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The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*

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Summary

Methionine-derived glucosinolates belong to a class of plant secondary metabolites that serve as chemoprotective compounds in plant biotic defense reactions and also exhibit strong anticancerogenic properties beneficial to human health. In a screen for the *trans*-activation potential of various transcription factors toward glucosinolate biosynthetic genes, we could identify the *HAG1* (*HIGH ALIPHATIC GLUCOSINOLATE 1*, also referred to as *MYB28*) gene as a positive regulator of aliphatic methionine-derived glucosinolates. The content of aliphatic glucosinolates as well as transcript levels of aliphatic glucosinolate biosynthetic genes were elevated in gain-of-function mutants and decreased in *HAG1* RNAi knock-down mutants. Pro_{HAG1}:GUS expression analysis revealed strong *HAG1* promoter activity in generative organs and mature leaves of *A. thaliana* plants, the main sites of accumulation of aliphatic glucosinolates. Mechanical stimuli such as touch or wounding transiently induced *HAG1/MYB28* expression in inflorescences of flowering plants, and *HAG1/MYB28* over-expression reduced insect performance as revealed by weight gain assays with the generalist lepidopteran herbivore *Spodoptera exigua*. Expression of *HAG1/MYB28* was significantly induced by glucose, indicating a novel transcriptional regulatory mechanism for the integration of carbohydrate availability upon biotic challenge. We hypothesize that HAG1/MYB28 is a novel regulator of aliphatic glucosinolate biosynthesis that controls the response to biotic challenges.

Keywords: biotic stress, glucose-responsive genes, glucosinolates, MYB transcription factors.

Introduction

Arabidopsis thaliana plants, along with other *Brassicaceae* species, produce an enormous diversity of aliphatic glucosinolates (Kliebenstein *et al.*, 2001; Mithen, 2001; Rask *et al.*, 2000), which play an important role in the defense against micro-organisms (Brader *et al.*, 2006; Chung *et al.*, 2005; Kliebenstein *et al.*, 2005a; Manici *et al.*, 2000; Mari *et al.*, 1996) and herbivores (Giamoustaris and Mithen, 1995; Levy *et al.*, 2005; Mewis *et al.*, 2005; Reymond *et al.*, 2004). The chemoprotective and anti-carcinogenic properties of glucosinolates, on the other hand, have produced an increasing interest in the biosynthesis and regulation of methionine-derived glucosinolates and their breakdown products (Gross *et al.*, 2000; Jeffery, 2005; Mithen *et al.*, 2003; Nho and Jeffery, 2004; Shapiro *et al.*, 2006). Furthermore, *Brassicaceae* cultivars are increasingly used as fumigants to suppress soil-borne pests and diseases in agricultural and horticultural crops (Vaughn *et al.*, 2005; Zasada and Ferris, 2004). In plants, glucosinolates form a defense system known as 'mustard oil bomb': upon tissue damage, non-toxic glucosinolates are hydrolysed by myrosinases into biologically active isothiocyanates, which have been shown to be highly toxic for both generalist and specialist herbivores (Kliebenstein *et al.*, 2005b; Rask *et al.*, 2000; Wittstock and Gershenzon, 2002).

The most prominent glucosinolate biosynthetic precursors are the amino acids methionine, tryptophan and

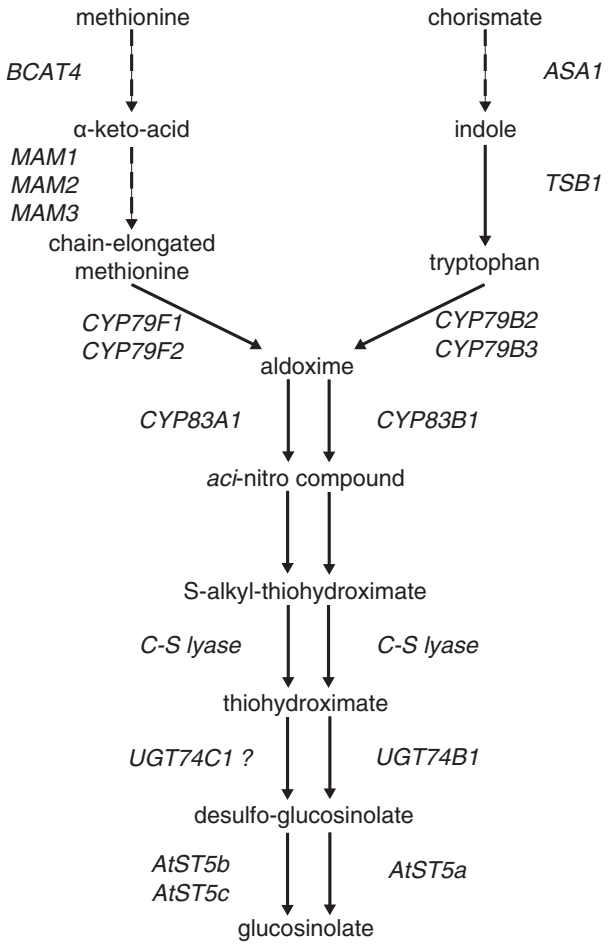

Figure 1. Proposed pathways for the biosynthesis of methionine-derived (left) and tryptophan-derived glucosinolates (right).

phenylalanine, leading to aliphatic, indolic and aromatic glucosinolates, respectively. The biosynthesis of glucosinolates proceeds in several steps (Figure 1) (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). First, methionine or other amino acids are transaminated by a cytoplasmic branched-chain aminotransferase (BCAT4) leading to corresponding α-keto-acids (Schuster *et al.*, 2006). Subsequent side-chain elongation of the amino acid involves the action of chloroplastic methylthioalkylmalate synthases (MAM1–3), followed by formation of the glucosinolate core structure mediated by cytochrome P450 mono-oxygenases, which catalyse the formation of aldoximes and the subsequent oxidation and conjugation of aldoximes with a sulfur donor to form *S*-alkyl thiohydroximates. In the case of methionine-derived glucosinolates, the cytochrome P450 mono-oxygenases CYP79F1, CYP79F2 and CYP83A1 are involved in these reactions (Chen *et al.*, 2003; Hemm *et al.*, 2003; Naur *et al.*, 2003; Reintanz *et al.*, 2001; Tantikanjana *et al.*, 2004). The resulting compounds are further metabolized to glucosinolates by consecutive actions of *C–S* lyases, *S*-glucosyltransferases and sulfotransferases (Grubb *et al.*, 2004; Klein *et al.*, 2006; Mikkelsen *et al.*, 2004; Piotrowski *et al.*, 2004). Secondary modifications then give rise to the considerable diversity of glucosinolates (Fahey *et al.*, 2001).

The glucosinolate pattern varies between tissues and organs during ontogenesis (Brown *et al.*, 2003; Petersen *et al.*, 2002), in response to environmental stimuli (Bednarek *et al.*, 2005; Brader *et al.*, 2001; Kliebenstein *et al.*, 2002; Mewis *et al.*, 2005; Mikkelsen *et al.*, 2003) or in different *A. thaliana* ecotypes (Kliebenstein *et al.*, 2001; Reichelt *et al.*, 2002). The highest glucosinolate concentrations have been found in reproductive organs, including inflorescences, flowers and siliques, followed by leaves and the root system. Indolic glucosinolates are mainly found in mature rosette leaves and roots, whereas aliphatic glucosinolates accumulate ubiquitously in plants, being predominantly present in generative organs.

The control of glucosinolate biosynthesis is poorly understood, and only recently have the first regulatory components of the glucosinolate biosynthetic pathways in *A. thaliana* been described. A novel calmodulin-binding nuclear protein, IQD1, was shown to be a positive regulator of aliphatic and indolic glucosinolate formation (Levy *et al.*, 2005). In addition, the transcription factor AtDof1.1 was identified as part of a regulatory network controlling CYP83B1 activity and glucosinolate accumulation (Skirycz *et al.*, 2006). The R2R3-MYB transcription factor HIG1/MYB51 has been recently shown to regulate specifically indolic glucosinolate biosynthesis (Gigolashvili *et al.*, in press). Another MYB factor, ATR1/MYB34, has previously been reported to regulate auxin and indolic glucosinolate homeostasis (Celenza *et al.*, 2005); however, *ATR1/MYB34* expression appears to be almost absent in leaves, the main site of indolic glucosinolate accumulation, and, moreover, *ATR1/MYB34* over-expression led to a high-auxin growth phenotype (Gigolashvili *et al.*, in press).

Remarkably, a specific regulator of aliphatic glucosinolate biosynthesis has not yet been described. Here, we demonstrate that the R2R3-MYB transcription factor HAG1/MYB28 represents a key component in the regulation of aliphatic methionine-derived glucosinolate biosynthesis in *A. thaliana*.

Results

HAG1/MYB28 activates genes of the aliphatic glucosinolate biosynthetic pathway

A co-transformation assay (Berger *et al.*, in press) was used as a tool to identify new regulatory genes of glucosinolate biosynthetic pathways. Reporter constructs, consisting of promoters of the aliphatic glucosinolate biosynthesis genes *MAM3*, *CYP79F1* and *CYP83A1*, and the indolic glucosino-

late biosynthesis genes *TSB1*, *CYP79B2* and *CYP79B3*, fused to the *uidA* (GUS) reporter gene were generated. Likewise, several effector constructs, i.e. putative transcription factors or nuclear-localized proteins (MYB factors, Dof1.1 and IQD1), putatively involved in the control of glucosinolate biosynthetic genes, were cloned into a binary vector under the control of the CaMV 35S promoter. Both reporter and effector constructs were simultaneously expressed in cultured *A. thaliana* cells using the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D (Koroleva *et al.*, 2005), and the *trans*-activation potential of the effector was subsequently assayed as GUS activity (Berger *et al.*, in press). Alternatively, a simple histochemical staining procedure using X-Gluc was applied.

As shown in Figure 2, cultured *A. thaliana* cells transiently expressing only the GUS reporter construct fused to promoters of putative target genes without an effector showed only weak GUS activity. However, three transcription factors (effectors), namely MYB28, MYB76 and MYB29, were shown to specifically *trans*-activate genes of the aliphatic glucosinolate biosynthetic pathway, i.e. *MAM3*, *CYP79F1*, and *CYP83A1*, but not those of the indolic glucosinolate biosynthetic pathway (*TSB1*, *CYP79B2*, *CYP79B3*). These three genes are therefore referred to as high aliphatic glucosinolate (*HAG*) genes: *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29*.

On the other hand, ATR1/MYB34 and HIG1/MYB51, which have been previously identified as regulators of the indolic glucosinolate biosynthetic pathway (Celenza *et al.*, 2005; Gigolashvili *et al.*, in press), exclusively *trans*-activated only promoters of genes involved in the indolic but not the aliphatic glucosinolate biosynthetic pathway (Figure 2). The co-transformation assay was validated by demonstrating that MYB12, involved in the control of the phenylpropanoid biosynthetic pathway, could not interact with any promoters of glucosinolate biosynthetic genes but strongly activated promoters of *CHS* (chalcone synthase) and *FLS* (flavone synthase) as previously demonstrated (Figure 2) (Berger *et al.*, in press; Mehrtens *et al.*, 2005;). Interestingly, the recently described regulators of glucosinolate biosynthesis, Dof1.1 and IQD1 (Levy *et al.*, 2005; Skirycz *et al.*, 2006), did not activate any tested promoter of glucosinolate biosynthetic genes in *trans*, and did not change the observed gene activation by HIG1/MYB51 and HAG1/MYB28 in triple assays (not shown).

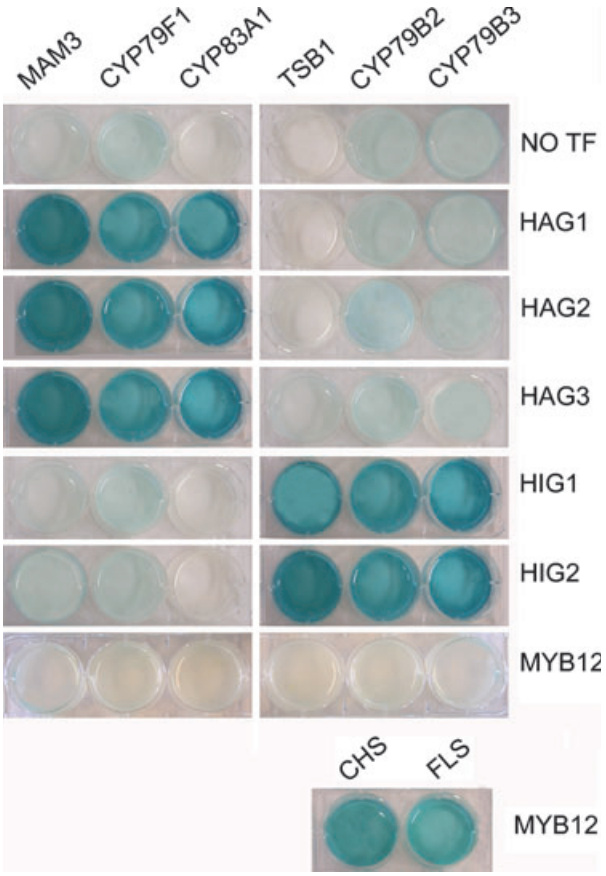

Figure 2. Identification of new regulators of methionine-derived glucosinolates in co-transformation assays using cultured *A. thaliana* cells.
Co-transformation assays for the determination of target gene specificity of various transcription factors (effectors) towards target promoters of aliphatic and indolic biosynthetic pathway genes are shown. The promoters of *MAM3*, *CYP79F1*, *CYP83A1*, *TSB1*, *CYP79B2* and *CYP79B3* genes were fused to the *uidA* (GUS) reporter gene (target promoter:GUS vectors). Cultured *A. thaliana* Col-0 cells were inoculated with the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D containing either only the reporter construct (target promoter:GUS:*pGWB3i*), or the reporter construct and, in addition, various effector constructs (Pro_{35S}:effector:*pGWB2*). GUS staining indicates *trans*-activation of a promoter by an effector. TF, transcription factor.

Properties of the HAG1/MYB28 protein

HAG1/MYB28 (At5g61420) is a member of the large family of R2R3-MYB transcription factors in *A. thaliana* (Stracke *et al.*, 2001). HAG1/MYB28 clusters into subgroup 12 of the *A. thaliana* MYB factors together with five other MYB factors (HIG1/MYB51, HIG2/MYB122, ATR1/MYB34, HAG2/MYB76 and HAG3/MYB29), all of which have been identified as regulators of either aliphatic or indolic glucosinolate biosynthetic genes (Celenza *et al.*, 2005; Gigolashvili *et al.*, in press; this work). The *HAG1/MYB28* gene structure of three exons and two introns was confirmed by isolation and sequencing of the full-length cDNA. The predicted protein is 336 amino acid residues in length, with a molecular mass of about 41.1 kDa and a calculated isoelectric point of 5.71. HAG1/MYB28 does not contain a typical nuclear localization signal (NLS) as revealed by PredictNLS (http://cubic.bioc.columbia.edu/cgi/var/nair/loctree/query/; Nair and Rost, 2005). However, an amino acid residue sequence LKKRL was detected (amino acid residues 112–116) that might act as a SV40-type NLS motif. Similar or identical motifs are also present in sequences of other members of the HAG1/MYB28 subfamily (LKKRL and LKKLL in HAG2/

MYB76 and HAG3/MYB29, respectively), suggesting that these motifs do actually serve as nuclear localization signals.

HAG1/MYB28 is directed to the nucleus of A. thaliana *cells*

To determine the subcellular localization of HAG1/MYB28 in plant cells, a co-translational *Pro$_{35S}$:HAG1*:GFP fusion plasmid was generated and transiently transferred into a homologous system, i.e. cultured *A. thaliana* cells.

As shown in Figure 3 (a,b), the nuclei showed a strong green fluorescence signal resulting from expression of the HAG1:GFP fusion protein, which co-localized with DNA-specific DAPI staining (Figure 3c), thus demonstrating the nuclear localization of the HAG1:GFP fusion protein. By contrast, the GFP signal was distributed uniformly in the cytosol when the cytosolic BCAT4 protein (Schuster *et al.*, 2006) was used as a control (Figure 3d).

Generation of Pro$_{35S}$*:* HAG1*-over-expressing and* HAG1 *RNAi plants*

To demonstrate that the accumulation of aliphatic glucosinolates can be triggered by HAG1/MYB28, several transgenic CaMV 35S over-expression and RNAi plants were created using Gateway-compatible vectors and *A. tumefaciens*-mediated transformation. About 60 primary transformants were analysed for both *HAG1/MYB28* over-expression and silencing constructs, and three representative over-expressing and RNAi lines with different steady-state *HAG1/MYB28* mRNA levels were analysed in more detail.

Constitutive over-expression of *HAG1/MYB28* led either to an almost unaltered growth phenotype (*Pro$_{35S}$:HAG1-12*), or plant growth was moderately (*Pro$_{35S}$:HAG1-15*) or strongly affected (*Pro$_{35S}$:HAG1-11*) (Figure 4). The observed growth phenotypes nicely correlated with the expression levels of *HAG1/MYB28* and decreased contents of both I3M (Figure 5d) and auxin (data not shown). *HAG1* RNAi plants did not show visible effects on plant morphology (Figure 4).

Metabolite profiling of HAG1/MYB28 *over-expressors and RNAi plants*

To analyse the levels of aliphatic glucosinolates in transformants with an altered expression of *HAG1/MYB28*, the glucosinolate pattern of *HAG1/MYB28* over-expressors and RNAi plants was determined. Glucosinolates (GS) were extracted from freeze-dried rosette leaves of 5-week-old

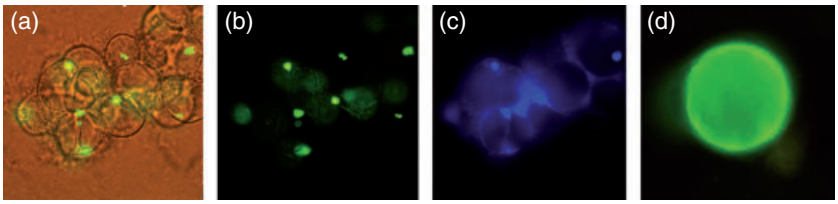

Figure 3. HAG1/MYB28 is localized to the cell nucleus.
Subcellular localization of a *Pro$_{35S}$:HAG1:*GFP translational fusion in cultured *A. thaliana* cells.
(a) The *Pro$_{35S}$:HAG1:*GFP construct is localized to the nucleus (bright-field with GFP).
(b) GFP filter only.
(c) DAPI staining of the nucleus.
(d) Cytosolic GFP distribution of the *Pro$_{35S}$:BCAT4:*GFP construct (Schuster *et al.*, 2006).

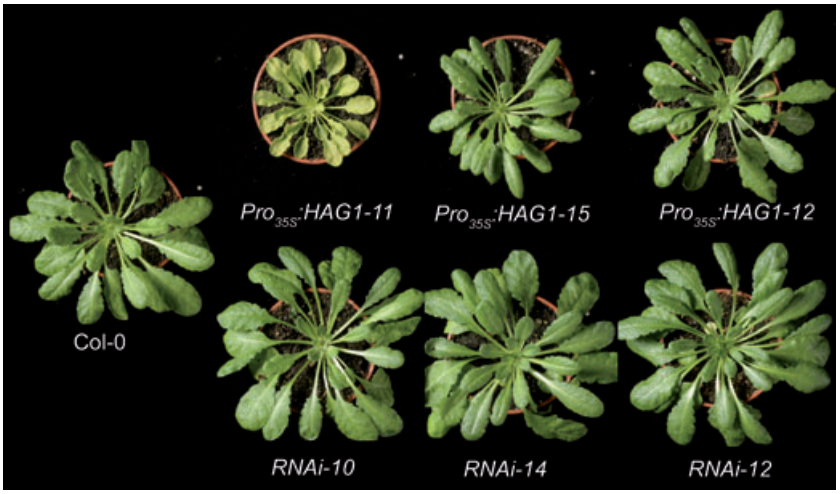

Figure 4. Growth phenotypes of *Pro$_{35S}$:HAG1* and *HAG1* RNAi mutants.
Various independent transgenic lines are shown. Upper row, from left to right: *Pro$_{35S}$:HAG1-11*, *Pro$_{35S}$:HAG1-15*, *Pro$_{35S}$:HAG1-12*. Lower row, from left to right: *HAG1-RNAi-10*, *HAG1-RNAi-14* and *HAG1-RNAi-12*. All transgenic lines are in the Col-0 wild-type background (furthest left).

plants, and the levels of 3-methylsulfinylpropyl-GS (3 MSOP), 4-methylsulfinylbutyl-GS (4MSOB), 5-methylsulfinylpentyl-GS (5MSOP), 4-methylthiobutyl-GS (4MTB), 8-methylsulfinyloctyl-GS (8MSOO), and of the indolic glucosinolates indol-3-ylmethyl-GS (I3M) and 4-methoxyindol-3-ylmethyl-GS (4MOI3M) were determined.

As shown in Figure 5, the HAG1/MYB28 over-expression lines *Pro$_{35S}$:HAG1-11*, *-15* and *-12* contained approximately two- to sevenfold higher levels of the main aliphatic glucosinolate 4MSOB as compared to the wild-type. Also, a two- to three-fold increase in the accumulation of other (minor) short-chain aliphatic glucosinolates such as 3MSOP and 5MSOP was observed, whereas the content of long-chain aliphatic glucosinolates such as 8MSOO remained almost unchanged. Also, the content of 4MTB did not significantly increase or decrease in loss-of-function or gain-of-function mutants, indicating that the levels of methylthioglucosinolates are presumably not regulated by HAG1/MYB28 (Figure 5b,f). The graduation in glucosinolate levels reflects well the corresponding *HAG1/MYB28* transcript levels, with *Pro$_{35S}$:HAG1-11* representing the strongest over-expressor line (Figure 5a). Remarkably, the increased accumulation of glucosinolates upon *HAG1/MYB28* over-expression was rather specific for methionine-derived glucosinolates. Synthesis of indolic glucosinolates was not influenced in the lines with a moderate or slight increase in the steady-state level of *HAG1* (lines *Pro$_{35S}$:HAG1-15* and *-12*). However, there was a decrease in the content of indolic glucosinolates in line *Pro$_{35S}$:HAG1-11* (Figure 5d) in which the methionine-derived glucosinolate biosynthetic pathway is highly activated.

Analysis of *HAG1* RNAi plants showed that these lines contained lesser amounts of both long- and short-chain aliphatic glucosinolates, namely 4MSOB, and also 3MSOP, 5MSOP and 8MSOO (Figure 5e–g). The levels of the indolic glucosinolates I3M and 4MOI3M were notably not affected in the RNAi lines (Figure 5h).

Transcription profiling of HAG1/MYB28 *over-expressors and* HAG1 *RNAi plants*

Real-time RT-PCR assays were used to assess whether the steady-state mRNA levels of genes encoding enzymes involved in aliphatic glucosinolate metabolism are affected in *HAG1/MYB28* over-expressors and *HAG1* RNAi knock-down plants. For this, samples from rosette leaves of wild-type plants, *Pro$_{35S}$:HAG1* plants (*Pro$_{35S}$:HAG1-11*, *-15* and *-12*) and RNAi plants (*HAG1-RNAi-10*, *-14* and *-12*) were analysed for relative mRNA levels. As shown in Figure 6, starting from the chloroplastidic MAM1 and MAM3 (MAML), enzymes involved in the side-chain elongation of methionine, to the last enzymes in the biosynthesis of the aliphatic glucosinolate core structure, the desulfoglucosinolate sulfotransferases AtST5b and AtST5c (Figure 1), *HAG1/MYB28*-over-expressing lines had consistently higher steady-state mRNA levels of aliphatic glucosinolate biosynthetic genes than the wild-type (Figure 6a).

The increase in mRNA transcript levels of putative target genes corresponded nicely with the increased levels of *HAG1/MYB28* transcript in the particular over-expression lines, i.e. the strongest over-expression line showing the strongest growth phenotype (*Pro$_{35S}$:HAG1-11*) also contained the highest transcript levels of biosynthetic genes. Remarkably, the transcript level of *CYP79B2* (and other genes of the indolic glucosinolate biosynthetic pathway including *AtST5a*, data not shown) remained unchanged in all *Pro$_{35S}$:HAG1* over-expression lines compared to the wild-type (Figure 6a).

In contrast, *HAG1/MYB28* RNAi knock-down plants showed a significant decrease in the transcript levels of aliphatic glucosinolate biosynthetic genes in leaves (Figure 6b). The same pattern was also observed in inflorescences (not shown). Again, the reduction in transcript levels corresponded well to the remaining levels of HAG1/MYB28 transcript in the respective RNAi lines, with line *HAG1-RNAi-10* being most strongly affected and line *HAG1-RNAi-12* showing only a partial decrease in the *HAG1/MYB28* transcript level. Notably, the transcript level of *CYP79B2*, a representative indolic glucosinolate biosynthetic gene, was slightly increased in all RNAi lines.

HAG1/MYB28*: tissue-specific expression and induction by mechanical stimuli*

To study the tissue-specific expression of *HAG1/MYB28*, the promoter region (−1995 to +157 bp) was used to generate a translational fusion construct with the *uidA* (GUS) reporter gene that was stably transferred to *A. thaliana* plants. About 25 transgenic *Pro$_{HAG1}$:uidA* lines were analysed in detail, most of them showing a similar tissue-specific pattern of *HAG1/MYB28* expression.

The analysis of GUS activity revealed the absence of reporter gene expression in cotyledons, hypocotyls and roots of 3–7-day-old seedlings (Figure 7). *HAG1/MYB28* promoter activity first appeared in stems, petioles and the main veins of true leaves in 10- to 14-day-old seedlings. Three-week-old seedlings showed increasing GUS expression, which was also detectable in lateral roots. Reporter gene activity gradually increased in expanding leaves, reaching a maximum in fully expanded leaves of non-flowering plants. However, during transition from the vegetative to the generative stage, GUS expression gradually decreased in leaves and appeared in inflorescences, being maintained during the whole generative stage. During senescence, GUS expression disappeared almost completely in leaves, but was still detectable in inflorescences.

Together, the highest *HAG1/MYB28* promoter activity could be detected in inflorescences of flowering plants

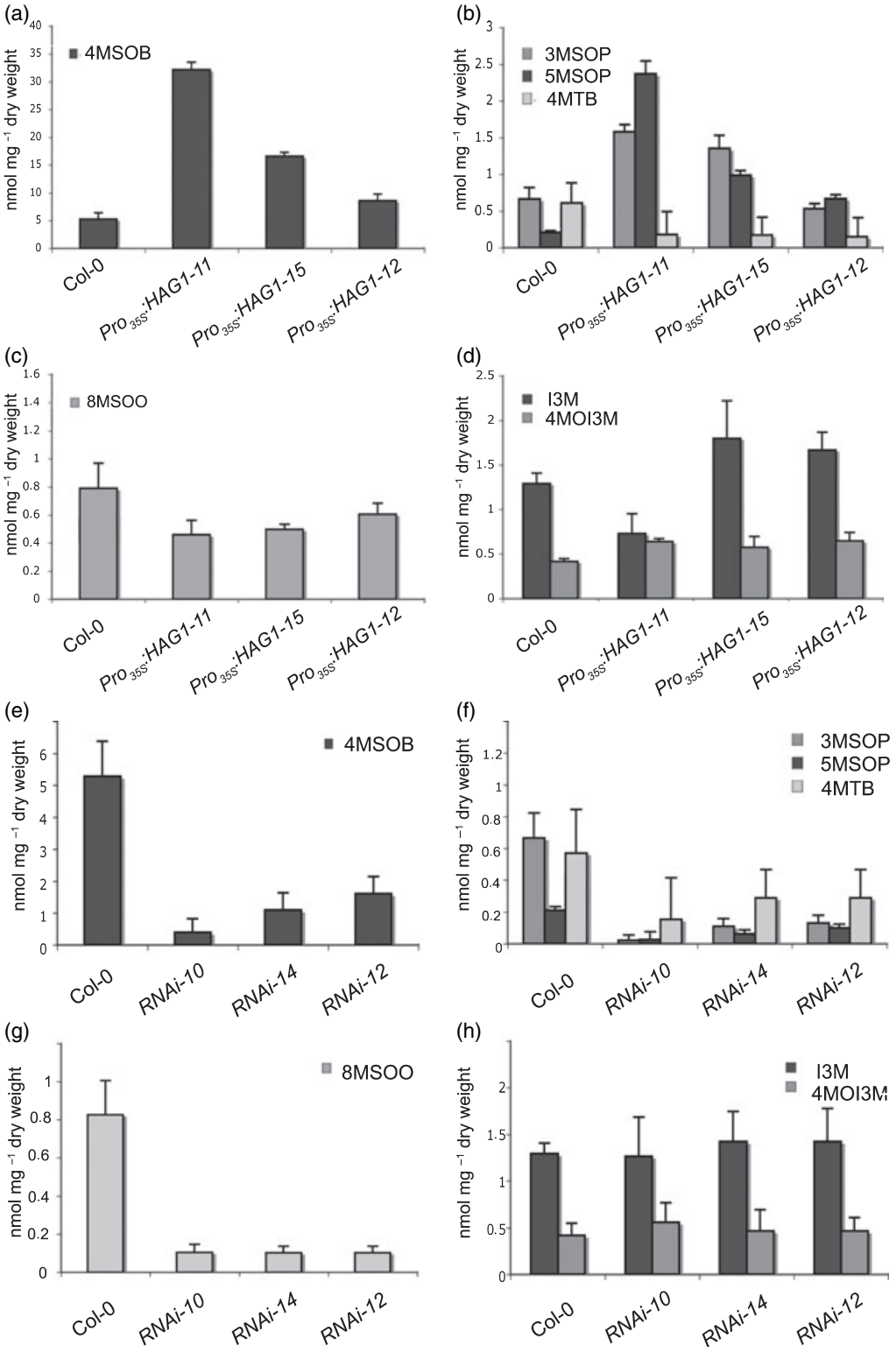

Figure 5. Glucosinolate contents in rosette leaves of 5-week-old *HAG1/MYB28* over-expression and RNAi plants.
(a–d) Glucosinolate contents (GS) in the *HAG1/MYB28*-over-expressing lines *Pro35S:HAG1-11*, *Pro35S:HAG1-15* and *Pro35S:HAG1-12*. Means and SD, $n = 3$.
(e–h) Glucosinolate contents (GS) in the *HAG1/MYB28* RNAi lines *HAG1-RNA1-10*, *-14* and *-12*. Means and SD, $n = 3$.
4MSOB, 4-methylsulfinylbutyl-GS; 3 MSOP, 3-methylsulfinylpropyl-GS; 5MSOP, 5-methylsulfinylpentyl-GS; 4MTB, 4-methylthiobutyl-GS; 8MSOO, 8-methylsulfinyloctyl-GS; I3M, indol-3-ylmethyl-GS; 4MOI3M, 4-methoxyindol-3-ylmethyl-GS.

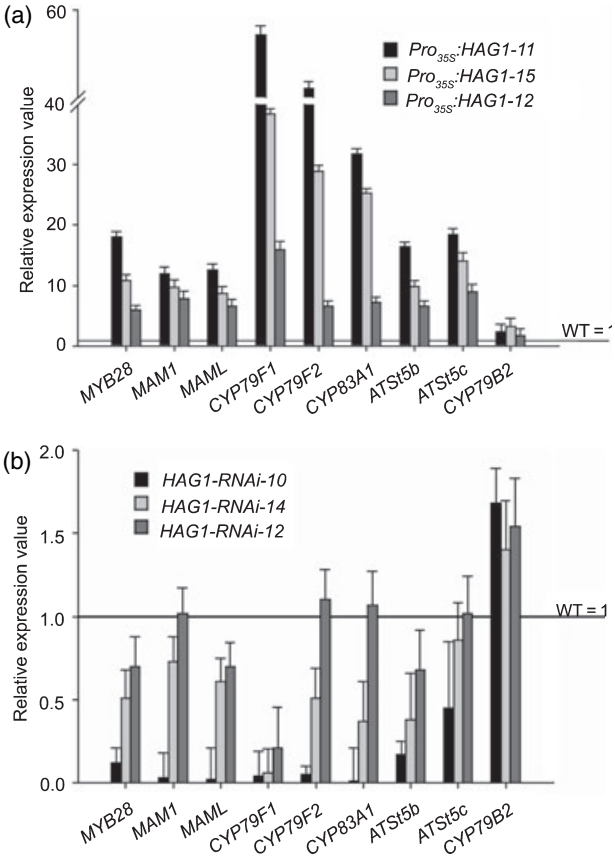

Figure 6. Transcript levels of glucosinolate pathway genes in rosette leaves of 5-week-old *HAG1/MYB28* over-expression and RNAi plants.
(a) Real-time RT-PCR analysis of aliphatic glucosinolate pathway genes measured in over-expression lines Pro_{35S}*:HAG1-11*, Pro_{35S}*:HAG1-15* and Pro_{35S}*:HAG1-12*.
(b) Real-time RT-PCR analysis of aliphatic glucosinolate pathway genes measured in RNAi plants *HAG1-RNAi-10, -14* and *-12*.
Relative gene expression values are given compared to the wild-type (= 1). Values are means and SD (n = 3).

and in adult leaves, the sites of accumulation of aliphatic glucosinolates. These observations are consistent with AtGenExpress data from the Genevestigator microarray database (Zimmermann *et al.*, 2004; http://www.genevestigator.ethz.ch/).

Interestingly, *HAG1/MYB28* expression was also induced by touch and wounding (Figure 7k,l) as has been previously reported for IQD1 (Levy *et al.*, 2005). To obtain more information about the time-dependent induction of *HAG1/MYB28* by mechanical stimuli, leaves and inflorescences were harvested 1, 5, 15, 60, 90 and 120 min after wounding, and used for real-time PCR analysis. As shown in Figure 8, *HAG1/MYB28* expression rapidly increased about threefold after wounding (by 1 min), and decreased within 15 min to below the level of unwounded plants. Similarly, an increased steady-state transcript level of *CYP79F1* was observed after approximately 5 min of induction by wounding, returning to its original level after 15 min.

HAG1/MYB28 *hinders performance of the generalist* Spodoptera exigua

Mechanical stimuli- and herbivory-induced tissue damage linked to myrosinase activity causes glucosinolate degradation and the release of bioactive molecules, which are known to act as repellents against generalist insects (Giamoustaris and Mithen, 1995; Kliebenstein *et al.*, 2002; Lambrix *et al.*, 2001; Levy *et al.*, 2005).

To test whether increased *HAG1/MYB28* expression correlates with enhanced resistance to a generalist lepidopteran herbivore, the performance of *Spodoptera exigua* larvae (Lepidoptera: Noctuidae), which are known to feed on cruciferous and other plant species, was studied. The weight gain of 5-day-old larvae, first kept on an artificial diet, was examined in feeding experiments on both wild-type and *HAG1/MYB28*-over-expressing plants over 5 days. As shown in Figure 9, larvae developing on *HAG1/MYB28* over-expressor plants were significantly smaller and gained about 70% less fresh weight than larvae on wild-type plants within 5 days, indicating that over-expression of *HAG1/MYB28* significantly reduces herbivore performance.

HAG1/MYB28 *expression is induced by glucose but not by plant hormones*

Because glucosinolates are involved in various biotic defense responses, their regulation by multiple signal transduction pathways such as methyl jasmonate (MeJa), salicylic acid (SA) or the ethylene precursor aminocyclopropane carboxylate (ACC) has been suggested (Brader *et al.*, 2001; Cipollini *et al.*, 2004; Devoto and Turner, 2005; Kliebenstein *et al.*, 2002; Mewis *et al.*, 2005; Mikkelsen *et al.*, 2003; Sasaki-Sekimoto *et al.*, 2005). Glucose is another important signaling molecule that may induce transcriptional regulatory mechanisms integrating carbohydrate availability and hormone action. We used Pro_{HAG1}*:uidA* plants to study whether or not *HAG1/MYB28* expression responds to these signaling molecules.

Two-week-old seedlings were transferred for 24 h to a sucrose-free medium for adaptation to autotrophic conditions. Subsequently, MeJa, SA, ACC or glucose were added, and plants were analysed for *HAG1/MYB28* expression after 4, 12 and 24 h of exposure. As shown in Figure 10, MeJa, SA and ACC did not exert any positive effect on *HAG1/MYB28* expression. Rather, exposure to SA even suppressed *HAG1/MYB28* expression after 4 h of treatment. On the other hand, *HAG1/MYB28* expression was significantly induced by glucose. Thus, glucose seems to be an important signalling molecule inducing *HAG1/MYB28* expression, indicating a special mechanism of transcriptional regulation of *HAG1/MYB28* in response to carbohydrate availability.

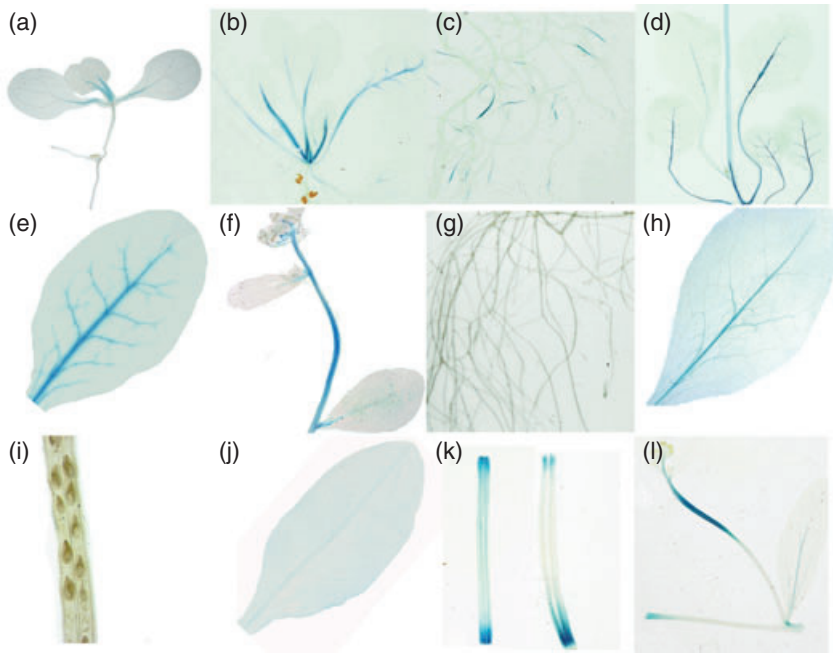

Figure 7. Histochemical GUS staining in tissues of Pro_{HAG1}:GUS plants.
(a) 14-day-old seedling. (b) Three-week-old plant. (c) Roots of three-to-four-week old plants. (d) Five-week-old plant. (e) Adult leaves. (f) Inflorescences of flowering plant. (g) Roots of adult plants. (h) Leaf of adult plant after transition from vegetative to generative stage. (i) Silique. (j) Leaf of senescent plants. (k) GUS induction at cut sites of inflorescences. (l) GUS induction at touch sites of inflorescences.

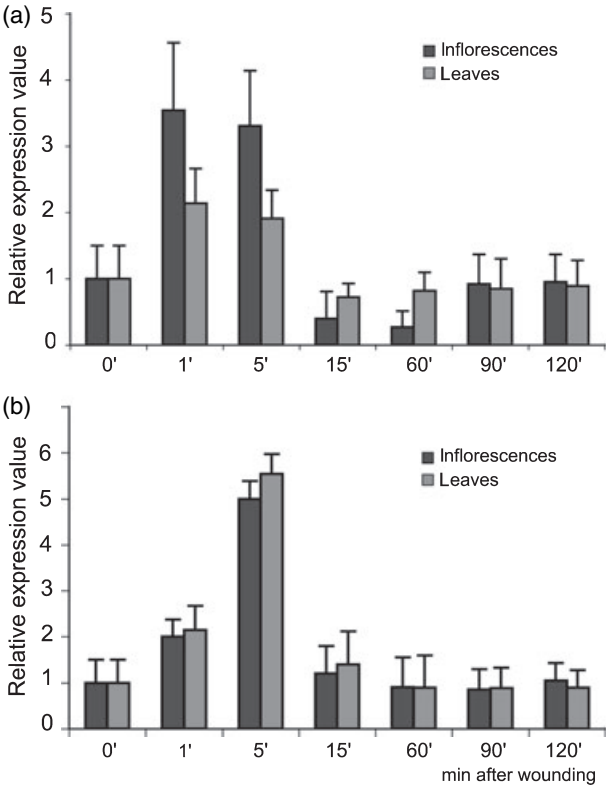

Figure 8. Transient expression of *HAG1/MYB28* (a) and *CYP79F1* (b) in leaves and inflorescences induced by wounding.
The plant material was punctured, and, after 1, 5, 15, 60, 90 and 120 min, used for real-time RT-PCR experiments (means and SD, $n = 3$). Relative expression values are given compared to non-wounded plants (= 1).

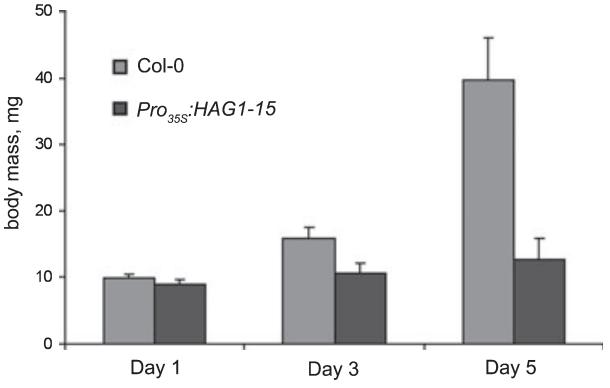

Figure 9. *HAG1/MYB28* over-expression reduces insect performance.
Weight gain assays of *Spodoptera exigua* larvae on 5-week-old *A. thaliana* plants. Larvae were kept on an artificial diet for 5 days and second-instar larvae were transferred to plants. Values are the mean fresh weight (and SE) of larvae feeding for 5 days on *A. thaliana* wild-type (Col-0) and Pro_{35S}:*HAG1-15* lines. Day 1 (Col-0 and Pro_{35S}:*HAG1-15*: $n = 15$ per line; *t* test, $P = 0.45$); day 3 (Col-0 and Pro_{35S}:*HAG1-15*: $n = 12$; *t* test, $P = 0.042$); day 5 (Col-0 and Pro_{35S}:*HAG1-15*: $n = 9$; *t* test, $P = 0.0016$).

Discussion

HAG1/MYB28 functions as a regulator of aliphatic glucosinolate biosynthesis and plant defense against herbivores

The biosynthetic pathways leading to the great diversity of glucosinolates have been elucidated in the past years, including identification of almost the whole set of corresponding biosynthetic genes (Grubb and Abel, 2006; Halkier

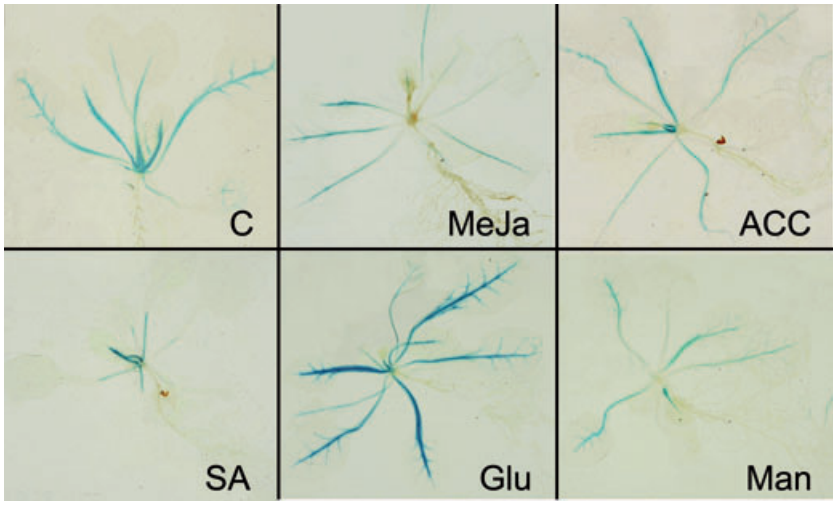

Figure 10. *HAG1/MYB28* expression is induced by glucose.
Elicitor-treated Pro_{HAG1}*:uidA* plants are shown. C, water control; MeJa, 0.45 mM methyl jasmonate; ACC, 10 μM 1-aminocyclopropane carboxylate; SA, 0.2 mM salicylic acid; Glu, 1% glucose. Mannitol (Man, 1%) was used as an osmotic control.

and Gershenzon, 2006). However, the first regulatory factors directly or indirectly activating glucosinolate pathway genes have only recently been described, e.g. IQD1, ATR1/MYB34, AtDof1.1 and HIG1/MYB51 (Levy *et al.*, 2005; Celenza *et al.*, 2005; Skirycz *et al.*, 2006; Gigolashvili *et al.*, in press). HIG1/MYB51 was shown to specifically regulate indolic glucosinolate biosynthesis (Gigolashvili *et al.*, in press), whereas the other genes are either involved in the control of both aliphatic and indolic glucosinolates or activate genes of the indolic glucosinolate pathway along with the biosynthesis of auxin, mainly because the indolic glucosinolate and indole-3-acetic acid (IAA, auxin) biosynthetic pathways share indole-3-acetaldoxime as a common intermediate. Nothing was known about the regulation of the aliphatic methionine-derived glucosinolates that play important roles in the plant defense response against herbivores and pathogens.

Here, we show that HAG1/MYB28 is involved in the specific regulation of aliphatic glucosinolates. Several lines of evidence indicate that HAG1/MYB28 functions as a key regulator of aliphatic glucosinolate biosynthesis and plant defense against herbivores.

First, an increase and/or decrease in glucosinolate accumulation nicely corresponded to the level of *HAG1/MYB28* transcripts in both the various over-expression lines as well as the RNAi lines (Figures 5 and 6). *HAG1/MYB28*-over-expressing lines contained up to sevenfold higher levels of short-chain aliphatic glucosinolates, especially of the major aliphatic glucosinolate, 4MSOB. These effects were restricted to aliphatic methylsulfinylglucosinolates; the levels of methylthioglucosinolates and indolic glucosinolates remained almost unaffected in plants with moderate expression of HAG1/MYB28 (Figure 5). However, lines strongly over-expressing *HAG1/MYB28* and containing high levels of methionine-derived glucosinolates (Pro_{35S}*:HAG1-11*, Figure 5d) showed a decrease in the content of the main indolic glucosinolate I3M. This observation may result from metabolic cross-talk between the different branches of glucosinolate biosynthesis. Alternatively, and as proposed by Grubb and Abel (2006), the competition of cytochrome P450 mono-oxygenases involved in either the aliphatic or indolic glucosinolate biosynthetic pathways for electrons could be the reason for reciprocal negative feedback regulation between both branches of glucosinolate biosynthesis. Interestingly, the *CYP79F1* null mutant (*bus1-1f*) that completely lacks short-chain aliphatic glucosinolates accumulated increased levels of indolic glucosinolates (Chen *et al.*, 2003; Reintanz *et al.*, 2001). These observations provide additional evidence for an interplay between both biosynthetic pathways.

The levels of indolic glucosinolates in *HAG1* RNAi plants were also unchanged, but those of aliphatic glucosinolates were significantly reduced. In contrast to mutants detective in CYP79F1 or CYP79F2 functions (Tantikanjana *et al.*, 2004), HAG1 RNAi plants did not completely lack aliphatic glucosinolates. This may be due to either the only partial knock-down of *HAG1/MYB28* in *HAG1* RNAi plants and/or complementation of HAG1/MYB28 function by other transcription factors with similar roles, e.g. HAG2/MYB76 or HAG3/MYB29. However, both HAG2/MYB76 and HAG3/MYB29 were apparently unable to fully compensate for the reduced HAG1/MYB28 activity in *HAG1* RNAi lines. A number of scenarios may be considered to explain this observation. The three transcription factors may have distinct sites of expression or could functionally act only in concert with other (transcription) factors and may thus differ in their *trans*-activation potential toward glucosinolate biosynthetic genes. As revealed by the Genevestigator database (Zimmermann *et al.*, 2004), HAG2/MYB76 shows a similar expression pattern as HAG1/MYB28, and should, in the case of functional redundancy, be able to complement the deficiency in HAG1/MYB28 activity. However, and in contrast to HAG1/MYB28, HAG2/MYB76 does not appear to

be co-regulated with glucosinolate biosynthetic genes such as *BCAT4*, *MAM1*, *CYP79F1* and *CYP83A1* (Toufighi *et al.*, 2005, http://bbc.botany.utoronto.ca). It may be functional as an enhancer of the expression of aliphatic glucosinolate biosynthetic genes, but only in concert with other factors, e.g. HAG1/MYB28. Future work will focus on understanding of the regulatory mechanism underlying the spatial and temporal *trans*-activation potential of HAG2/MYB76 and HAG3/MYB29 in comparison with that of HAG1/MYB28.

Second, using co-transformation assays, it could be demonstrated that HAG1/MYB28 specifically activates genes of the aliphatic glucosinolate biosynthetic pathway, i.e. *MAM1*, *MAM3*, *CYP79F1*, *CYP79F2*, *CYP83A1*, *AtST5b* and *AtST5c*, without affecting genes involved in the indolic glucosinolate pathway, e.g. *TSB1*, *CYP79B2*, *CYP79B3* (Figures 2 and 6). Thus, this list of target genes encompasses genes involved in the first reactions of aliphatic glucosinolate biosynthesis up to the very last steps (Figure 1). It may be noted that the expression patterns of these genes overlap with those of *HAG1/MYB28* (http://bbc.botany.utoronto.ca; Toufighi *et al.*, 2005). *HAG1* RNAi plants, on the other hand, contained lower transcript levels of aliphatic glucosinolate pathway genes. Remarkably, AtDof1.1 and IQD1 did not possess any *trans*-activation potential towards promoters of aliphatic and indolic glucosinolate biosynthetic genes, indicating that these proteins are not able to directly activate glucosinolate biosynthetic genes, i.e. that additional factors are required to exert activation of glucosinolate biosynthesis. It may be noted that the recently discovered regulators of the indolic glucosinolate pathway, ATR1/MYB34 and HIG1/MYB51 (Celenza *et al.*, 2005; Gigolashvili *et al.*, in press), specifically activate indolic but not aliphatic glucosinolate biosynthetic genes (Figure 2).

Third, *HAG1/MYB28* expression overlaps well with the expression of aliphatic glucosinolate biosynthetic genes (Figure 7). For example, *BCAT4* and *CYP79F1* show a strong expression in the vasculature of young and mature rosette leaves and in stems (Chen *et al.*, 2003; Reintanz *et al.*, 2001; Schuster *et al.*, 2006). This observation is consistent with the role of *HAG1/MYB28* as a positive regulator of this pathway. However, expression of glucosinolate biosynthesis genes often appeared to be present in non-photosynthetic organs such as flowers and seeds, in which *HAG1/MYB28* is not expressed. To provide reproductive organs with glucosinolates, long-distance transport of glucosinolates from photosynthetic tissues to siliques is envisaged (Brudenell *et al.*, 1999; Chen and Andreasson, 2001). Remarkably, the expression of *HAG1/MYB28* overlaps not only with the expression of glucosinolate biosynthetic genes, but also covers the main sites of methionine-derived glucosinolate accumulation. Young rosette leaves exhibit a high concentration of aliphatic glucosinolates, which declines during maturation and flowering, resulting in a predominance of indolic glucosinolates in rosette leaves (Brown *et al.*, 2003). This distribution correlates well with the switch in *HAG1/MYB28* expression during transition from the vegetative to the generative phase of development. Starting with flowering, aliphatic glucosinolates are found mainly in generative organs, which are also the main sites of *HAG1/MYB28* expression.

Finally, *HAG1/MYB28* expression is very rapidly induced by mechanical stimuli, resulting in increased levels of aliphatic glucosinolates (Figure 8). Moreover, weight gain assays using larvae of the generalist herbivore *Spodoptera exigua* showed that larvae fed with *HAG1/MYB28*-over-expressing plants gained markedly less weight as compared to larvae fed with wild-type plants (Figure 9). Thus, over-expression of *HAG1/MY28* reduces herbivore performance.

Pro$_{35S}$*:HAG1* plants exhibited, dependent on the strength of over-expression, retardations in growth, whereas *HAG1* RNAi plants with lowered levels of aliphatic glucosinolates have a wild-type appearance (Figure 4). We occasionally observed that some *HAG1/MYB28* over-expression lines with extremely increased levels of aliphatic glucosinolates and decreased levels of I3M and auxin exhibited a drastic retardation in growth. These plants were unable to produce seeds and could not be further analysed.

HAG1/MYB28 *expression is induced by glucose but not by MeJa, SA and ACC*

Environmental stimuli such as herbivore attack, wounding or hormone treatment (e.g. MeJa, SA, ACC) are known to have an impact on glucosinolate regulation, and several glucosinolate biosynthesis pathway genes have been shown to be induced by these treatments (Brader *et al.*, 2001; Kliebenstein *et al.*, 2002; Mewis *et al.*, 2005; Mikkelsen *et al.*, 2003). Although *HAG1/MYB28* expression clearly responds to mechanical stimuli, as has also been shown for *IQD1*, *BCAT4* and *MAM1* (Levy *et al.*, 2005; Schuster *et al.*, 2006), none of the tested plant hormones were able to induce *HAG1/MYB28* expression (Figure 10), indicating that *HAG1/MYB28* may function independently of these hormones. However, *HAG1/MYB28* expression clearly responded to sugars, e.g. glucose, suggesting that HAG1/MYB28 is a component of regulatory mechanisms integrating signals for carbohydrate availability (Li *et al.*, 2006; Zimmermann *et al.*, 2004). Glucose is released during myrosinase action on glucosinolates, thus possibly generating a signal for *HAG1/MYB28* induction. It has been generally shown that biotic stress, wounding or infection by viruses, bacteria and fungi can modulate the carbohydrate status of plant tissues. An extracellular invertase appears to play a prominent role in carbon partitioning and to integrate sugar, stress and hormone signals (Roitsch, 1999; Rolland *et al.*, 2002; Sonnewald *et al.*, 1991; Sturm, 1999). As shown previously, sugars can regulate expression of the wound-inducible proteinase inhibitor II, lipoxygenase genes (Johnson and

Ryan, 1990; Sadka *et al.*, 1994) and pathogenesis-related genes (Herbers *et al.*, 1996; Xiao *et al.*, 2000). Remarkably, analysis of microarray data for *A. thaliana* seedlings led to the identification of *HAG1/MYB28* as one of the very rapidly up-regulated genes in response to glucose treatment (Li *et al.*, 2006). In accordance with this observation, the *HAG1/MYB28* promoter contains several glucose regulated motifs (e.g. AAACCCTAA, GTTAGGTT, RCCGAC), as listed by Li *et al.* (2006).

HAG1/MYB28 is obviously a key player in a signaling pathway connecting external signals such as mechanical stimuli to glucose availability and further to the activation of transcriptional machinery for the production of aliphatic glucosinolates, promoting sustained plant resistance against herbivores.

Experimental procedures

Plant materials and growth conditions

Plants (*Arabidopsis thaliana* ecotype Columbia) and corresponding gain-of-function and loss-of-function mutants were grown in a temperature-controlled greenhouse under a 16 h light/8 h dark regime or in a growth chamber under a 8 h light/16 h dark regime at day/night temperatures of 21°C/18°C and at 40% humidity. For selection of kanamycin-resistant plants, seeds were surface-sterilized and plated on agar-solidified half-strength Murashige and Skoog (MS) medium containing 2.15 g/l MS salts, 0.5% sucrose, pH 5.8. Plates were grown in a culture chamber (16 h light periods, 75% humidity, 21°C).

Arabidopsis thaliana *cell culture*

An *A. thaliana* Col-0 suspension-cultured cell line generated from roots was maintained in 50 ml of *A. thaliana* (AT) medium (Mathur *et al.*, 1998). The AT medium contained 4.3 g/l MS basal salt medium (Duchefa; http://www.duchefa.com), 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 4 ml vitamin B5 mixture (Sigma; http://www.sigmaaldrich.com/) and 30 g/l sucrose (pH 5.8). Cells were gently agitated at 160 rpm in the dark at 22°C.

Co-transformation assays

To generate reporter constructs, the promoter regions of the aliphatic glucosinolate biosynthetic genes *MAM1* (−1739 to +270 bp), *CYP79F1* (−1388 to +45 bp) and *CYP83A1* (−919 to +63 bp), and of indolic glucosinolate biosynthesis genes *TSB1* (−2320 to +132), *CYP79B2* (−1383 to +81 bp) and *CYP79B3* (−1353 to +84 bp) were amplified from genomic DNA of *A. thaliana* plants and cloned into the pDONOR-207 vector (Invitrogen Life Technologies; http://www.invitrogen.com/). To drive *Agrobacterium*-mediated expression of the *uidA* (GUS) gene under the control of these promoters, the binary plant transformation vector pGWB3i containing an intron within the GUS gene was generated. A 189 bp intron fragment was amplified from pPCV 6NFHyg GUS Int vector (Vancanneyt *et al.*, 1990) using a proof-reading polymerase. The blunt-ended PCR product was ligated to the pGWB3 vector using the *Sna*BI restriction site. The pGWB3i vector was recombined with the pDONOR-207 vector containing corresponding promoter sequences using LR reactions (Invitrogen Life Technologies). Gateway entry clones for creating reporter constructs for *CHS* and *FLS* and for the generation of an effector construct for MYB12 were kindly provided by R. Stracke (University of Bielefeld, Germany).

To generate effector constructs, the coding sequences of *HAG1/MYB28*, *HAG2/MYB76*, *HAG3*/MYB76, *HIG1/MYB51* and *ATR1/MYB34* were amplified by RT-PCR and cloned into the pDONOR207 vector. To recombine the insert from the entry clone into the destination vector, LR reactions betweenpDONOR207 and pGWB2 clones were performed. The reporter and effector constructs were used to transform the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D.

For transient expression assays, *Agrobacteria* containing effector constructs, the anti-silencing 19 K protein (Voinnet *et al.*, 1999) and each of the reporter constructs were taken from fresh YEB plates, grown overnight, resuspended in 1 ml of *A. thaliana* (AT) medium and used for the transformation of dark-grown cultured *A. thaliana* Col-0 cells generated from roots (Mathur *et al.*, 1998). To estimate the *trans*-activation potential, three clones of *Agrobacterium*, containing effector, reporter and anti-silencing constructs (19 K) were mixed in a 1:1:1 ratio, and 75 μl of this suspension was added to 3 ml of cultured *A. thaliana* cells, grown for 3–5 days in the dark and stained afterwards with X-Gluc. GUS activity could be analysed earlier, but reliable results were obtained at later time points.

Generation of transgenic HAG1/MYB28 *over-expression and RNAi plants*

To generate *HAG1/MYB28* over-expression plants, the *Pro$_{35S}$:HAG1* construct, also used in *trans*-activation assays, was transformed into *A. tumefaciens* (strain GV3101) by electroporation and further into *A. thaliana* plants by vacuum infiltration.

Constructs for silencing of the *HAG1/MYB28* gene were generated using a Gateway-based pJawohl17 vector kindly provided by I. Somssich (Max Planck Institute for Plant Breeding Research, Cologne, Germany). First, a suitable target region for silencing of the *HAG1/MYB28* gene was selected. Two complementary 5′ → 3′ and 3′ → 5′ *HAG1/MYB28* fragments were amplified from a wild-type cDNA using Gateway *att*B1- and *att*B2-extended primers (attB1-TTAATGGCTTCACTGAGCAGATTC; attB2-TGATGAGACTTC-TTGGGAAACATC) and cloned into the pDONOR-207 vector. To recombine inserts from the entry clone into the pJawohl17 destination vector supplied with two recombination cassettes, LR reactions between two pDONR-207 clones and the pJawohl17 destination clones were performed. The entire *HAG1-pJawohl17* silencing construct was transformed into the Gateway-compatible *A. tumefaciens* strain GV3101 by electroporation and into *A. thaliana* plants by vacuum infiltration. All transformants were selected with kanamycin, and the *HAG1/MYB28* transcript level was monitored by quantitative real-time PCR analysis.

Construction of the Pro$_{35S}$*:HAG1:GFP fusion plasmid and transformation of cultured* A. thaliana *cells*

To generate an expression clone with the *Pro$_{35S}$:HAG1:*GFP fusion, the pDONOR-207 vector containing the *HAG1/MYB28* coding sequence was recombined with the pGWB5 destination vector using an LR reaction. Transformation of dark-grown cultured *A. thaliana* cells was performed using the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D (kindly provided by Dr Memelink, University of Leiden, The Netherlands) as described by Koroleva

et al. (2005). GFP expression patterns and DAPI (4,-6-diamidino-2-phenylindole) staining of the nucleus were recorded using a fluorescence microscope (Eclipse E800, Nikon; http://www.nikon.de).

Histochemical analysis of transgenic plants expressing the Pro$_{HAG1}$*:uidA fusion construct*

The *HAG1/MYB28* promoter region (−1995 to +157 bp) was amplified from genomic DNA of *A. thaliana* plants and cloned into the pDONOR-207 vector. To drive expression of the *uidA* gene under the control of the *HAG1/MYB28* promoter, the binary Gateway-compatible plant transformation vector pGWB3 was recombined with the *HAG1/MYB28* entry clone using an LR reaction. The *Pro$_{HAG1}$:uidA* clone in pGWB3 was transformed into the GV3101 *Agrobacterium* strain and *A. thaliana* wild-type plants.

Histochemical localization of GUS in several independent transgenic lines harboring the *Pro$_{HAG1}$:uidA* construct was performed as described by Jefferson *et al.* (1987) with some modifications. Sample tissues were infiltrated with the reaction buffer [50 mM Na_2HPO_4-NaH_2PO_4, pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, containing 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) as substrate] under vacuum, and incubated at 37°C overnight. Plant pigments were destained with 80% ethanol, and the GUS staining patterns were recorded under a binocular microscope (SMZ-U, Nikon).

Preparation of methanolic extracts and HPLC analysis of glucosinolates

Leaves (50–100 mg) were placed into a 2 ml reaction tube and frozen in liquid nitrogen. Frozen leaf samples were lyophilized and homogenized in an MM301 mill (Retsch; http://www.retsch.com). Glucosinolates were extracted in 80% methanol after addition of 20 μl of 5 mM benzyl glucosinolate as an internal standard (http://www.glucosinolates.com). Extracts were applied onto a DEAE Sephadex A25-column (0.1 g powder equilibrated in 0.5 M acetic acid/NaOH, pH 5). Glucosinolates were converted to desulfoglucosinolates by overnight incubation with a purified sulfatase (E.C. 3.1.6.1) designated 'type H-1, from *Helix pomatia*, 16 400 units/g solid' (Sigma). For analysis of desulfoglucosinolates, samples were subjected to HPLC analysis on a Hewlett-Packard 1100 series chromatograph with a quaternary pump and a 1040M diode array detector and using a Supelco C-18 column (Supelcosil LC-18, 250 × 4.6 mm, 5 μm internal diameter; http://www.sigmaaldrich.com). Elution was accomplished with a gradient of 0–5% solvent B (10 min), 5–38% solvent B (24 min), followed by a cleaning cycle (38–100% solvent B in 4 min, 6 min hold, 100 to 0% solvent B in 5 min, 7 min hold) (solvent A, water; solvent B, methanol). Peaks were quantified by the peak area at 229 nm (bandwidth 4 nm) relative to the area of the internal standard peak, applying the response factors as used by Brown *et al.* (2003). The identities of the desulfo derivatives of glucosinolates were confirmed by LC-ESI-MS as described previously (van Dam *et al.*, 2003; Müller and Martens, 2005). Three different technical replicates and two independent sets of plants were used for the analyses.

Reverse transcriptase-mediated first-strand synthesis and real-time RT-PCR analysis

Total RNA was extracted from rosette leaves of adult plants from different mutant lines using TRIsure buffer (Biolone; http://www.biocompare.com) followed by treatment with RNase-free DNase (Roth; http://www.carl-roth.de) to remove genomic DNA contaminations. Total RNA (7–10 μg) was reverse-transcribed using the First-Strand cDNA Synthesis SSII Kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

The expression of glucosinolate biosynthesis genes was analysed by real-time quantitative RT-PCR analysis using the fluorescent intercalating dye SYBR Green in a GeneAmp® 5700 sequence detection system (Applied Biosystems; http://www.appliedbiosystems.com/). The Arabidopsis *ACTIN2* gene was used as a standard. 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 according to the manufacturer's instructions. Subsequently, the cDNAs were used as a template in real-time PCR experiments with gene-specific primers (for primer sequences, see Table 1). Real-time PCR was performed using the SYBR Green master kit system (Applied Biosystems) according to the manufacturer's instructions. The C_t, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is detected, is used as a measure of the starting copy number of the target gene. Relative quantification of expression levels was performed using the comparative C_t method (manufacturer's instructions, bulletin 2, Applied Biosystems). The relative value for the expression level of each gene was calculated by the equation $Y = 2^{-\Delta\Delta Ct}$, where ΔC_t is the difference between control and target products ($\Delta C_t = C_{t(GENE)} - C_{t(ACT)}$), and $\Delta\Delta C_t = \Delta C_{t(mutant)} - \Delta C_{t(wt)}$. Thus, the calculated relative expression values are normalized to the wild-type expression level (wild-type = 1). The efficiency of each primer pair was tested using wild-type (Col-0) cDNA as a standard template, and the RT-PCR data were normalized according to the relative efficiency of each primer pair. Three different technical replicates and two independent sets of plants were used for the analyses.

Elicitor treatments

Several independent transgenic plants harboring the *Pro$_{HAG1}$:*GUS construct were grown for 7 days on a half-strength Murashige and Skoog agar plates containing 0.5% of sucrose in a 22–24°C growth chamber with a 16 h light/8 h dark cycle. Before elicitor induction, plants were adapted to autotrophic growth conditions in sucrose-

Table 1 Primers used in real-time RT-PCR experiments

Primer name	Primer sequence (5′ → 3′)
RL-MYB28sh F	TCCCTGACAAATACTCTTGCTGAAT
RL-MYB28sh R	CATTGTGGTTATCTCCTCCGAATT
RL-MAM1 F	CGGCTGAAAGAGTGGGGATATGA
RL-MAM1 R	CGTTAGCGCCGTTTAATTTCTC
RL-MAM3 F	GAGAAATTGAACGCTGTCTTCTCAC
RL-MAM3 R	AGCCGTTAGACTTTAAACCGTTAGC
RL-CYP79F1 F	CCATACCCTTTTCACATCCTACTAGTCT
RL-CYP79F1 R	GTAGATTGCCGAGGATGGGC
RL-CYP79F2 F	ACTAGGATTTATCGTCTTCATCGCA
RL-CYP79F2 R	CTAGGACGAGTCATGATTAGTTCGG
RL-CYP83A1 F	TTCAAGAGGTTGTCAATGAGACGC
RL-CYP83A1 R	CTACAATATCCAAGATGACGGCTTT
RL-AtST5b F	GGAATCCAAAACCATAAACGACG
RL-AtST5b R	CGGATCTTTTGGTCTCCAGCC
RL-AtST5c F	CCCTACCGAGTCACGACGAGA
RL-AtST5c R	GGTAGCCACCAGTAACCACCATACT
Actin 2 F	TAACTCTCCCGCTATGTATGTCGC
Actin 2 R	CCACTGAGCACAATGTTACCGTAC

free liquid MS culture. For induction, methyl jasmonate (MeJA, 0.45 mM), 1-aminocyclopropane carboxylate (ACC, 10 μM), salicylic acid (SA, 0.2 mM), glucose (1% w/v) or mannitol (1% w/v) were added. After 4, 12 and 24 h, the plants were histochemically analysed for GUS activity.

Weight gain assays with the generalist herbivore Spodoptera exigua

Eggs of the lepidopteran herbivore, *Spodoptera exigua* (Lepidoptera: Noctuidae), were obtained from Bayer CropScience (Monheim, Germany), and larvae were kept on an artificial diet for 5 days. Second-instar larvae (15 per line) were taken and transferred to 5-week-old plants of either wild-type or *HAG1/MYB28*-over-expressing plants that had been grown in soil under short-day conditions (8 h light/16 h dark). Larvae were kept on plants at 27°C and a 12 h light/12 h dark cycle. After 1, 3 and 5 days of feeding, the fresh weights of larvae were individually determined. Student's *t*-tests were performed to compare larval weights on both plant lines.

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