 2011). Furthermore, together with the impaired interaction of bHLH05D94N with JAZs, an improved interaction of bHLH05D94N with MYB34 was observed (Fig. 6B). This Gly to Lys exchange in the N-terminal JID/MIR interaction region of bHLH28 is most probably also the reason for its increased interaction with MYBs (Fig. 3).

Altogether, the data demonstrate the importance of the N-terminal part of bHLH05 protein for the interaction with JAZ and MYB, which can thereby affect the regulatory ability of these proteins. Importantly, similar mutations in the N-terminal MYB-interacting region of the bHLH proteins DELILA (DEL from *Antirrhinum majus*) and Myc-RP (from *Perilla frutescens*) positively affect anthocyanin biosynthesis (Pattanaik et al., 2006, 2008).

An Analysis of Single and Multiple *bhlh* Knock-Out Mutants Reveals a Special Role of bHLH04 and bHLH05 in the Regulation of GSL in the Absence of JA

To study the role of bHLH04, bHLH05, bHLH06, and bHLH28 in the regulation of GSL, single and multiple *bhlh* mutants were generated (for details, see the "Materials and Methods") and analyzed. None of the T-DNA insertion lines (*bhlh04, bhlh05, bhlh06,* and *bhlh28*) or double and triple homozygous loss-of-function mutants (*bhlh04/05, bhlh05/06, bhlh04/06*, and *bhlh04/05/06*) showed an obvious phenotype. The GSL levels in leaves of single *bhlh04*, *bhlh05, bhlh06*, and *bhlh28* or double *bhlh04/06* and *bhlh05/06* mutants were comparable with those of wild-type plants (Fig. 7A; Supplemental Fig. S7). This contrasted with the significantly reduced GSL levels in double *bhlh04/05* and triple *bhlh04/05/06* mutants (Fig. 7A; Supplemental Fig. S7), because *bhlh04/05* contained only 15% of wild-type GSL levels and *bhlh04/05/06* accumulated almost no GSL. These data reveal an essential role for bHLH04, bHLH05, and bHLH06 and a minor role for bHLH28 in the regulation of GSL accumulation.

Previous studies have shown an important role of bHLH04, bHLH05, and bHLH06 in multiple JA signaling processes (Cheng et al., 2011; Fernández-Calvo et al., 2011; Niu et al., 2011). To understand more about bHLH-mediated IG regulation in response to JA, we treated the single and multiple *bhlh* mutants with methyl jasmonate (MeJA) and analyzed IG and AG in wild-type and mutant seedlings (Fig. 8; Supplemental Fig. S8). In agreement with the previously reported JA inducibility of *bHLHs*, the dramatic increase in total IG of Col-0 and single *bhlh* mutants and only negligible changes in the triple *bhlh04/05/06* mutant were detected. Remarkably, the residual levels of IG in the triple *bhlh04/05/06* and *myb34/51/122* mutants were only marginally affected by JA treatment (Frerigmann and Gigolashvili, 2014; Fig. 8), implying the importance of both bHLH and MYBs in the production of IGs. Furthermore, all analyzed double *bhlh* mutants (with only single active *bHLH04, bHLH05*, or *bHLH06*) were attenuated in JA-induced IG biosynthesis to a similar extent (Fig. 8), underlining the importance of all three bHLH proteins in JA-induced GSL biosynthesis. Furthermore, bHLH06 was able to complement the low IG level of *bhlh04/05* under these conditions, implying a specific role of bHLH06 in MeJA signaling.

The data here reveal the specific importance of bHLH04 and bHLH05 in the regulation of both IGs and AGs in the absence of JA. Under standard growth conditions, bHLH06 only slightly contributes to the production of GSL, because its function is more important in response to JA. Despite the interaction with MYBs, bHLH28 appears to play only a minor role in GSL regulation in both conditions and further analysis of this protein is required to understand its possible role in GSL biosynthesis.

Attenuated Expression of GSL Pathway Genes in the Triple *bhlh04/05/06* Mutant

Because IG and AG levels are lower in the triple *bhlh04/05/06* mutant, we measured the expression of genes involved in the biosynthesis of IG, AG, or both pathways by real-time quantitative reverse transcription PCR (qRT-PCR). As shown in Figure 7B, the transcript analysis of the triple *bhlh04/05/06* mutant revealed the dramatically reduced expression of genes involved in the biosynthesis of IGs (*CYP79B2, CYP79B3,* and *CYP83B1*), AGs (*CYP79F2* and *CYP83A1*), as well as transcript levels of genes involved in the biosynthesis of both IGs and AGs (*C-S lyase, Sulfotransferase16* [*SOT16*], and *PAPS-Transporter1* [*PAPST1*]).

Notably, the expression of *MYB34* and *MYB122*, which regulate the production of IG (Celenza et al., 2005; Gigolashvili et al., 2007a; Frerigmann and Gigolashvili, 2014) was hardly altered (Supplemental Fig. S9). However, the steady-state level of *MYB51* was approximately 3-fold higher in *bhlh04/05/06* compared with Col-0. The low levels of GSL in this triple mutant could be sensed by plants, which can increase the GSL production via a negative feedback loop (Mugford et al., 2009) in the *bhlh04/05/06* mutant.

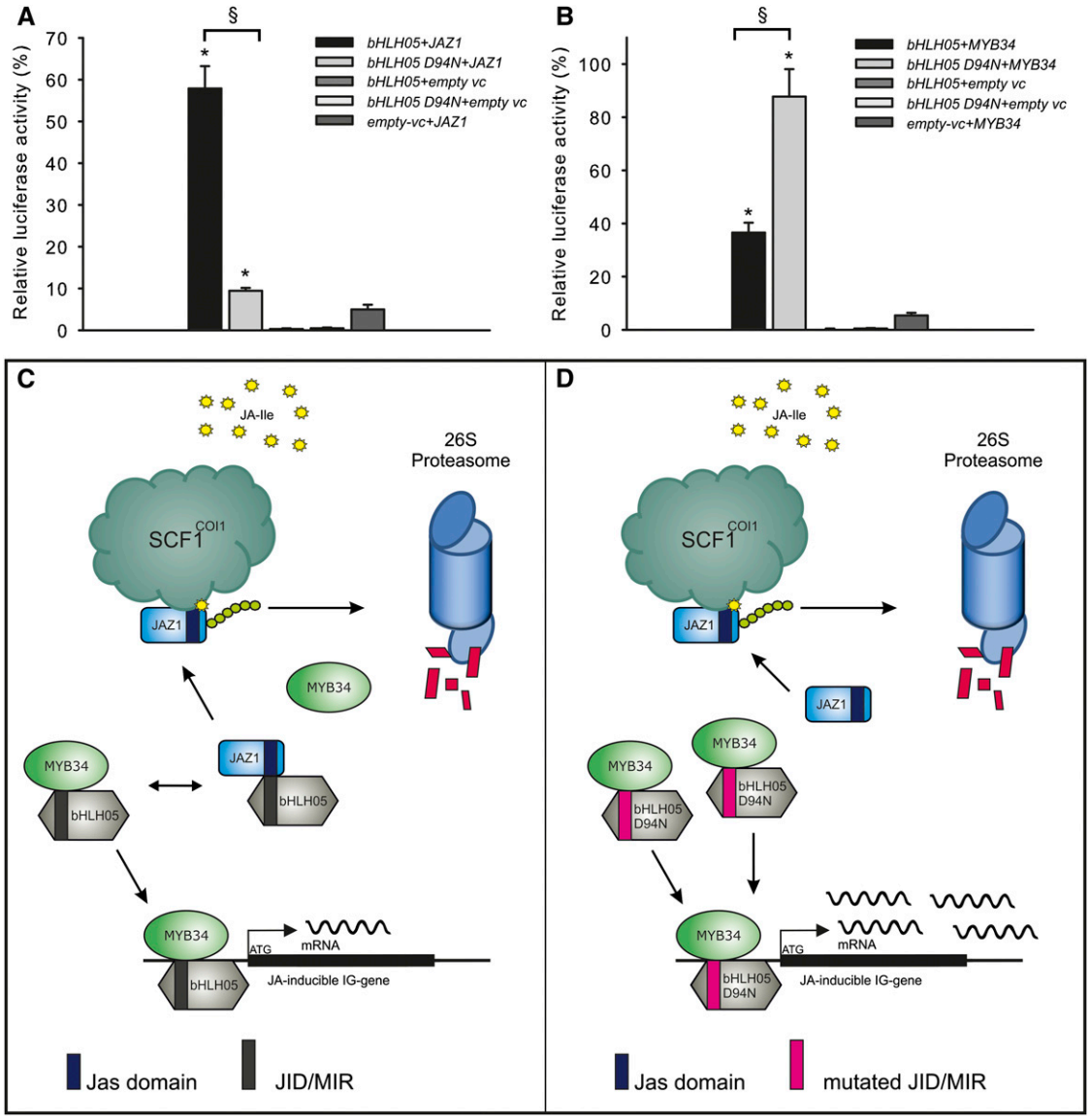

Figure 6. Mutation D94N in the N-terminal part of bHLH05 prevents interaction of bHLH05 with JAZ1 and stimulates interaction with MYB34. A and B, LUMIER assays for the interaction of native bHLH05 and the mutated bHLH D94N (Asn at position 94) proteins with JAZ1 (A) and MYB34 (B) are shown. Interaction between luciferase-tagged MYB34 and JAZ1 on one side and the protein A-tagged bHLH05 and bHLH05 D94N on the other side are shown. The proteins were coexpressed in human cells (HEK293TN) and coimmunoprecipitated with IgG Dynabeads. Values marked with asterisks are significantly different from both empty vector controls (empty vc) without coding sequence fusion (Student's *t* test; $P < 0.05$). Bars marked with § are significantly different from each other (Student's *t* test; $P < 0.001$). C and D, Model for protein-protein interactions of native bHLH05 protein (C) and mutated bHLH05D94N protein (D) with MYB34 and JAZ1 proteins (without JA addition). C, The interaction of native bHLH05 with JAZ1 and MYB34 is balanced and bHLH05 interacts with both proteins, allowing production of IG only at basal (wild-type) levels. D, The mutation bHLH05D94N impairs the interaction with JAZ1 and triggers the strong interaction of bHLH05D94N with MYB34 followed by enhanced transcription of IG pathway genes and an increased accumulation of IG levels. The JAZ1 protein interacts less with bHLH05D94N and is therefore free for ubiquitinylation by CORONATINE INSENSITIVE1 (COI1) and degradation by the 26S proteasome. The D94N mutation of bHLH05 mimics the presence of JA. Jas domain, The interaction domain of JAZ proteins used during the physical interaction with bHLH proteins; JID/MIR, a JAZ Interaction Domain/MYB Interaction Domain (a part of bHLH proteins, which allows the physical interaction with JAZ and MYB proteins).

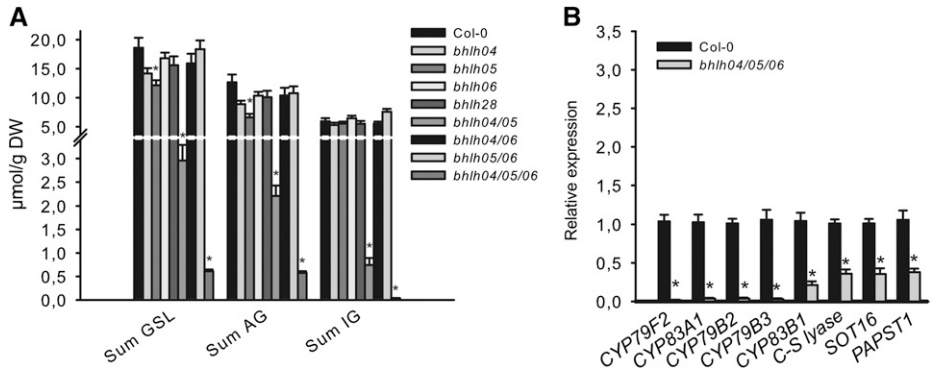

Figure 7. GSL accumulation is significantly reduced in rosette leaves of double *bhlh04/05* and triple *bhlh04/05/06* mutants. A, GSL levels in single and multiple *bhlh* mutants are shown. For GSL analysis, leaves of 6-week-old plants were harvested and the sum of the three major IGs (I3M, 4MO-I3M, and 1MO-I3M), the sum of the four main AGs (3MSOP, 4MSOB, 5MSOP, and 8MSOO), and the sum of IGs and AGs are shown as Sum GSL. Results are means ± SE from three independent experiments with four biological replicates each ($n = 12$). Values marked with asterisks are significantly different from Col-0 (Student's t test; $P < 0.01$). B, Expression of GSL biosynthetic genes in the triple *bhlh* mutant is shown. Relative expression values of biosynthesis genes for AG (*CYP79F2* and *CYP83A1*), IG (*CYP79B2*, *CYP79B3*, *CYP83B1*, and *SOT16*) and both AG and IG (*C-S lyase* and *PAPST1*) are shown. Relative expression was measured in leaves of 6-week-old *bhlh04/05/06* knock-out lines (Col-0 = 1). Data are means ± SE from three independent experiments with three biological replicates each ($n = 9$). Values marked with asterisks are significantly different from control plants (Student's t test; $P < 0.05$). DW, Dry weight.

Transactivation Assays with bHLH Proteins and *promoter-uidA* Constructs of GSL Biosynthesis Genes via Transient Expression with Cultured Arabidopsis Cells

It was previously shown that R2R3 MYBs that regulate IG biosynthesis can activate the transcription of numerous promoter-reporter constructs with GSL biosynthesis genes via transient expression of cultured Arabidopsis cells (Gigolashvili et al., 2007a, 2008). Here, we showed that bHLHs interact with MYBs to control GSL production, and that the triple *bhlh04/05/06* mutant contains less than 1% of the GSL present in wild-type plants. This suggests that the GSL pathway might be under transcriptional control by bHLH proteins or by MYB-bHLH complexes. To test the hypothesis that genes involved in the biosynthesis of GSL are controlled by bHLHs or MYB-bHLH complexes, we used a cotransformation assay (Berger et al., 2007; Gigolashvili et al., 2007a, 2008) to identify TFs that bind to promoters of GSL synthesis genes. For this, constructs containing promoters of the IG biosynthetic genes *ANTHRANILATE SYNTHASE ALPHA1*, *CYP79B3*, and *CYP83B1* fused to the *uidA* (*GUS*) reporter gene were used as reporter constructs, and *bHLH05* or a mixture of *bHLH05* and *MYB51* were used as effector constructs. Reporter and effector constructs were coexpressed in cultured Arabidopsis cells and the transactivation potential of these TFs toward promoters of GSL biosynthesis genes was estimated histochemically, using 5-bromo-4-chloro-3- indolyl-β-D-glucuronic acid (X-Gluc) staining (Fig. 9A) or was determined by measurements of GUS activity (Fig. 9B). In the absence of an effector construct, the reporter construct containing the promoters of GSL biosynthetic genes revealed only faint GUS activity (Fig. 9, A and B). The MYBs could induce the transcription of all tested promoters of GSL biosynthetic pathway in trans, indicating a direct role of MYB51 in the binding and activation of GSL biosynthesis genes. However, this was not the case for bHLH proteins, because we did not detect any induced transcription of reporter gene expression after coexpression with bHLHs. Furthermore, coexpression of MYB and bHLH with the GSL promoters did not reveal any increase in GUS activity, but even attenuated the positive transactivation effect observed for MYBs (Fig. 9, A and B). Finally, not only were the expression levels of reporter constructs not positively affected by bHLH, but the accumulation of GSL was also not increased in these Arabidopsis wild-type cells overexpressing bHLH proteins (Fig. 9B). These data support our finding that plants overexpressing *bHLH05* (Supplemental Fig. S5) or containing the constitutively active form of this protein (*bHLH05D94N*; Fig. 5) failed to accumulate more IGs than the wild type. By contrast, the overexpression of MYB51 in cultured cells was followed by both the increased transcription of promoter-GUS constructs and production of GSL in this transient expression assay.

DISCUSSION

Protein-protein interactions play a central role in the regulation of scores of cellular processes (Martinez, 2002; Istrail and Davidson, 2005). Despite the identification of numerous TFs involved in plant regulatory networks (Lee et al., 2007; Benhamed et al., 2008; Kaufmann et al., 2009; Morohashi and Grotewold, 2009; Oh et al., 2009), our understanding of the molecular mechanisms of these protein complexes in the regulation of plant gene expression remains poor, including the regulation of GSL biosynthesis. Given the

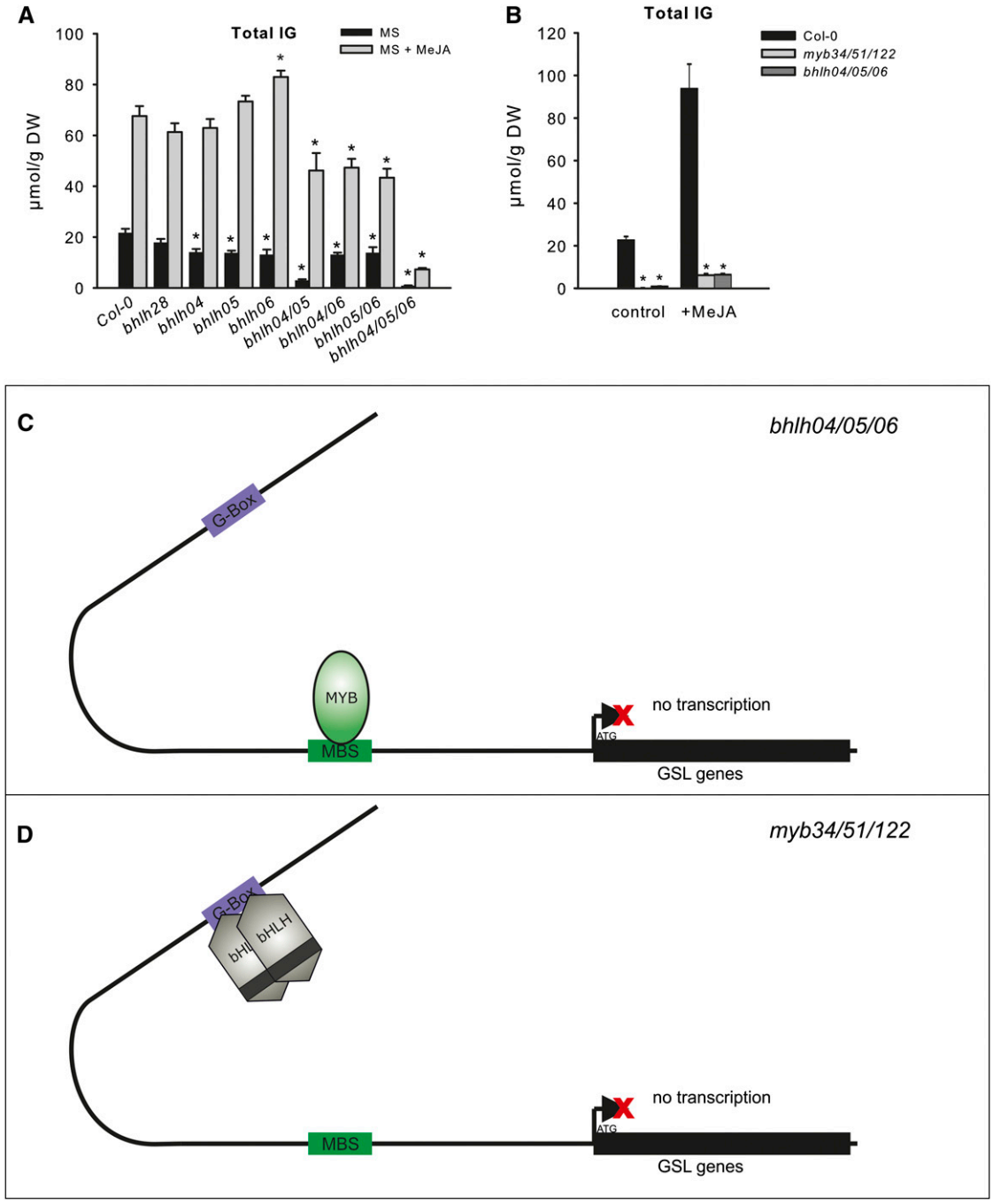

Figure 8. Production of IGs in single and multiple *bhlh* mutants in response to MeJA treatment. Model for the IG production in *bhlh04/05/06* and *myb34/51/122* mutants. A, The IG content of single and multiple *bhlh* mutants after MeJA treatment is shown. For GSL analysis, the mutant and wild-type (Col-0) plants were grown on MS plates for 2 weeks followed by transfer to plates supplemented with 50 μM of MeJA. The sum of the three major IGs (I3M, 4MO-I3M, and 1MO-I3M) are shown. Results are means ± SE from four two independent experiments with four biological replicates each ($n = 8$). Values marked with asterisks are significantly different from Col-0 (Student's *t* test; $P < 0.01$). B, Comparison of IG content in *bhlh04/05/06* and *myb34/51/122* in the presence or absence of MeJA. Mutant and wild-type (Col-0) plants were grown on MS plates free of additives or MS supplemented with 50 μM of MeJA. The sum of the three major IGs (I3M, 4MO-I3M, and 1MO-I3M) is presented. Results are means ± SE from three independent experiments with four biological replicates each ($n = 12$). Values marked with asterisks are significantly different from Col-0 (Student's *t* test; $P < 0.05$). C and D, A model for the biosynthesis of GSL by MYB and bHLH in Arabidopsis in triple *bhlh04/05/06* and *myb34/51/122* mutants is shown. Biosynthesis of GSL is blocked in the absence of both bHLH (C) and MYB (D) proteins; thus, both types of TFs are necessary to produce GSLs in planta. DW, Dry weight.

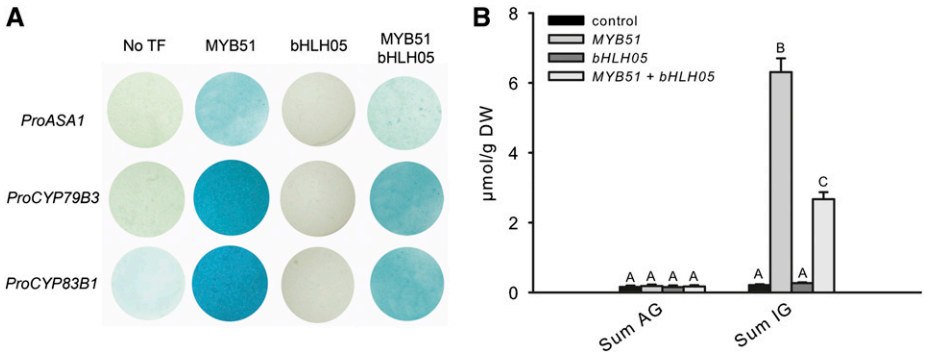

Figure 9. Transactivation assay of GSL biosynthesis promoters with bHLH TFs. TF coexpression assay to determine the DNA binding and transactivation potential of *bHLH05* toward target promoters of the GSL biosynthesis pathway genes *ASA1, CYP79B3,* and *CYP83B1*. A, The promoters of *ASA1, CYP79B3,* and *CYP83B1* were fused to the *uidA* (GUS) reporter gene. Cultured Arabidopsis Col-0 cells were inoculated with the supervirulent *A. tumefaciens* strain LBA4404.pBBR1MCS.virGN54D containing either only the reporter construct or the reporter construct and in addition, *Pro-35S:MYB51*, *Pro-35S:bHLH05*, and the 1:1 mixture of *Pro-35S:MYB51* and *Pro-35S:bHLH05* effector constructs. The GUS staining indicates transactivation of a promoter by an effector. Histochemical GUS staining of cultured cells was performed with X-Gluc 4 d after transfection. B, Accumulation of GSL in cultured cells transfected with *Pro-35S:MYB51, Pro-35S:bHLH05*, and the 1:1 mixture of *Pro-35S: MYB51 Pro-35S:bHLH05*. The sum of the three major IGs (I3M, 4MO-I3M, and 1MO-I3M) and the sum of the four main AGs (3MSOP, 4MSOB, 5MSOP, and 8MSOO) are shown. Results are means ± SE from three independent biological replicates (n = 3). Values marked with asterisks are significantly different from Col-0 (Student's t test; $P < 0.01$). DW, Dry weight.

importance of transcriptional regulation in the generation of diverse types of GSL under various environmental conditions, only few regulatory proteins that are crucial for the production of GSL biosynthesis in Arabidopsis have been described. In this study, we identified a new class of TFs, which together with R2R3-MYBs, are crucial for the production of GSL in vivo. Using a yeast two-hybrid assay, we identified bHLH05 as an interacting partner of MYB51 and showed that bHLH05, bHLH04, bHLH06, and bHLH28 interact with R2R3-MYB proteins both in vitro and in vivo. This specific MYB-bHLH interaction plays an essential role in the transcriptional activation of GSL biosynthesis genes and the production of these secondary metabolites in planta.

bHLH Proteins Are Regulators of GSL Biosynthesis in Arabidopsis

The bHLH04, bHLH05, and bHLH06 proteins control JA-dependent responses, such as root growth inhibition, defense against bacterial pathogens, and insect herbivory (Fernández-Calvo et al., 2011). These TFs have almost identical DNA-binding specificities to the canonical G-box (CACGTG), suggesting their importance in the recognition of target genes involved in JA responses (Fernández-Calvo et al., 2011). Furthermore, a recent chromatin immunoprecipitation sequencing (ChIP-seq) analysis revealed that bHLH06 is able to bind the promoter of GSL biosynthesis genes in vivo, all containing canonical G-boxes (Schweizer et al., 2013a). In this study, we identified the GSL biosynthesis pathway as a target of these bHLH proteins and dissected the specific role of bHLH04, bHLH05, and bHLH06 in the regulation of GSL biosynthesis.

Gene expression analysis of the triple *bhlh04/05/06* mutant revealed that GSL biosynthetic genes are downregulated in the *bhlh04/05/06* triple mutant (Fig. 7B). However, not only the GSL biosynthetic genes, but also the production of IG and AG is impaired in the *bhlh04/05/06* triple mutant (Fig. 7A), pointing to a crucial role of bHLH04, bHLH05, and bHLH06 in the regulation of GSL biosynthesis.

In contrast with the GSL profile of the triple *bhlh04/05/06* mutant, the single *bhlh* mutants and *bhlh04/06* and *bhlh05/06* double mutants showed similar GSL levels to those in wild-type plants, indicating functional redundancy between bHLH04, bHLH05, and bHLH06. However, unlike *bhlh04/06* and *bhlh05/06* mutants, the double *bhlh04/05* mutant was strongly affected in the production of GSL (Fig. 7), pointing to a special role of bHLH04 and bHLH05 in the production of GSL in the absence of JA. In agreement with the low GSL levels of the double *bhlh04/05* mutant, the latter was reported to be more susceptible against generalist herbivores than *bhlh04/06* and *bhlh05/06* (Fernández-Calvo et al., 2011). Further analysis of GSL levels in multiple *bhlh* mutants revealed the inability of bHLH06 to fully complement GSL biosynthesis in the *bhlh04/05* mutant under standard growth conditions (Fig. 7). This contrasted with GSL biosynthesis levels in seedlings of *bhlh04/05* after MeJA treatment, because *bhlh04/05* can be fully complemented by bHLH06 (Fig. 8). Notably, IG levels of double *bhlh05/06* and *bhlh04/06* mutants treated with MeJA treatment were comparable with IG levels of MeJA-treated *bhlh04/05*, pointing to involvement of all three bHLH proteins in JA-triggered GSL biosynthesis (Fig. 8). Based on this observation, it can be assumed that mainly bHLH06 and secondarily bHLH04 and bHLH05 are important in GSL regulation via JA signaling, which

differs from the regulation of constitutive levels of GSL mediated mainly by bHLH04 and bHLH05.

The mRNA microarray analysis of *bhlh06/myc2* identified a list of bHLH06-regulated genes, including genes involved in wound/insect responses, flavonoid biosynthesis, and oxidative stress tolerance (Dombrecht et al., 2007). The authors found that the *bhlh06/myc2* mutant accumulated more IG in response to MeJA (Fig. 8) and the genes involved in IG biosynthesis were also upregulated. Dombrecht et al. (2007) also suggested that bHLH06 is a negative regulator of GSL accumulation. However, this is apparently not the case, because bHLH06 as well as bHLH04 and bHLH05 are positive regulators of IG biosynthesis, and bHLH04 and bHLH05 compensate for the absence of bHLH06 by inducing the production of IGs in *bhlh06* in response to MeJA (Guo et al., 2013b).

Jasmonate, bHLHs, and GSL Regulation

Glucosinolate metabolism in the Brassicaceae has evolved to optimize plant fitness in changing environments (Giamoustaris and Mithen, 1995; Brader et al., 2001; Hiruma et al., 2010). Diverse pathogens trigger the production of GSL by inducing the JA, ethylene, abscisic acid, salicylic acid, or Glc signaling pathways (Kiddle et al., 1994; Doughty et al., 1995; Brader et al., 2001; León et al., 2001; Kliebenstein et al., 2002; Mikkelsen et al., 2003; Farnham et al., 2004; Lorenzo et al., 2004; Dombrecht et al., 2007; Yan and Chen, 2007; Erb et al., 2011; Guo et al., 2013a). This can modulate GSL accumulation via MYBs (Hirai et al., 2007; Sønderby et al., 2007; Gigolashvili et al., 2007a, 2007b; Frerigmann and Gigolashvili, 2014) and other regulatory proteins in GSL biosynthesis. JA is an important regulator of GSL synthesis, which affects the expression of MYB TFs and GSL biosynthesis genes (Doughty et al., 1995; Brader et al., 2001; Mikkelsen et al., 2003; Dombrecht et al., 2007; Frerigmann and Gigolashvili, 2014). Glc signaling, which also positively regulates GSL biosynthesis, acts synergistically with JA, because Glc-induced GSL biosynthesis can be enhanced by the addition of JA (Guo et al., 2013a).

We identified the bHLH proteins using yeast two-hybrid screening, which were known previously from the JA signaling pathway to regulate the JA-inducible genes (Fernández-Calvo et al., 2011; Schweizer et al., 2013a, 2013b). We also showed that bHLH04, bHLH05, and bHLH06 are regulators of GSL biosynthesis in response to MeJA in Arabidopsis. In the absence of MeJA, the bHLH proteins form fewer protein complexes with MYBs and significantly more protein dimers with JAZ proteins (Fernández-Calvo et al., 2011). The bHLH proteins that interact with JAZ are inactive and the production of GSL in the absence of JA is attenuated. In the presence of JA, the JAZ proteins are degraded by the Skp1–Cullin–F-box protein - COI1 complex, triggering the physical interaction of bHLH with MYBs and the formation of active MYB-bHLH complexes, which activate GSL production.

Notably, in the absence of JA, wild-type plants accumulate basal levels of GSL, owing to the formation of less-abundant but still active MYB-bHLH pairs. bHLH04 and bHLH05 especially appear to form active protein complexes with MYBs in standard growth conditions, because the loss of function of both TFs has a dramatic effect on GSL production (Fig. 7). In contrast with bHLH04 and bHLH05, the role of bHLH06 in the production of GSL is particularly important in the presence of JA, because mutation of *bhlh06* has no negative effect on GSL production in the absence of JA (Fig. 7), whereas bHLH06 can complement the GSL-deficient *bhlh04/05* mutant upon JA treatment (Fig. 8).

Both MYB and bHLH TFs Are Necessary to Regulate GSL Biosynthesis in Arabidopsis

In this study, we demonstrated that the GSL biosynthetic pathway is not only under transcriptional control by MYBs as was previously shown (Hirai et al., 2007; Sønderby et al., 2007; Gigolashvili et al., 2007a, 2007b, 2008; Beekwilder et al., 2008; Malitsky et al., 2008; Sønderby et al., 2010a; Li et al., 2013; Frerigmann and Gigolashvili, 2014), but is also tightly regulated by bHLHs. This is supported by the following observations. First, bHLH04, bHLH05, bHLH06, and bHLH28 interact with known R2R3-MYB regulators of IG and AG biosynthesis (Figs. 1 and 2). Second, the amount of IGs in the triple *bhlh04/05/06* mutant is as strongly impaired as in the triple *myb34/51/122* mutant (Figs. 7 and 8; Frerigmann and Gigolashvili, 2014). Third, combinatorial gain of function of MYB34 and bHLH05 proteins has an additive effect on IG biosynthesis in Arabidopsis. Thus, the IG levels in the *MYB34-1D bHLH05 D94N* gain-of-function allele are twice as high as that in *MYB34-1D* and 20 times greater than that in *bHLH05 D94N* and Col-0 (Fig. 5). Similarly, MeJA treatment of *MYB34-1D* caused IG levels to double (Supplemental Fig. S10), supporting the importance of bHLH proteins released from the JAZ complex to activate the production of IGs. Fourth, analysis of IG levels in double *myb51 bhlh05* mutants (Fig. 4) revealed synergistic/additive effects of both MYB51 and bHLH05 in the accumulation of IG in seedlings. Fifth, treatment of *myb34/51/122* with MeJA, which triggers the release of bHLHs from the JAZ complex, does not lead to an increased production of IGs in mutants that lack MYBs (Fig. 8; Frerigmann and Gigolashvili, 2014). Finally, it is not possible to induce the production of IG in *bhlh04/05/06* by solely overexpressing *MYB51* (Supplemental Fig. S11), demonstrating the importance of both bHLH and MYB proteins for the production of GSL.

Our findings on the importance of bHLH in GSL regulation agree with ChIP-seq experiments, which showed that the G-boxes of GSL biosynthesis genes are enriched in the chromatin of MeJA-treated bHLH06:FLAG plants (Schweizer et al., 2013a); thus, bHLH06 binds DNA. Similar DNA-binding activities to GSL

biosynthesis genes are therefore predicted for bHLH04 and bHLH05. Although regulatory elements of GSL biosynthesis genes can be bound by bHLH06, the role of bHLH proteins in the direct transcriptional control of GSL genes remains controversial. The transactivation assay with promoter-reporter constructs of GSL biosynthesis genes and *35S:bHLH05* revealed no ability of these bHLHs to activate the transcription of GSL genes in trans (Fig. 9). In contrast with the behavior of bHLH proteins, the MYBs strongly induced the promoters of GSL biosynthesis pathway genes in trans. Furthermore, transactivation activity of bHLHs cannot be achieved even in the presence of both bHLHs and R2R3-MYB in the same cell (Fig. 9, A and B), pointing to an alternative role to the transcriptional control of GSL genes for bHLH proteins. Notably, bHLH05 protein not only failed to activate transiently introduced promoter-reporter constructs (Fig. 9A), and to positively affect GSL production in gain-of-function *bHLH05D94N* (Fig. 5) and *35S:bHLH05* plants (Supplemental Fig. S5), but it additionally inhibited the GSL accumulation in the bHLH05 overexpressor plants and cultured Arabidopsis cells containing both *bHLH05* and *MYB* constructs (Fig. 9; Supplemental Fig. S5). The inhibitory effect of bHLH05 protein observed in these experiments could be explained by its recently reported ability of bHLH to activate the transcription of JAZ proteins (Niu et al., 2011). It could be speculated that the newly transcribed JAZ proteins not only inactivate the transgenic bHLH05, but also sequester endogenous bHLH05 from the functional bHLH05-MYBx complex by replacing it with unfunctional bHLH05-JAZx. Thus, the inability of bHLHs to activate both transient promoter-reporter constructs and endogenous GSL genes cannot be attributed to limitations of the Arabidopsis cell-culture system, because promoters of GSL biosynthetic genes harboring both G-boxes and MYB binding motifs (shown in Supplemental Text S1 for CYP79B2, CYP79B3, and CYP81B1; Higo et al., 1999) were activated by MYB51 in trans. In addition, analysis of GSL levels of cultured Arabidopsis cells revealed that the promoters of GSL biosynthesis genes could be manipulated by overexpressing MYBs, but not bHLH proteins (Fig. 9A).

Combinatorial Gene Regulation by MYB-bHLH Complexes

The physical interaction between bHLH and MYB factors in plants plays an important role in different plant processes and has become a paradigm in plant combinatorial gene regulation (Feller et al., 2011). Several studies that aimed to understand transcriptional gene regulation by the MYB-bHLH complex have addressed this in trichome patterning and the differentiation of epidermal cells (Glover et al., 1998; Bernhardt et al., 2005; Pesch and Hülskamp, 2009; Pesch et al., 2013) in Arabidopsis. Furthermore, the MYB-bHLH protein complexes involved in the regulation of flavonoid genes in Arabidopsis and also in other species such as rice (*Oryza sativa*), maize (*Zea mays*), cotton (*Gossypium hirsutum*), apple (*Malus domestica*), strawberry (*Fragaria* spp.), snapdragon (*Antirrhinum majus*), beefsteakplant (*P. frutescens*), gerbera (*Gerbera hybrida*), and clustered gentian (*Gentiana triflora*; Martin et al., 1991; Grotewold et al., 2000; Hu et al., 2000; Aharoni et al., 2001; Sompornpailin et al., 2002; Elomaa et al., 2003; Humphries et al., 2005; Schwinn et al., 2006; Sweeney et al., 2006; Espley et al., 2007; Furukawa et al., 2007; Nakatsuka et al., 2008; Brueggemann et al., 2010; Lin-Wang et al., 2010) have been rigorously characterized.

In this study, we identified the GSL biosynthesis pathway as a target of the MYB-bHLH complex. We monitored the transcriptional activation of transiently introduced or endogenous GSL biosynthesis genes by MYBs and bHLH, and addressed the special role of bHLH proteins for GSL biosynthesis. The role of bHLHs in the MYB-bHLH complex remains open, because it is not clear why the interaction of bHLH with MYBs is required, or why the simultaneous overexpression of both bHLH and MYB proteins does not induce the transcription of promoter-reporter constructs in transiently transformed Arabidopsis cells. To explain this, an alternative role for bHLHs other than the direct activation of gene transcription can be assumed. Such an indirect activation of gene transcription is also the case for bHLH-mediated gene regulation in flavonoid biosynthesis, which we consider below.

Similar to the R2R3-MYBs that regulate GSL biosynthesis, the C1 protein, which belongs to the R2R3 MYB group and regulates flavonoid biosynthesis in maize, possesses a functional transcription activation domain at its C terminus (Sainz et al., 1997a), which can bind DNA in vitro and activate gene transcription (Sainz et al., 1997b). However, the function of R2R3 MYBs in flavonoid biosynthesis is dependent on bHLH proteins, which is also true for R2R3 MYBs in GSL biosynthesis. It was initially thought that the requirement of C1 to interact with R1 (bHLH protein) is a consequence of a low affinity of C1 for DNA (Sainz et al., 1997b); however, it was shown that even when the DNA-binding ability of this protein increased (via the mutated C1 protein), C1 remains dependent from bHLH (Hernandez et al., 2004). This appears to be the case not only in flavonoid and GSL biosynthesis, because two other proteins belonging to R2R3-MYB TF family known as GLABRA1 and WEREWOLF, which contain transcription activation domains and are able to bind DNA, are also dependent on the bHLH factors GLABRA3/ENHANCER OF GLABRA3 (Lee and Schiefelbein, 2001; Zhang et al., 2003). In summary, the R2R3 MYBs can bind and activate their target genes both transiently and in planta, whereas bHLHs are essential for the regulation of target genes in planta but are dispensable for the activation of transiently expressed promoter-reporter constructs in wild-type Arabidopsis cells.

One hypothesis that can explain the requirement of bHLHs for MYBs is the inhibition hypothesis. It can be

suggested that MYBs that regulate GSL biosynthesis can interact with other inhibitor proteins that retain the ability of MYBs to activate GSL biosynthesis genes. Thus, the bHLH-mediated release of MYBs from inhibition will be followed by the MYB-mediated activation of GSL biosynthesis genes. Interestingly, several independent research groups have recently demonstrated that the bHLH subgroup IIId TFs (bHLH03, bHLH13, bHLH14, and bHLH17) are targets of JAZ proteins and therefore inhibitors of JA signaling, which can indeed serve as antagonists of the bHLH subgroup IIIe (bHLH04, bHLH05, and bHLH06) analyzed in this study (Nakata et al., 2013; Sasaki-Sekimoto et al., 2013; Song et al., 2013; Fonseca et al., 2014). Furthermore, these bHLH subgroup IIId TFs function redundantly to negatively regulate JA-mediated plant defense; therefore, it is highly probable that bHLH03, bHLH13, bHLH14, and bHLH17 factors are transcriptional repressors of GSL biosynthesis and antagonize bHLH04, bHLH05, and bHLH06. To verify this hypothesis, it needs to be shown that bHLH03, bHLH13, bHLH14, and bHLH17 interact with MYBs regulating GSL biosynthesis or bind the promoters of GSL biosynthesis genes. However, the existence of inhibitors alone cannot explain the absolute requirement of bHLH proteins for the regulation of GSL biosynthesis by the bHLH-MYB complex. If bHLH04, bHLH05, and bHLH06 were only needed to out-sequester these inhibitor proteins, then the simultaneous overexpression of bHLH05 and MYB51 in vitro would have caused an increase in transcription of GSL promoter-reporter constructs (Fig. 9) by sequestering the bHLH03, bHLH13, bHLH14, and bHLH17 proteins from the unfunctional MYB51-bHLHx complex and replacing it with MYB51-bHLH05.

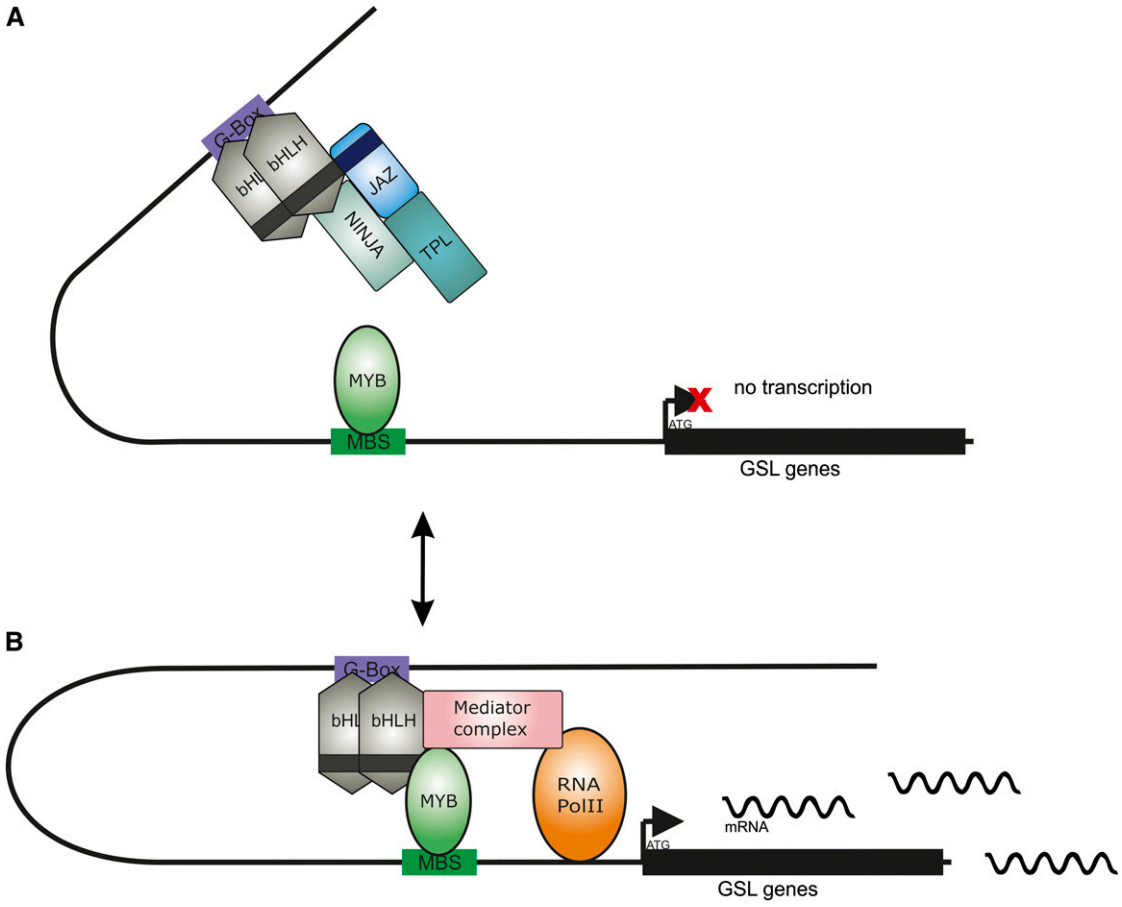

Figure 10. Working model for regulation of GSL by MYB and bHLH in Arabidopsis. In the absence of JA, JAZ repressors bind to bHLH and inhibit the interaction between the bHLH proteins and R2R3-MYBs, attenuating the potential of the MYB-bHLH complex to activate the promoters of GSL pathway genes. Competitive binding to bHLH proteins by JAZs and MYBs as well as preferential binding of some bHLH proteins (mainly bHLH05 and bHLH04) to MYBs rather than to JAZ allows the basal transcriptional activity of the bHLH-MYB complex and allows the production of the moderate level of GSL present in wild-type plants. In the presence of JA, the bHLH proteins form more protein complexes with MYBs. Once the MYBs and bHLH are simultaneously recruited to the promoter of GSL genes, they activate the transcription of GSL biosynthesis genes. MYBs binds to MYB-boxes present in GSL genes, whereas bHLH proteins bind to G-boxes present in the same genes and the transcriptional of GSL genes is activated. The function of bHLHs is fulfilled by the tethering of the mediator complex, and probably the chromatin-modifying factors to DNA, which unwind chromatin, make it accessible to the MYBs and RNA polymerase II.

An alternative hypothesis that can additionally explain the requirement of bHLHs for MYBs and can reconcile all findings presented in this study (including the contradictory results on the dispensability of bHLH in wild-type cultured cells and indispensability in planta) relates to the role of mediator proteins and/or chromatin in the bHLH-mediated regulation of GSL. To assist this regulatory role, bHLH requires an additional interaction partner that can modify chromatin. The protein R-interacting factor1 (RIF1) can modify chromatin in flavonoid biosynthesis (Hernandez et al., 2007). The RIF1 protein is a nuclear, AGENET domain-containing EMSY-like protein, which is recruited by the bHLH protein R to modify chromatin, whereas R2R3-MYBs can proceed with transcriptional gene activation. Thus, it can be speculated that the function of bHLHs in GSL biosynthesis might link the transcriptional activation of genes with chromatin functions by tethering chromatin-modifying proteins to DNA and thereby allowing the modulation of gene expression by histone-modifying (acetylation/methylation) proteins. To facilitate the tethering of RIF-like proteins to DNA, the bHLH proteins need either to be able to bind DNA or to be kept in close proximity to DNA by interacting with MYBs. Notably, this appears to be true for GSL-regulating bHLHs, because ChIP-seq experiments with bHLH06-overexpressing plants revealed the binding of bHLH06 to G-boxes of GSL genes in the presence of R2R3 MYBs (Schweizer et al., 2013a). Whether bHLH proteins can bind the promoters of GSL genes in the absence of MYBs that regulate IGs or AGs needs to be addressed in the future. Interestingly, chromatin immunoprecipitation experiments were unable to detect DNA binding of bHLH protein to one of the pathway gene promoters in the absence of MYB genes (Hernandez et al., 2007). This suggests that R2R3-MYBs may influence the recruitment of bHLH-like factors to DNA, perhaps by conformational changes that expose the bHLH domain for dimerization and DNA binding (Feller et al., 2011).

Emerging evidence has revealed the interaction of the Mediator25 (MED25) protein with bHLH04, bHLH05, and bHLH06 as well as involvement of the Mediator complex in the JA-induced transcriptional regulation (Kidd et al., 2009; Çevik et al., 2012; Chen et al., 2012). Notably, among bHLH proteins interacting with MED25, several important TFs (AP2/ERF1, ORA59, and TDR1/ERF98) known to function in the control of JA-associated gene expression were also identified (Çevik et al., 2012; Chen et al., 2012). Mediator is a conserved protein complex known to promote the transcription of protein-coding genes by RNA polymerase II in eukaryotes. Mediator is highly conserved in a wide range of eukaryotes (Chadick and Asturias, 2005; Malik and Roeder, 2005; Bäckström et al., 2007; Chen et al., 2012). Remarkably, several recent studies demonstrated that besides interacting directly with polymerase II, Mediator has multiple functions and can interact with and coordinate the action of numerous other coactivators and corepressors, including those acting at the level of chromatin (Kidd et al., 2010; Malik and Roeder, 2010; Borggrefe and Yue, 2011; Chen and Roeder, 2011; Conaway and Conaway, 2011; Hentges, 2011; Kidd et al., 2011; Kim and Chen, 2011; Ries and Meisterernst, 2011; Chen et al., 2012). Altogether, we suggest that the Mediator complex could be an integrative point that coordinates GSL biosynthesis and JA signaling through interaction with multiple TFs. Proteins, such as MED25, which is known to act downstream of JA signaling and to interact with bHLH proteins, are probably essential to regulate GSL biosynthesis via the MYB-bHLH complex. Further investigation of individual subunits of the Mediator complex and their interaction partners could provide the potential for discovering regulatory proteins functioning in association with bHLH-MYB complex to regulate GSL biosynthesis. These could be proteins involved in chromatin modification like RIF1, as well as proteins important for function of RNA polymerase II transcription apparatus binding to promoter regions of GSL genes.

We propose a model that we believe occurs in planta (Fig. 10), in which MYBs and bHLHs are recruited to the promoters of GSL genes to activate the transcription of GSL biosynthesis genes; MYB proteins bind to MYB-boxes present in GSL genes, whereas bHLH proteins bind to G-boxes in the same genes. The function of bHLHs is probably to tether the mediator complex and chromatin-modifying factors to DNA, which unwind chromatin and make it accessible to MYBs and the RNA polymerase II transcription apparatus, which can then activate the transcription of GSL biosynthesis genes.

MATERIALS AND METHODS

Arabidopsis Loss-of-Function Mutants Used in This Study

The Arabidopsis (*Arabidopsis thaliana*) loss-of-function mutants used in this study are all in the Col-0 genetic background. For *bHLH04, bHLH05*, and *bHLH28*, the following insertion lines were obtained from the Nottingham Arabidopsis Stock Centre: At4g17880/bHLH04 (GK491E10), At5g46760/bHLH05 (GK445B11), and At5g46830/bHLH28 (SALK_119765C). Homozygous lines were identified by PCR for all three mutants and the position of the insertions was confirmed by sequencing. The absence of mRNA transcripts was additionally confirmed by RT-PCR. The *bhlh06/jin1.9* mutant (AT1G32640/bHLH06/MYC2) was kindly provided by Dombrecht et al. (2007). The T-DNA insertion mutants for *MYB34* and *MYB51* were previously described and are known as *myb34* (AT5G60890; WiscDsLox424F3; Frerigmann and Gigolashvili, 2014) and *myb51/hig1* (AT1G18570; GK228B12; Gigolashvili et al., 2007a). For the sake of simplicity, the lines will be herein named as *bhlh04*, *bhlh05*, *bhlh06*, *bhlh28*, *myb34*, and *myb51*. The multiple mutants *bhlh04/05*, *bhlh04/06*, *bhlh05/06*, *bhlh04/05/06*, *bhlh04/myb34*, *bhlh04/myb51*, *bhlh05/myb34*, *bhlh05/myb51*, *bhlh06/myb34*, and *bhlh06/myb51* were generated by crossing the corresponding parental homozygous lines and genotyping F2 segregating progenies to select homozygous mutations (primers for genotyping are listed in Supplemental Table S1). The homozygosity of mutants containing *bhlh06* was confirmed by analyzing the population for long roots on Murashige and Skoog (MS) plates supplemented with 50 μM of MeJA, compared with the respective control lines. The isolation of triple and quadruple mutants with *bhlh28* was not achieved due to the close linkage of the bHLH05 (At5g46760) locus to bHLH28 (At5g46830).

Arabidopsis Gain-of-Function Alleles and Overexpression Lines Used in This Study

For the generation of *MYB51* and *bHLH05* overexpression lines, the respective full-length coding sequences were amplified with gene-specific primers (Supplemental Table S2) and cloned into the Gateway pENTR/D-TOPO vector.

The final destination clones were generated by LR (Invitrogen) recombination with *pGWB2* (*Pro::35S*; kindly provided by Nakagawa et al., 2007) and/or *pBatTL-K-GFP* (Jörgens et al., 2010) followed by *Agrobacterium tumefaciens*-mediated transformation into wild-type (Col-0) plants. Successful transformed lines were selected using the respective marker genes and were analyzed for elevated transcript levels by qRT-PCR (PCR primers listed in Supplemental Table S3).

The gain-of-function alleles *MYB34-1D* (*atr1D*, AT5G60890; Bender and Fink, 1998) and *bHLH05-D94N* (*atr2D*, AT5G46760; Smolen et al., 2002) and the double gain-of-function allele *MYB34-1D bHLH05-D94N* (*atr1D atr2D*; Smolen et al., 2002) were kindly provided by the respective authors. The *MYB51-1D* (*HIG1-1D*) activation-tagged line and *35S:MYB51* overexpression plants were available from a previous study (Gigolashvili et al., 2007a)

Protein-Protein Interaction Analysis Using BiFC

To test the protein-protein interaction in planta, BiFC was used (Walter et al., 2004). The coding sequences of MYB51 and bHLH05 were transferred from the entry clones into the gateway pCL112 YFP N-terminal and pCL113 YFP C-terminal vectors, respectively (kindly provided by Sean Chapman, SCRI, and recently described by Pesch et al., 2013). The nuclear-localized factor bHLH133, which was cloned into the pCL113 YFP C-terminal vector, was used as a negative control for the interaction with MYB51. All plasmids of interest were transformed into *A. tumefaciens* and used for transient expression in tobacco via leaf infiltration. YFP fluorescence in tobacco was monitored 3.5 d after infiltration via a Leica DMRE fluorescence microscope (excitation at 514 nm and emission at 520-560 nm).

Protein-Protein Interaction Analysis Using LUMIER

The LUMIER assay is based on the techniques of Barrios-Rodiles et al. (2005) for mammalian proteins and Pesch et al. (2013) for plant proteins. The LUMIER assay was performed as described by Pesch et al. (2013), with slight modifications. To fuse luciferase from *Renilla reniformis* or protein A from *Staphylococcus aureus* to MYB and bHLH proteins, the two destination vectors pcDNA3-Rluc-GW and pTREX-dest30-ntPrA were recombined by LR reaction with the respective entry clones. The entry clone containing the gain-of-function protein D94N bHLH05 was generated by amplifying the full-length coding sequence from the *bHLH05-D94N* mutant (*atr2D*, AT5G46760; Smolen et al., 2002) followed by cloning into the Gateway pENTR/D-TOPO vector. The entry clone for the native bHLH05 was generated as described above (Arabidopsis gain-of-function alleles and overexpression lines used in this study).Generated constructs were transiently coexpressed in HEK293TN cells (BioCat/SBI LV900A-1) as hybrid proteins. About 0.4 μg of each plasmid DNA was cotransfected into 1×10^5 HEK293TN cells in 48-well plates using 1 μL of Lipofectamine 2000 (Invitrogen). After 48 h, the medium was removed and cells were washed with phosphate-buffered saline (PBS). The cells were collected by centrifugation (600*g*, 15 min) and then lysed on ice in 180 μL of ice-cold lysis buffer (20 mM Tris, pH 7.5, 250 mM NaCl, 1% Triton X-100, 10 mM EDTA, 10 mM dithiothreitol, protease inhibitor cocktail [catalog no. 11697498001, Roche] and phosphatase inhibitor cocktail [catalog no. 04693116001, Roche]) for 1 h. Subsequently, lysates were centrifuged at 21,380*g* for 15 min at 4°C, and the 50 μL of supernatant was mixed with 3 μL of PBS-prewashed sheep antirabbit IgG-coated magnetic beads (2 mg/mL final concentration, Dynabeads M280; Invitrogen) and incubated for 1 h on ice. The beads were than washed five times with cold PBS using a magnetic plate washer (Hydro Flex; Tecan) and luminescence was measured with 200 μL of luciferase buffer (1.1 M NaCl, 2.2 mM EDTA, 200 mM $KxPO_4$, 0.44 mg/mL bovine serum albumin, and 2.5 μM coelenterazine) in a microtiter plate reader (Tecan). For the luciferase input control, 10 μL of the lysate was diluted to 50 μL with PBS and measured accordingly. Luciferase activity was determined for the input and the pull-down. Lysis buffer, PBS, and untransformed cells served as a negative control. Pull-down efficiency was calculated by the following: [relative luminescence unit pull-down/(relative luminescence unit input $\times$ 5)] $\times$ 100.

RNA Extraction and Expression Analysis by qRT-PCR

Total RNA was extracted from rosette leaves or seedlings of different mutants using TRIzol buffer (Life Technologies) followed by RNase-free DNAse (Roth) treatment to remove genomic DNA contamination. The expression of GSL biosynthesis genes was analyzed by real-time qRT-PCR analysis using the fluorescent intercalating dye SYBR-Green in a GeneAmp 5700 Sequence Detection System (Applied Biosystems). The Arabidopsis *ACTIN2* gene was used as an endogenous control. A two-step RT-PCR analysis was performed. First, total RNAs (10 μg per reaction) were reverse transcribed into cDNAs, using the First-Strand cDNA Synthesis SSII Kit (Life Technologies) according to the manufacturer's instructions. Subsequently, the cDNAs were used as a template in real-time PCR experiments with the SYBR-Green Master Kit System (Life Technologies) and gene-specific primers (for primer sequences, see Supplemental Table S3) according to the manufacturer's instructions. The relative quantification of expression levels was performed using the comparative Δ cycle threshold method, and the calculated relative expression values were normalized to *ACTIN2* and compared with the expression level in wild-type plants (Col-0 = 1). If not specified in the figure legend, three technical replicates and three biological replicates from independently grown plants were analyzed.

Generation and Analysis of probHLH05-uidA Plants

For in planta expression analysis of *bHLH05*, two different *ProbHLH05* constructs were generated. The first construct included the first two exons, intron, and -1325 bp of the promoter region. The second construct comprised only the promoter region from -1325 to -4 bp and no sequences from the coding part of the gene. Both pieces of DNA were cloned into an entry vector and subsequently transferred by LR into the *pGWB3* vector containing a *uidA* (GUS) fusion. All stably transformed plants containing only a -1325 to -4 bp part of promoter region exhibited similar tissue-specific expression patterns although the intensity of blue staining slightly differed from plant to plant. The promoter fusion construct including -1325 to -4 bp, the translation initiation codon, and the first two exons did not show any GUS activity in stably transformed plants and were therefore eliminated from further analysis. Histochemical detection of GUS activity was performed using X-Gluc as a substrate. Incubation in 80% (v/v) ethanol was performed to remove chlorophyll.

HPLC Analysis of desulpho-GSL

The isolation and analysis of GSL content was performed by using the desulpho-GSL method (Thies, 1979) on an ultra-performance liquid chromatography device (Eschborn; Waters) as previously described (Gigolashvili et al., 2012).

Promoter Transactivation Assay with bHLH05 and MYB51 and Promoters of GSL Biosynthesis Genes in Cultured Arabidopsis Cells

An Arabidopsis suspension-culture cell line was maintained in 50 mL of Arabidopsis (AT) medium. The AT medium contained 4.3 g/L MS basal salts (Duchefa), 1 mg/L 2,4-dichlorophenoxyacetic acid, 4 mL of vitamin B_5 mixture (Sigma-Aldrich), and 30 g/L Suc (pH 5.8). Cells were gently agitated at 160 rounds per minute in the dark at 22°C. Promoters of the GSL biosynthetic genes *ASA1*, *CYP79B3*, and *CYP83B1* were generated as reported in Gigolashvili et al. (2007a). The overexpression constructs for *bHLH05* and *MYB51* generated as described above (Arabidopsis gain-of-function alleles and overexpression lines used in this study) together with GSL promoter-reporter constructs were transferred into the supervirulent *A. tumefaciens* strain LBA4404.pBBR1MCS.virGN54D.

To assess the transactivation potential of bHLH05 and MYB51 proteins against promoters or GSL genes, the effector constructs with TFs and the promoter-reporter *uidA* constructs driven by the promoters of GSL biosynthesis genes were used. Thus, the effector constructs in the supervirulent *A. tumefaciens* strain, the antisilencing *A. tumefaciens* strain 19K (Voinnet et al., 1999), and each of the reporter constructs were taken from fresh yeast extract broth plates, grown overnight, resuspended in 1 mL of AT medium and used for cotransfection. To estimate the transactivation potential, three clones of *A. tumefaciens* containing effector, reporter, and antisilencing constructs (19K) were mixed in a 1:1:1 ratio and 75 μL of this suspension was added to 3 mL of cultured Arabidopsis cells. When the combinatorial effect of two effectors (bHLH05 and MYB51) was desired, two effector constructs, one promoter-reporter construct, and 19K strains were mixed in a 1:1:1:1 ratio and used for the contransfection of cells. After 4 to 5 d of coculturing (in the dark, 22°C, 160 rpm), cells were treated with 100 μL X-Gluc solution for 1 h to overnight at 37°C or cells were centrifuged, the proteins isolated and GUS activity determined using 4-methylumbelliferyl-β-D-glucuronide as a substrate. The amount of 4-methylumbelliferone formed was recorded fluorometrically (excitation, 340 nm; emission, 465 nm). For this, 200 μL of substrate buffer (protein extraction buffer with 1 mM 4-methylumbelliferyl-β-D-glucuronide) and 25 μL of protein extract were mixed, incubated at 37°C in the dark, and measured every 10 to 30 min on a multiwell plate reader. The slope of the increase in 4-methylumbelliferone was determined in the linear range and divided by the amount of protein.

Plant Growth Conditions and MeJA Treatment

Seeds of Arabidopsis Col-0 and of mutant lines were stratified for 2 to 7 d in the dark at 4°C to break seed dormancy. Plants were grown in growth cabinets with a 8-h/16-h light/dark cycle and 21°C/18°C day/night temperature, 40% humidity, and a mean photon flux density of 150 μmol m^{-2} s^{-1}. A minimum of 100 mg of rosette material was harvested from 4- to 5-week-old plants, immediately frozen in liquid nitrogen, and kept at −80°C until DNA and RNA extraction or GSL analysis.

For MeJA treatment, wild-type and mutant plants were grown vertically on sterile MS plates for 2 weeks and then transferred onto MS plates supplemented with 50 μM of MeJA or without any supplementation. Seedlings were grown for an additional 2 d or 1 week on these media until they were harvested and used for transcript or GSL analysis.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Biosynthesis of aliphatic and indolic glucosinolates in *Arabidopsis*.

Supplemental Figure S2. BiFC reveals an interaction of indolic MYB51, MYB34 and MYB122 but also of MYB28 and MYB29 with bHLH05 and bHLH04.

Supplemental Figure S3. Expression level of bHLH subgroup IIIe is comparable in roots and shoots.

Supplemental Figure S4. Levels of individual IG and AG in seedlings of various double *myb bhlh* mutants.

Supplemental Figure S5. GSL accumulation in rosette leaves of *Arabidopsis* is not positively affected by the overexpression of *35S:bHLH05*.

Supplemental Figure S6. Levels of individual IG and AG in single and double gain-of-function alleles of *MYB34* and *bHLH05*.

Supplemental Figure S7. Levels of individual IGs and AGs in single and multiple *bhlh* mutants.

Supplemental Figure S8. Levels of individual AG and IGs in the *bhlh04/05/06* mutant treated with MeJA.

Supplemental Figure S9. Expression of *MYB34*, *MYB51* and *MYB122* genes is not reduced in *bhlh04/05/06* mutant.

Supplemental Figure S10. Induction of indolic glucosinolates in *MYB34-1D* by MeJA treatment.

Supplemental Figure S11. Accumulation of indolic GSLs in rosette leaves of triple *bhlh04/05/06* is not positively affected by the overexpression of *MYB51*.

Supplemental Table S1. Primer sequences for qPCR analysis.

Supplemental Table S2. Primers used for the screening of T-DNA insertion lines.

Supplemental Table S3. Primer sequences for cloning.

Supplemental Data Text S1. Putative Cis Elements for MYB and bHLH detected by PLACE in CYP79B2, CYP79B3 and CYP83B1.

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