HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*

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Summary

• In a previous *trans*activation screen, two *Arabidopsis thaliana* R2R3-MYB transcription factors, HAG2/MYB76 and HAG3/MYB29, along with the already characterized HAG1/MYB28, were identified as putative regulators of aliphatic glucosinolate biosynthesis.

• Molecular and biochemical characterization of HAG2/MYB76 and HAG3/MYB29 functions was performed using transformants with increased or repressed transcript levels. Real-time PCR assays, cotransformation assays and measurements of glucosinolate contents were used to assess the impact of both MYB factors on the steady-state level of glucosinolate biosynthetic genes and accumulation of aliphatic glucosinolates.

• Both HAG2/MYB76 and HAG3/MYB29 were shown to be positive regulators of aliphatic glucosinolate biosynthesis. Expression of promoter-β-glucuronidase (GUS) fusions indicated GUS activities in both vegetative and generative organs, with distinct characteristics for each MYB factor. HAG1/MYB28, HAG2/MYB76 and HAG3/MYB29 reciprocally *trans*activated each other in the control of aliphatic glucosinolate biosynthesis and downregulated the expression of genes involved in the control of indolic glucosinolate biosynthesis, pointing to a reciprocal negative regulation of these two pathways.

• All three HAG transcription factors exert a coordinated control on aliphatic glucosinolate biosynthesis.

Key words: glucosinolates, MYB transcription factors, methyl jasmonate-responsive genes, regulatory network, *trans*activation.

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Introduction

Glucosinolates comprise a wide array of amino acid-derived secondary metabolites mainly produced in plants of the family *Brassicaceae*, including *Arabidopsis thaliana* (Kliebenstein *et al.*, 2001; Mithen, 2001). These compounds and their breakdown products help to protect plants against generalist herbivores (Giamoustaris & Mithen, 1995; Reymond *et al.*, 2004; Mewis *et al.*, 2005) and microorganisms (Mari *et al.*, 1996; Manici *et al.*, 2000; Chung *et al.*, 2005; Brader *et al.*, 2006). In *A. thaliana*, the two most abundant glucosinolate types are aliphatic glucosinolates, derived from methionine, and indolic glucosinolates, derived from tryptophan. Aliphatic glucosinolates make up the majority of the glucosinolate pool in *A. thaliana* tissues, with the exception of roots and late-stage rosette leaves, where the indolic glucosinolates make up nearly half the pool (Brown *et al.*, 2003).

Aliphatic and indolic glucosinolate metabolism has been studied extensively in *A. thaliana* and most of the enzymes in the biosynthetic pathway are now characterized. Aliphatic

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glucosinolate biosynthesis begins in the cytosol where methionine is deaminated by BCAT4 (Schuster *et al.*, 2006). The resulting α-keto acid is then imported into the chloroplast, where it reacts with acetyl-CoA in a condensation reaction, catalysed by MAM enzymes, followed by isomerization and oxidative decarboxylation, yielding a homoketo acid with one additional carbon in the side chain. This homoketo acid can then enter a new condensation cycle, thus creating homoketo acids of increasing side-chain length (Grubb & Abel, 2006; Halkier & Gershenzon, 2006). Upon side-chain elongation, the methionine derivatives are transaminated to form chain-elongated methionine.

Synthesis of the methionine (Met)-derived glucosinolate core structure proceeds in a series of reactions catalysed by cytochrome P_{450} monooxygenases, *C–S* lyases, *S*-glucosyl-transferases and sulfotransferases (reviewed by Grubb & Abel, 2006). Subsequent side-chain modifications of the glucosinolates further contribute to the large variability in this group of molecules (Fahey *et al.*, 2001). In addition to most of the core catalytic enzymes, both global and specific regulators of glucosinolate biosynthesis have been characterized. IQD1 is a calmodulin-binding regulator that upregulates genes in the indolic glucosinolate pathway after wounding or mechanical stimuli of the plant tissue (Levy *et al.*, 2005). By contrast, SLIM1 negatively regulates both aliphatic and indolic biosynthesis pathways under sulfur-limiting conditions. SLIM1 also mediates downregulation of *ATR1/MYB34* (Maruyama-Nakashita *et al.*, 2006) which, like *HIG1/MYB51* and *HIG2/MYB122*, code for specific regulators of the indolic glucosinolate pathway. ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122 directly upregulate several of the main enzymes of the indolic glucosinolate pathway (Celenza *et al.*, 2005; Gigolashvili *et al.*, 2007a). Conversely, HAG1/MYB28 is a transcription factor that directly activates genes of the aliphatic glucosinolate pathway, as also suggested for HAG3/MYB29 (Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007; these authors named MYB28 and MYB29 as PMG1 and PMG2, Production of Met-derived Glucosinolates). Another characterized regulator is Dof1.1, which upregulates expression of *CYP83B1* and promotes glucosinolate accumulation (Skirycz *et al.*, 2006). The four MYB factors described above (HAG1/MYB28, HAG3/MYB29, ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122) all belong to subgroup 12 of *A. thaliana* R2R3-MYB transcription factors (Stracke *et al.*, 2001).

Here we describe the characterization of two proteins from this subgroup, HAG3/MYB29 and HAG2/MYB76. We show that they both enhance aliphatic glucosinolate production by activating key biosynthetic genes of this pathway both *in trans* and *in planta*. Furthermore, the low basic transcript level of *HAG2/MYB76* was considerably induced by HAG1/MYB28, HAG3/MYB29 and by HAG2/MYB76 itself, and additionally in response to wounding, whereas *HAG3/MYB29* transcription was less induced by HAG1/MYB28 and HAG2/MYB76, but responded to methyl jasmonate (MeJA) treatment. We therefore suggest that *HAG2/MYB76* integrates the different stress responses in aliphatic glucosinolate regulation, and that *HAG3/MYB29* is more important for keeping a basic level of aliphatic glucosinolates both in nonstress situations and in response to environmental cues.

Materials and Methods

Plant growth and cell culture cultivation

Wild-type seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia were sterilized and plated on half-strength Murashige and Skoog (MS) medium with agar (2.15 g l^{-1} MS salts, 0.5% sucrose, 0.8% agar pH 5.8), cold-treated at 4°C for 3 d in the dark, then germinated in a culture chamber at 16 : 8 h light : dark cycle, 75% humidity, 21°C. Seedlings were transferred to soil and grown under long-day (16 h light, 8 h dark) or short-day conditions (8 h light, 16 h dark) at 22–25°C and 40% humidity. Transgenic plants were selected by germination on half-strength MS medium with 50 μg ml^{-1} kanamycin and subsequently treated as wild-type plants.

Arabidopsis Col-0 suspension culture was grown in 50 ml *A. thaliana* (AT) medium (4.3 g l^{-1} MS basal salt media (Duchefa), 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 4 ml of a vitamin B5 mixture (Sigma) and 30 g l^{-1} sucrose pH 5.8). Suspension cell culture was diluted weekly to 1 : 4 or 1 : 5 with fresh AT media and gently agitated at 150 rpm in the dark at 22°C.

Generation of *Pro$_{35S}$:HAG2* and *Pro$_{35S}$:HAG3* gain-of-function plants and isolation of *hag2* and *hag3* T-DNA insertion mutants

The coding sequences (CDS) of the At5g07690 gene encoding MYB29 and of the At5g07700 gene encoding MYB76 were amplified by RT–PCR using *A. thaliana* leaf cDNA as a template. PCR products were then cloned into the Gateway Entry vectors (Invitrogen, Karlsruhe, Germany). The inserts were transferred from Entry clones into the pGWB2 destination vector (provided by T. Nakagawa, Shimane University, Japan) by LR recombination as described by the manufacturer (Invitrogen). The generated *Pro$_{35S}$:HAG2:pGWB2* and *Pro$_{35S}$:HAG3:pGWB2* constructs were transformed by electroporation into *Agrobacterium tumefaciens* strain GV3101 and finally into wild-type Col-0 plants using vacuum infiltration. About 55–60 transformants were selected on media containing kanamycin and verified by RT–PCR.

T-DNA insertion mutants of *HAG2/MYB76* (SALK line N55242 with the insertion in the first exon) and *HAG3/MYB29* (GABI-KAT line GK-040H12 harbouring T-DNA insertion in the third exon) were isolated, and the transcript level of disrupted genes verified by RT–PCR. Homozygous mutants were found to be complete knockouts of corresponding genes, as no *HAG2/MYB76* and *HAG3/MYB29* transcripts were detectable in these lines.

Glucosinolate extraction and HPLC/UPLC analysis of desulfoglucosinolates

Glucosinolates were extracted from approx. 100 mg thoroughly homogenized freeze-dried rosette leaves by washing twice with 1 ml 80% (v/v) methanol with one addition of 20 µl 5 mM benzyl glucosinolate as an internal standard. The two methanol extractions were combined and applied to DEAE Sephadex A-25 columns equilibrated with 0.5 M acetic acid/NaOH pH 5, and washed with 10 ml water and 4 ml 0.02 M acetic acid/NaOH pH 5. After addition of 50 µl purified *Helix pomatia* sulfatase (EC 3.1.6.1, type H-1, 16 400 U g^{-1}, Sigma, Deisenhofen, Germany), columns were sealed and left for overnight digestion. The resulting desulfoglucosinolates were eluted with 6 × 1 ml HPLC water. The eluate was lyophilized and resuspended in 300 µl HPLC water. Samples were analysed by HPLC on a 1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) or by an Acquity UltraPerformance LC (UPLC) system (Waters, Eschborn, Germany). For the HPLC analysis, 20 µl desulfoglucosinolates were applied on a Supelco C-18 column (Supelcosil LC-18, 5 µm, 250 × 4.6 mm; Hewlett-Packard) and eluted using the following elution program with solvents A (water) and B (methanol): 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, 100–0% solvent B in 5 min, 7 min hold). For UPLC analysis, 5 µl of sample was applied to an Acquity UPLC system (Waters) and separated on a BEH C18 column (1.7 µm; 2.1 × 150 mm; Waters) under a linear gradient elution program with solvent A (10% acetonitril in water) and solvent B (90% acetonitrile in water): 0–47% solvent B (6.5 min), 47–95% solvent B (6.6 min), hold 95% solvent B (6.7 min), and 100% solvent A (7 min). Elution was operated at 0.225 ml min^{-1} flow and a column temperature of 35°C. Detection was performed at 229 nm and quantified based on response factor and internal benzyl glucosinolate standard, as described previously (Gigolashvili *et al.*, 2007a).

Isolation of RNA and real-time RT–PCR

The expression of glucosinolate biosynthetic genes was analysed by real-time quantitative RT–PCR using the fluorescent intercalating dye SYBR Green in a GeneAmp 5700 sequence detection system (Applied Biosystems, Darmstadt, Germany). The Arabidopsis *ACTIN2* gene was used as a standard. First, total RNA was isolated using TRIsure (Bioline, Luckenwalde, Germany) and reverse-transcribed into cDNA, using the FirstStrand cDNA Synthesis SSII kit (Bioline) according to the manufacturer's instructions. Subsequently, the cDNA was used as a template in real-time PCR experiments with gene-specific primers (for primer sequences see Table 1 in Gigolashvili *et al.*, 2007b). Real-time PCR was performed using the SYBR Green master mix system (Applied Biosystems) according to the manufacturer's instructions. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is detected, was used as a measure of the transcript level of the target gene. Relative quantification of expression levels was performed using the comparative Ct method (manufacturer's instructions, bulletin 2, Applied Biosystems).

Cloning of promoters of glucosinolate biosynthesis genes and *trans*activation assays in cultured *A. thaliana* cells

To generate reporter constructs, the promoter regions of the aliphatic glucosinolate biosynthetic genes *MAM3* (–1739 to +270 bp), *MAM1* (–3074 to +429), *CYP79F1* (–1388 to +45 bp), *CYP79F2* (–562 to +1841), *CYP83A1* (–919 to +63 bp), C-S *lyase* (–2935 to +104) and of indolic glucosinolate biosynthesis genes *CYP79B2* (–1383 to +81 bp) and *ASA1* (–1210 to +96) were amplified from genomic DNA of *A. thaliana* plants and cloned into the Gateway entry vector (Invitrogen). The promoter sequences were then subcloned into the binary plant transformation vector pGWB3i (Berger *et al.*, 2007), resulting in reporter constructs. As effectors, the constructs *Pro_{35S}:HAG2:pGWB2* and *Pro_{35S}:HAG3:pGWB2* were used in addition to the previously described constructs *Pro_{35S}:HIG1:pGWB2* and *Pro_{35S}:HAG1:pGWB2* (Gigolashvili *et al.*, 2007a, 2007b). The reporter and effector constructs were used to transform the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D (kindly provided by Dr Memelink, University of Leiden, the Netherlands). For transient expression assays in the cell culture, *Agrobacteria* containing the effector constructs, the antisilencing 19-K protein or one of the reporter constructs were taken from fresh yeast extract broth (YEB) plates, grown overnight, and resuspended in 1 ml AT medium. The Agrobacteria were mixed in a 1 : 1 : 1 ratio, and 75 µl of this suspension was added to 3 ml cultured *A. thaliana* root cells, which were then grown for 3–5 or 7 d in the dark and subsequently used for GUS activity measurements or staining.

Transient cotransformation experiments using *Nicotiana benthamiana* plants

To estimate the *trans*activation potential of HAG1/MYB28, HAG2/MYB76 and HAG3/MYB29 towards promoters of *HAG* genes and of indolic glucosinolate regulators, transient coexpression experiments were performed with *N. benthamiana* plants. To generate reporter constructs, the promoter regions of aliphatic glucosinolate regulators *HAG1/MYB28* (–1995 to +157), *HAG2/MYB76* (–1726 to +275), *HAG3/MYB29* (–2368 to +81) and of indolic glucosinolate regulators *HIG1/MYB51* (–1676 to +342), *HIG2/MYB122* (–2332 to +1458), *ATR1/MYB34* (–2501 to +12) were recombined from Gateway Entry clones into the pGWB3i destination vector using LR clonase (Invitrogen). As effectors, *Pro_{35S}:HAG1:pGWB2*,

Pro$_{35S}$:HAG2:pGWB2 and *Pro$_{35S}$:HAG3:pGWB2* constructs, also used in *trans*activation assays in *A. thaliana* cells, were used.

Supervirulent Agrobacteria containing effector and reporter constructs and the antisilencing Agrobacteria strain 19K were taken from fresh YEB plates, grown overnight, sedimented, resuspended in 10 mM $MgCl_2$, 10 mM 2-(*N*-morpholine)-ethanesulphonic acid pH 5.6, and adjusted to an OD_{600} of 0.7–0.8. Two working solutions were prepared for each promoter. Working solution 1 contained a suspension with effector and reporter constructs together with the Agrobacteria strain 19K in a 1 : 1 : 1 ratio. Working solution 2 contained a suspension with an empty pGWB2 vector (without effector), reporter and the 19K Agrobacteria strain in a 1 : 1 : 1 ratio. Acetosyringon was added (0.15 mM, final concentration) and the suspensions were incubated for 2–4 h at 30°C. Three to five leaves of 1–2-month-old *N. benthamiana* plants were infiltrated with each working solution into abaxial side air space using a syringe, and sampled after 3–5 d infiltration for GUS activity measurements.

Generation of *Pro$_{HAG2}$:GUS* and *Pro$_{HAG3}$:GUS* plants and their histochemical analysis

To study tissue-specific gene expression *in planta*, the *Pro$_{HAG2}$:GUS:pGWB3i* and *Pro$_{HAG3}$:GUS:pGWB3i* constructs were transformed into *A. thaliana* plants using vacuum infiltration. Histochemical localization of GUS in several independent transgenic lines harbouring the *Pro$_{HAG2}$:GUS* and *Pro$_{HAG3}$:GUS* constructs 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-b-D-glucuronic acid (X-Gluc) as substrate) under vacuum, and incubated at 37°C overnight. Plant pigments were destained with 80% ethanol and the histochemical pattern was analysed using a binocular microscope (SMZ-U, Nikon).

Plant hormone induction and wounding experiments

Arabidopsis thaliana wild-type seedlings (Col-0) were grown on half-strength MS media with 0.5% agar and 0.5% sucrose for 10 d in a growth chamber at 21°C. Afterwards they were transferred to half-strength MS liquid media containing MeJA (10 μM) or salicylic acid (SA, 10 μM) and treated for 0, 2, 5, 15, 120 min or 24 h. Afterwards seedlings were placed in 2-ml reaction tubes, frozen in liquid nitrogen, and used for RNA isolation. Three independent sets of plants induced by these elicitors were used for analysis by real-time PCR.

For wounding experiments, inflorescences of Col-0 plants were slightly cut with a scalpel or blade. Samples were collected after 1, 5 15, 30, 60 and 120 min after treatment, immediately frozen in liquid N, and used for RNA isolation and real-time PCR analysis.

Results

Metabolite profiling of *A. thaliana* plants overexpressing *HAG2/MYB76* and *HAG3/MYB29*

To study the role of *HAG2/MYB76* (At5g07700) and *HAG3/MYB29* (At5g07690) *in planta*, 35S CaMV overexpression *A. thaliana* plants were generated. Ten independent transgenic lines with stable gene expression were analysed, and three representative overexpression lines for both *HAG2/MYB76* (*Pro$_{35S}$:HAG2-5*, *-6*, *-23*) and *HAG3/MYB29* (*Pro$_{35S}$:HAG3-6*, *-12*, *-42*) with various steady-state mRNA levels (Fig. 1a,b) are presented in more detail. The transcript levels were increased two- to 13-fold in the case of *HAG3/MYB29* and 15- to 100-fold in the case of *HAG2/MYB76*, respectively. In wild-type plants, *HAG3/MYB29* expression is about eightfold higher than *HAG2/MYB76*, but lower than *HAG1/MYB28* (Fig. 1c).

Plants overexpressing both MYB factors exhibited altered levels of glucosinolates in comparison with the wild-type (Fig. 2). *HAG3/MYB29* overexpression plants showed increased levels of both short- and long-chained aliphatic glucosinolates (Fig. 2a). The content of the main short-chained aliphatic glucosinolate 4MSOB was increased about two to four times, and that of 3MSOP and 5MSOP about two to six times. Accumulation of the long-chained aliphatic glucosinolate 8MSOO was dependent on the transcription factor expression level, and was observed only in lines *Pro$_{35S}$:HAG3-6* and *Pro$_{35S}$:HAG3-12*, but not in line *Pro$_{35S}$:HAG3-42*, which has only a twofold increase in the *HAG3/MYB29* transcript level (Fig. 2a). The level of the main indolic glucosinolate I3M was decreased in the strong overexpression line *Pro$_{35S}$:HAG3-12* with a 13-fold increase in the transcript level, but was not significantly changed in other moderate overexpression lines.

Likewise, *HAG2/MYB76* overexpression lines contained increased levels of both short-chained and long-chained aliphatic glucosinolates, and this increase correlated well with the *HAG2/MYB76* expression level (Fig. 2b). However, as compared with HAG3/MYB29, much higher transcript levels are required to obtain these increases. For example, line *Pro$_{35S}$:HAG2-6* exhibiting 100-fold higher transcript levels compared with the wild-type showed only three- to fourfold higher contents of 4MSOB.

Surprisingly, and in contrast to *HAG3/MYB29* overexpression lines, the elevated content of aliphatic glucosinolates was accompanied by a two- to fourfold increase in the content of the indolic glucosinolate I3M in *HAG2/MYB76* overexpression lines (Fig. 2b).

Taken together, overexpression of both transcription factors resulted in increased contents of aliphatic glucosinolates. In the case of *HAG3/MYB29*, a moderate increase in transcript levels already resulted in pronounced increases in the content of aliphatic glucosinolates, effects that could be brought about only by very strong overexpression of *HAG2/MYB76*.

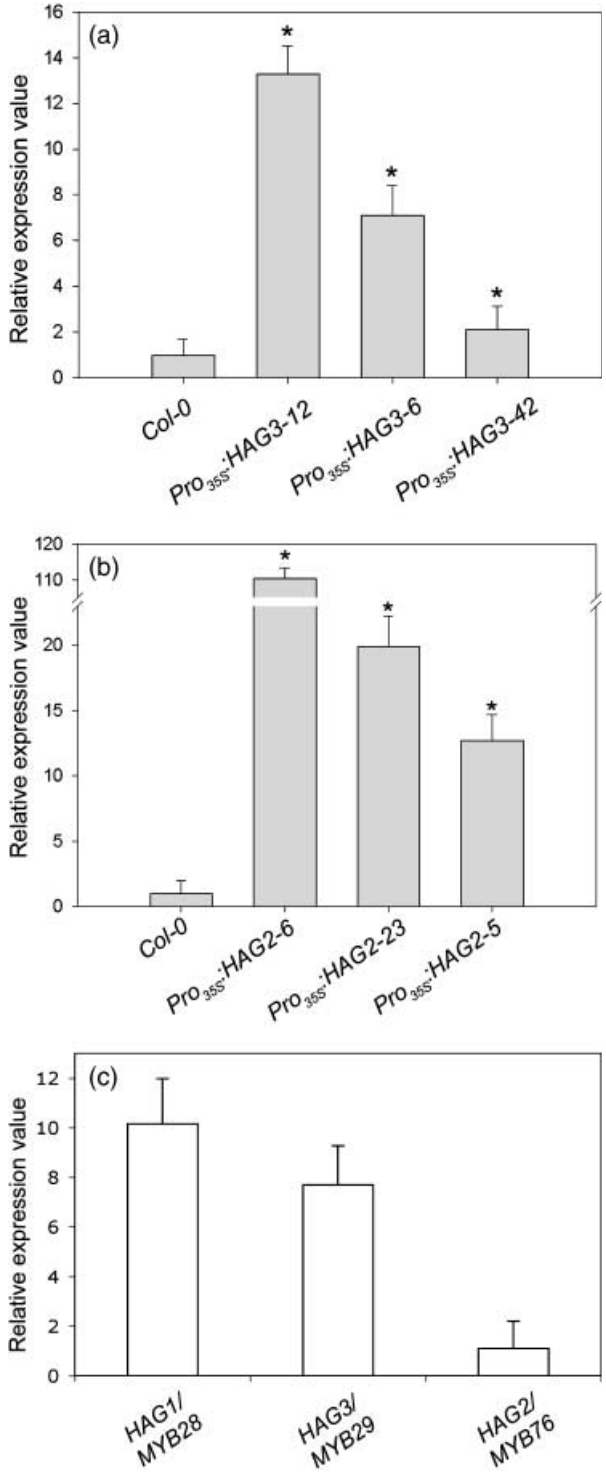

Fig. 1 Relative gene expression levels of *HAG* genes in rosette leaves of 5-wk-old overexpression and wild-type *Arabidopsis thaliana* plants. (a) *HAG3/MYB29* transcript levels in Pro_{35S}*:HAG3* overexpression lines (wt = 1). (b) *HAG2/MYB76* transcript levels in Pro_{35S}*:HAG2* overexpression lines (wt = 1). (c) Relative gene expression levels of *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29* in wild-type plants (Col-0). Data are presented in relation to the level of *Actin2* expression (Actin2 = 100). *, Significantly different (Student's test, $P < 0.05$) in comparison the wild type.

HAG2/MYB76 and HAG3/MYB29 *trans*activate glucosinolate biosynthetic pathway genes

To analyse steady-state transcript levels of genes encoding aliphatic glucosinolates biosynthetic enzymes, real-time RT–PCR assays were performed. Samples from rosette leaves of wild-type plants and three independent *HAG2/MYB76* overexpression lines (Pro_{35S}*:HAG2-5*, *-6*, *-23*) and *HAG3/MYB29* overexpression lines (Pro_{35S}*:HAG3-6*, *-12*, *-42*) were analysed for relative mRNA levels of seven different genes, all encoding enzymes of the aliphatic glucosinolate biosynthetic pathway. As shown in Fig. 3, both transcription factors were able to activate all aliphatic glucosinolate biosynthetic genes tested: *MAM1*, *MAML*, *CYP79F1*, *CYP79F2*, *CYP83A1*, *AtSt5b* and *AtSt5c*. Furthermore, the increase in the steady-state levels of these transcripts correlated well with the expression levels of *HAG2/MYB76* and *HAG3/MYB29* in the different lines: lines showing the highest accumulation of transcripts also possessed the highest *trans*activation potential towards the target genes. Notably, *HAG3/MYB29* overexpression lines showed a stronger *trans*activation potential than *HAG2/MYB76* lines.

Transient *trans*activation assays were used to assess directly the activation potential of the MYB factors (Berger *et al.*, 2007). Cultured *A. thaliana* Col-0 cells were transformed with a supervirulent *Agrobacterium* strain carrying either one of the *HAG* constructs as effector and/or the *uidA* (GUS) gene driven by one of the promoters of different putative target genes (*MAM1*, *MAML*, *CYP79F1*, *CYP79F2*, *CYP83A1*, C–S *lyase* and *CYP79B2*) as reporter.

As shown in Fig. 4, *A. thaliana* cells transiently expressing only the reporter constructs showed only weak GUS activity, whereas cotransformation with Pro_{35S}*:HAG3* and Pro_{35S}*:HAG2* led to a significant increase in GUS activity. Thus *HAG2/MYB76* and *HAG3/MYB29*, along with the previously described *HAG1/MYB28* (Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007), are able to activate all tested Met-glucosinolate biosynthetic genes (Fig. 4a,c). By contrast, previously described regulators of glucosinolate biosynthesis, IQD1 and Dof1.1 (data not shown), as well as the regulator of indolic glucosinolate biosynthesis, HIG1/MYB51 (Fig. 4b), were not able to activate these genes even after 5–7 d of transient expression.

It is also evident from Fig. 4 that the *trans*activation capacity towards *MAM1* and *MAML*, as well as *CYP79F1* and *CYP79F2*, differed for HAG2/MYB76 and HAG3/MYB29. It has been shown previously that MAM1 is involved in the biosynthesis of short-chained aliphatic glucosinolates, whereas MAML/MAM3 is primarily responsible for the generation of long-chained aliphatic glucosinolates (Textor *et al.*, 2007). *MAM1* was strongly activated by *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29*, indicating that all three MYB factors contribute to the production of short-chained aliphatic glucosinolates. Compared with *MAM1*, the promoter fragment of *MAML* was generally less activated by all three MYB factors. *HAG1/MYB28* conferred the strongest *trans*activation potential towards the

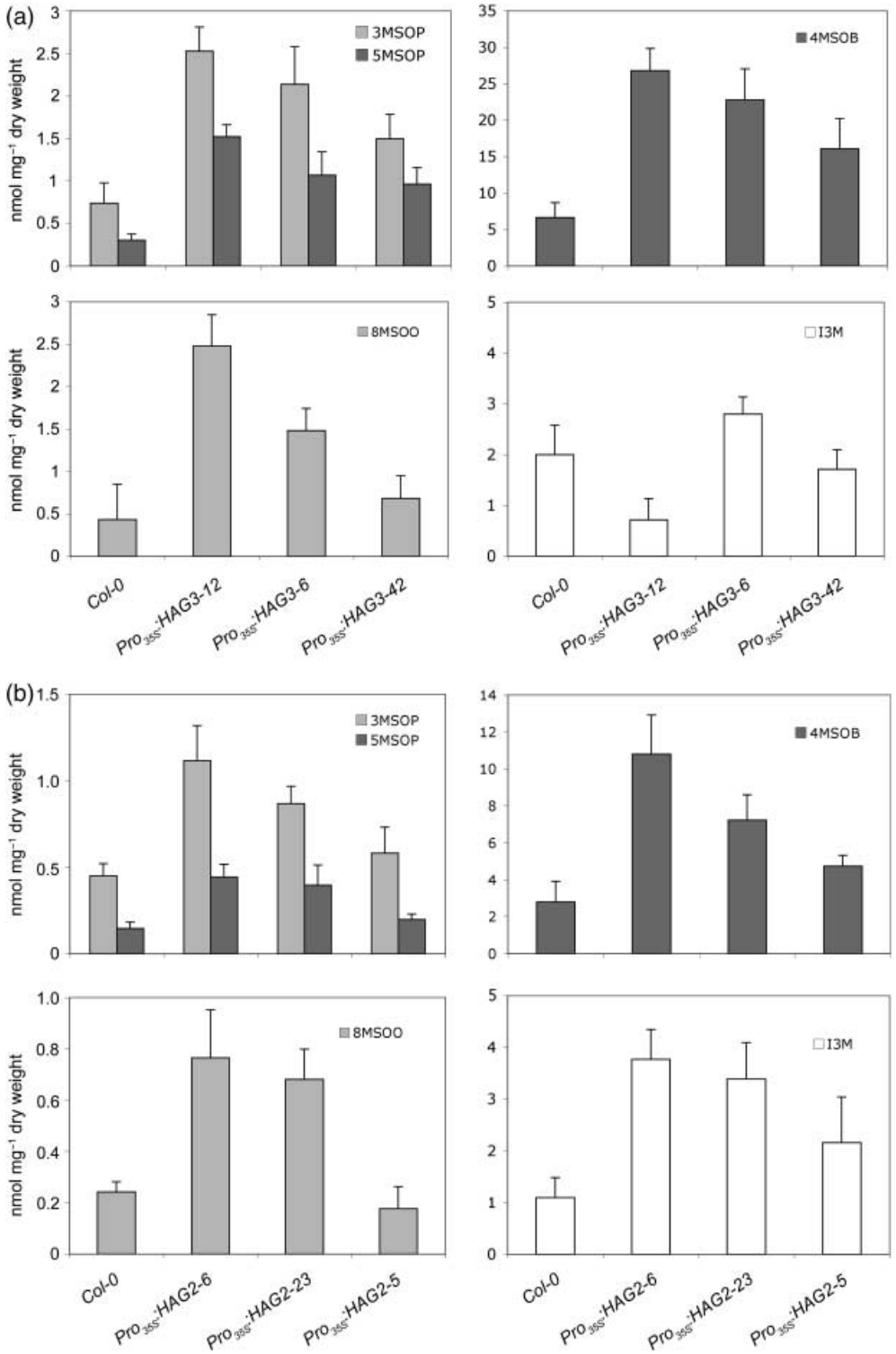

Fig. 2 Glucosinolate contents in rosette leaves of 5-wk-old *HAG3/MYB29* and *HAG2/MYB76* overexpression *Arabidopsis thaliana* plants. (a) Glucosinolate contents in *HAG3/MYB29* overexpression plants Pro_{35S}*:HAG3-12* Pro_{35S}*:HAG3-6* and Pro_{35S}*:HAG3-42* (means ± SD, $n = 5$). (b) Glucosinolate contents in *HAG2/MYB76* overexpression plants Pro_{35S}*:HAG2-6*, Pro_{35S}*:HAG2-23* and Pro_{35S}*:HAG2-5* (means ± SD, $n = 5$). 4MSOB, 4-methylsulfinylbutyl-GS; 3MSOP, 3-methylsulfinylpropyl-GS; 5MSOP, 5-methylsulfinylpentyl-GS; 8MSOO, 8-methylsulfinyloctyl-GS; I3M, indol-3-yl-methyl-GS.

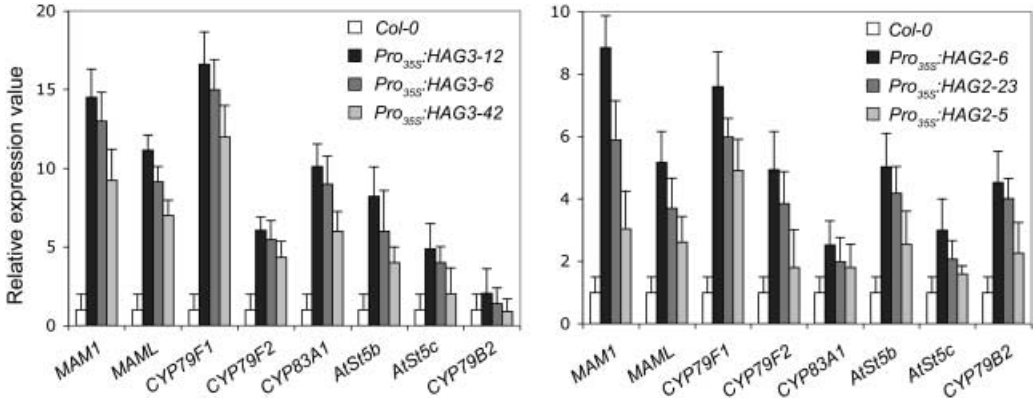

Fig. 3 *HAG2/MYB76* and *HAG3/MYB29* activate the glucosinolate biosynthetic pathway genes. Real-time PCR analysis of steady-state mRNA levels of glucosinolate biosynthetic pathway genes in wild-type (Col-0) and three independent transgenic lines overexpressing *HAG3/MYB29* (Pro_{35S}*:HAG3-12*, *-6* and *-42*) and *HAG2/MYB76* (Pro_{35S}*:HAG2-6*, *-23* and *-5*). Relative gene expression values are shown compared with the wild-type Col-0 (wt = 1). Total RNA was prepared from rosette leaves of 5-wk-old *Arabidopsis thaliana* plants and, after first-strand biosynthesis, gene-specific primers for glucosinolate biosynthetic genes were used. Each PCR assay was repeated three times with two independent sets of plants.

MAML promoter, whereas *HAG3/MYB76* and *HAG2/MYB28* were also able to activate *MAML*, but to a lesser extent. Also, *CYP79F1* and *CYP79F2* were differentially activated by the MYB factors: *CYP79F1*, catalysing the production of short-chained aliphatic glucosinolates, was equally well activated by HAG2/MYB76 and HAG3/MYB29, whereas *CYP79F2*, catalysing the production of long-chained aliphatic glucosinolates, was *trans*activated to a greater extent by HAG1/MYB28 and HAG3/MYB29, and less by HAG2/MYB76 (Fig. 4a,c).

If the transient expression assays were left for > 4 d following transformation, all three *HAG* genes were shown to induce *CYP79B2*, involved in the biosynthesis of indolic glucosinolates, but not *ASA1* (Fig. 4b). This observation is not caused by a redundancy of regulatory elements in *CYP* genes, as constitutive overexpression of *HIG1/MYB51*, a regulator of indolic glucosinolate biosynthesis, did not deregulate any promoters of aliphatic glucosinolate biosynthesis genes, even after 5–7 d of transformation (Fig. 4b).

Phenotypic appearance of Pro_{35S}*:HAG2* and Pro_{35S}*:HAG3* overexpression lines

All *HAG2/MYB76* overexpression lines possessed an unchanged growth phenotype, as demonstrated by the three representative lines used for metabolite and transcript analysis (Fig. 5). By contrast, overexpression of *HAG3/MYB29* led to the development of moderate or strong growth phenotypes (Fig. 5). A moderate overexpression of *HAG3/MYB29* caused a slight growth retardation (lines Pro_{35S}*:HAG3-12*, *-6*, *-42*), whereas strong overexpression led to a dramatic restriction in plant growth (Pro_{35S}*:HAG3-1st* and Pro_{35S}*:HAG3-2st*; Fig. 5, bottom line), as is the case for plants strongly overexpressing *HAG1/MYB28*. The phenotype of strong *HAG3/MYB29* overexpression plants resembled the bushy (or supershoot) growth phenotype of *HAG1/MYB28* overexpression plants (Gigolashvili *et al.*, 2007b); Fig. 5, bottom line): plants were retarded in growth, flowered earlier than the wild type, and had small, cup-shaped leaves and elongated internodes. Moreover, *HAG1/MYB28* overexpression lines were also impaired in gravitropic response, as shown in Fig. 5 (Pro_{35S}*:HAG1-1st* and Pro_{35S}*:HAG1-2st*).

The glucosinolate chemotype of *hag2* and *hag3* knockout mutants

To study further the role of *MYB* genes in the biosynthesis of aliphatic glucosinolates, homozygous loss-of-function alleles in both *HAG2/MYB76* (SALK line N55242 harbouring a T-DNA insertion in the first exon of *HAG2/MYB76*) and *HAG3/MYB29* (GABI-Kat line GK04OH12 harbouring a T-DNA insertion in the third exon of *HAG3/MYB29*) were isolated and analysed. The mutants showed no visible effects on plant morphology under the given growth conditions (Fig. 5); however, the *hag3* mutant contained significantly reduced levels of short-chained aliphatic glucosinolates (3MSOB, 4MSOB and 5MSOB) in leaves, but unaltered levels of long-chained aliphatic glucosinolates (8MSOO; Table 1). By contrast, the *hag2* mutant showed no significant changes in glucosinolate contents, whereas *hag1* knockdown RNAi mutants showed a reduction in both short- and long-chained glucosinolates (Gigolashvili *et al.*, 2007b).

Altogether, analysis of the knockout mutants indicates that *HAG3/MYB29* plays an important role in the regulation of short-chained aliphatic glucosinolates, whereas a defect in the *HAG2/MYB76* gene had no significant effect on the glucosinolate content.

HAG2/MYB76 and *HAG3/MYB29* are *trans*activated by HAG1/MYB28, HAG3/MYB29 and HAG2/MYB76

As shown above, overexpression of the three *HAG* genes resulted in increased levels of aliphatic glucosinolates. The

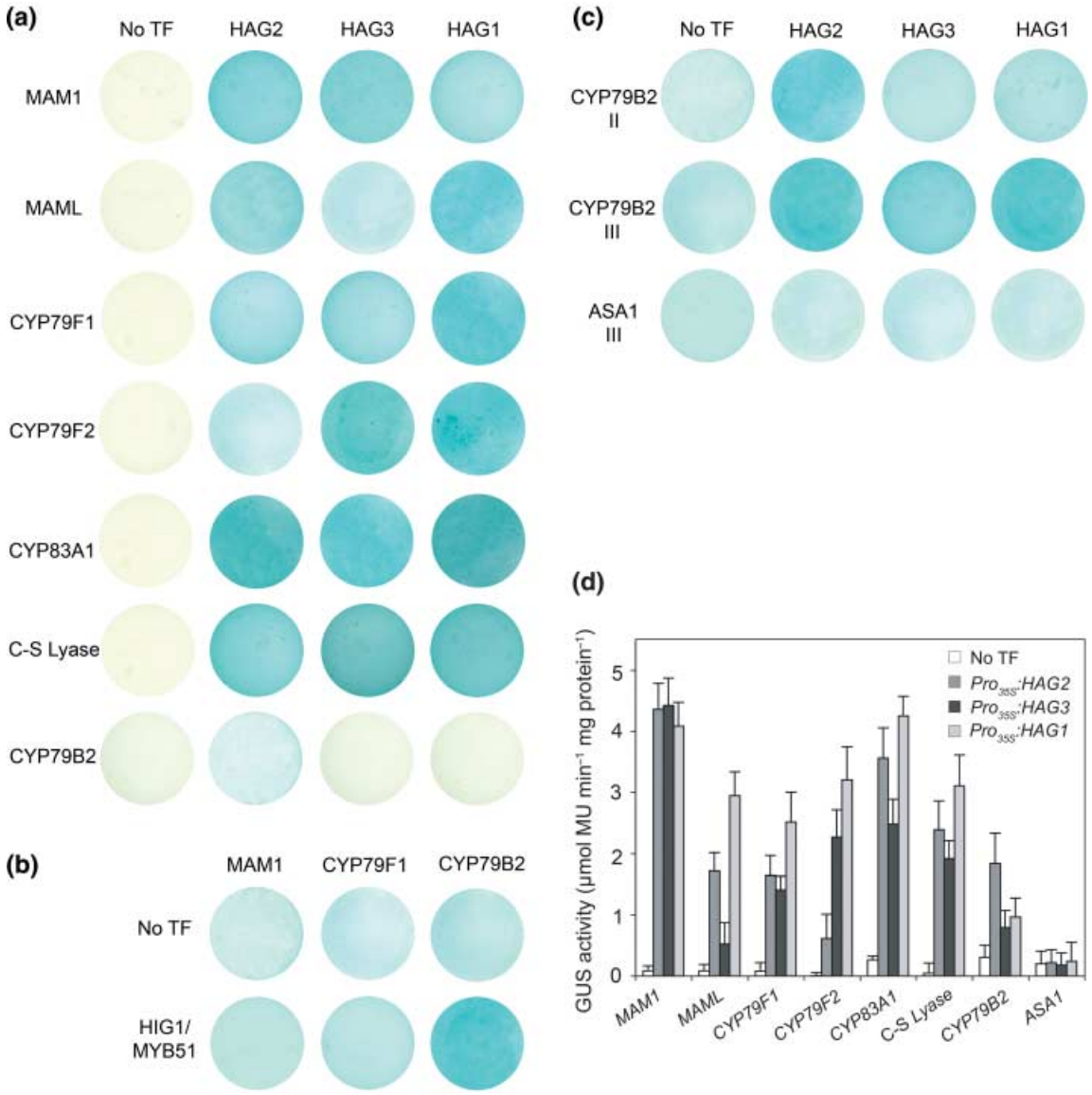

Fig. 4 Cotransformation assays to determine the target gene specificity of *HAG3/MYB29* and *HAG2/MYB76* (effectors) towards target promoters of glucosinolate biosynthetic pathway genes. The promoters *of MAM1*, *MAML*, *CYP79F1*, *CYP79F2*, *CYP83A1*, C-S *lyase*, *CYP79B2* and *ASA1* genes were fused to the *uidA* (*GUS*) reporter gene (*TargetPromoter:GUS* vectors) (a–c). The regulator of indolic glucosinolates, HIG1/MYB51 does not show an activation of aliphatic glucosinolate biosynthetic genes *MAM1* and *CYP79F1* (b). Cultured *Arabidopsis thaliana* cells were transformed with the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D containing either only the reporter construct (*TargetPromoter:GUS:pGWB3i*) or the reporter construct (*TargetPromoter:GUS:pGWB3i*) and, in addition, the effector constructs (Pro_{35S}*:HAG2:pGWB2*, Pro_{35S}*:HAG3:pGWB2*, Pro_{35S}*:HAG1:pGWB2 or* Pro_{35S}*:HIG1:pGWB2*). (a–c) Histochemical GUS staining of cultured cells. GUS staining was performed 2–3 d after transformation (a); after 4–5 d (b); or after 4–5 d (II) or 7 d (III) in (c). (d) Quantitative evaluation of GUS activity 2–3 d after transformation. White bars represent expression of only the TargetPromoter:GUS constructs, light and dark grey bars represent the expression of TargetPromoter:GUS constructs cotransformed with the effectors. Means of GUS activity in µmol MU min^{-1} and mg protein ± SD, $n = 5$.

question arises whether these genes exert coordinated control on the aliphatic glucosinolate biosynthetic pathway. As shown in Fig. 6a, real-time PCR analysis demonstrated that, in lines overexpressing either *HAG1/MYB28*, *HAG2/MYB76* or *HAG3/MYB*29, the transcript levels of the other *HAG* genes are also enhanced, except for *HAG1/MYB28*, which is barely activated by the other two *HAG* genes. Furthermore, transient expression assays using Pro_{HAG2}*:GUS* as a reporter construct and Pro_{35S}*:HAG1*, Pro_{35S}*:HAG2* and Pro_{35S}*:HAG3* as effectors indicated that the expression of *HAG2/MYB76* was significantly induced by all three *HAG* genes (Fig. 6b). Thus, although results from the overexpression of *HAG2/MYB76* suggest that HAG2/MYB76 has only a minor control of the biosynthesis of aliphatic glucosinolates (Figs 1b,c, 2b; Table 1),

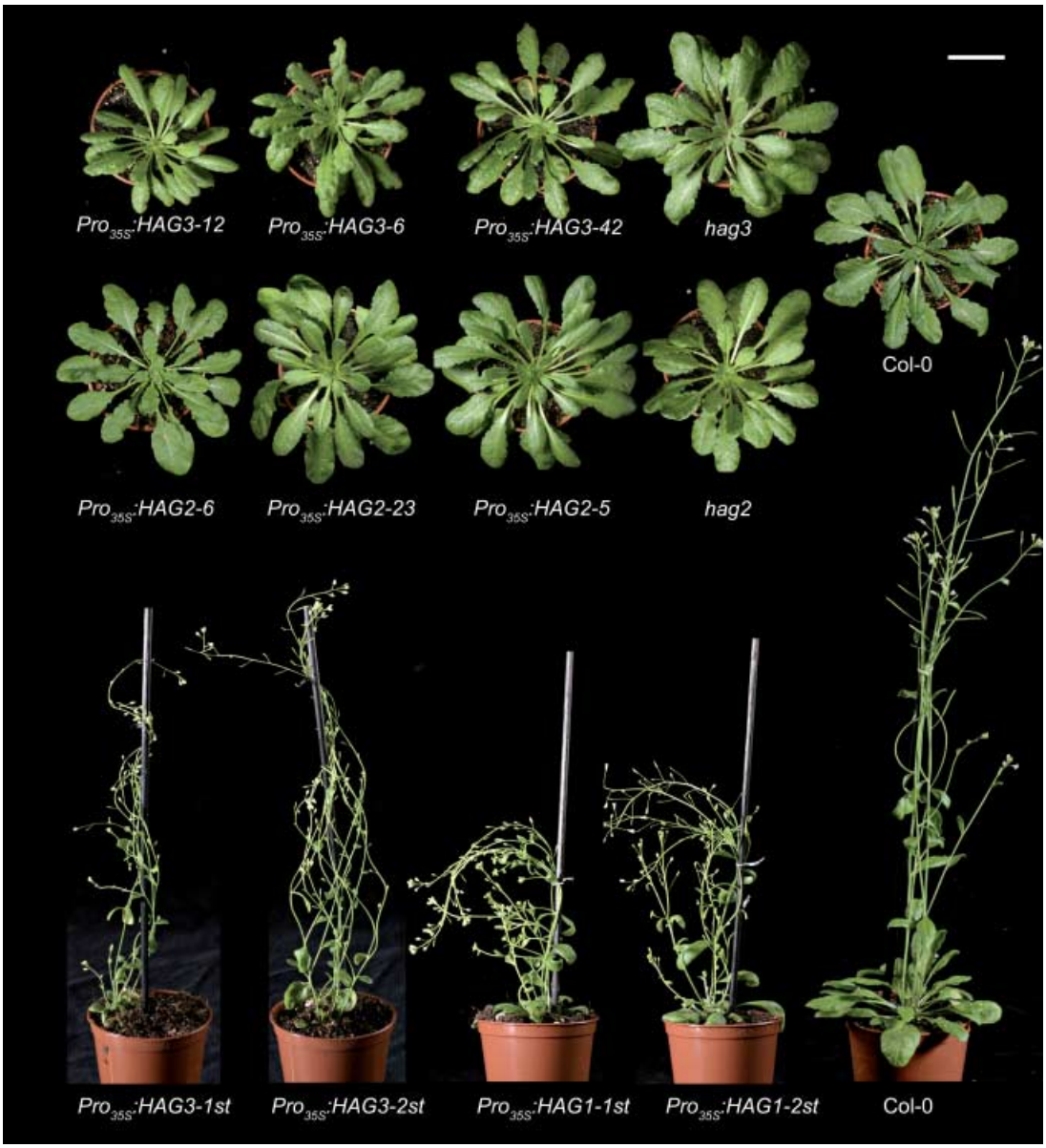

Fig. 5 Growth phenotypes of *HAG3/MYB29* and *HAG2/MY76* overexpression plants and of corresponding *hag* knockout mutants. All transgenic lines are in the Col-0 wild-type background (bar, 3 cm). Upper line, appearance of *HAG3/MYB29* overexpression plants and of the *hag3* knockout mutant. From left to right: Pro_{35S}*:HAG3-12*, Pro_{35S}*:HAG3-6*, Pro_{35S}*:HAG3-42* and *hag3*. Middle line, appearance of *HAG2/MYB76* overexpression plants and of the *hag2* knockout mutant. From left to right: Pro_{35S}*:HAG2-6*, Pro_{35S}*:HAG2-23*, Pro_{35S}*:HAG2-5* and *hag2*. Lower line, growth phenotype of strong (st) Pro_{35S}*:HAG3* overexpression lines exhibiting a bushy phenotype with elongated internodes and of st Pro_{35S}*:HAG1* overexpression lines with a bushy phenotype and altered gravitropism. From left to right: Pro_{35S}*:HAG3–1st*, Pro_{35S}*:HAG3–2st*, Pro_{35S}*:HAG1–1st*, Pro_{35S}*:HAG1–2st*. All these overexpression plants with a strong growth phenotype were partially or fully sterile.

HAG2/MYB76 can obviously be activated by both HAG1/MYB28 and HAG3/MYB29 and, in addition, by HAG2/MYB76 itself, thereby contributing to aliphatic glucosinolate biosynthesis in a complex regulatory network (Fig. 6c). In addition, *HAG3/MYB29* is activated by HAG2/MYB76 and HAG1/MYB28 (Fig. 6a,c, grey arrows), whereas HAG1/MYB28 seems to be hardly dependent on the function of the other two *HAG* genes.

Regulators of indolic glucosinolate biosynthesis are repressed by *HAG* genes

It has been shown previously that a shortage of the aliphatic glucosinolate pathway resulted in an increased accumulation of indolic glucosinolates (Reintanz *et al.*, 2001; Chen *et al.*, 2003; Hemm *et al.*, 2003). Likewise, an increased accumulation of indolic glucosinolates caused a repression of aliphatic

Table 1 Glucosinolate contents (means ± SD, $n = 8$) in rosette leaves of *hag2* and *hag3* knockout plants in comparison with the wild-type *Arabidopsis thaliana* (nmol mg^{-1} DW; means ± SD, $n = 8$)

	HAG3/MYB29				HAG2/MYB76				HAG1/MYB28†	
	WT	SD	*hag3*	SD	WT	SD	*hag2*	SD	WT	*HAG1-RNAi-10*
3MSOP	0.74	0.24	0.59*	0.10	0.45	0.07	0.39	0.09	0.66	0.02
4MSOB	6.97	2.03	5.08*	1.11	2.80	0.91	2.49	0.79	5.20	0.39
5MSOP	0.30	0.07	0.19*	0.06	0.15	0.04	0.10	0.06	0.21	0.02
8MSOO	0.43	0.05	0.42	0.04	0.24	0.04	0.23	0.07	0.82	0.10
I3M	1.60	0.34	2.31	0.39	1.11	0.39	1.32	0.26	1.29	1.26

†For comparison, glucosinolate contents in the *HAG1/MYB28* RNAi line *HAG1-RNA1-10* are listed (Gigolashvili *et al*., 2007b).
4MSOB, 4-methylsulfinylbutyl-GS; 3MSOP, 3-methylsulfinylpropyl-GS; 5MSOP, 5-methylsulfinylpentyl-GS; 8MSOO, 8-methylsulfinyloctyl-GS; I3M, indol-3-yl-methyl-GS.
*, $P < 0.05$.

glucosinolate biosynthetic genes (Levy *et al*., 2005; Gigolashvili *et al*., 2007a). Similarly, strong overexpression of *HAG1/MYB28* (Gigolashvili *et al*., 2007b) and of *HAG3/MYB29* (line Pro_{35S}*:HAG3-12*; Fig. 2a) resulted in a decreased indolic glucosinolate content. To address the issue of a cross-talk between regulators of aliphatic and indolic glucosinolate biosynthesis, Pro_{HIG1}*:GUS*, Pro_{HIG2}*:GUS* and Pro_{ATR1}*:GUS* reporter constructs were coexpressed with Pro_{35S}*:HAG1*, Pro_{35S}*:HAG2* and Pro_{35S}*:HAG3* constructs. As shown in Fig. 7, all *HAG* genes repressed the expression of the regulators of the indolic glucosinolate pathway, HIG1/MYB51, HIG2/MYB122 and ATR1/MYB34.

Tissue-specific expression of *HAG2/MYB76 and HAG3/MYB29*

It has been shown previously that *HAG1/MYB28* is expressed mainly in generative organs and mature leaves of *A. thaliana* plants (Gigolashvili *et al*., 2007b). To assess the tissue-specific expression profile of *HAG2/MYB76* and *HAG3/MYB29*, we analysed plants expressing Pro_{HAG2}*:GUS* and Pro_{HAG3}*:GUS* constructs. Expression of the reporter gene was driven by upstream regions of *HAG2/MYB76* (–1726 to +275 bp) and *HAG3/MYB29* (–1368 to +81 bp), respectively.

Reporter gene expression was detected for both *HAG2/MYB76* and *HAG3/MYB29* in seedlings, in the case of *HAG2/MYB76* mainly in the transition zone between roots and the foliar part and in stems (Fig. 8a,b), whereas expression of *HAG3/MYB29* was observed in stems and also in the midvein of leaves (Fig. 9a,b). In vegetative parts of 3-wk-old plants, GUS staining was faint for both *HAG2/MYB76* and *HAG3/MYB29*, and was primarily present around the midvein (Figs 8b,c, 9a,b). The gene expression level gradually increased in expanding leaves, reaching a maximum in fully expanded leaves of adult plants (Figs 8g, 9e). At this stage, *HAG3/MYB29* was expressed solely in the primary vein, and *HAG2/MYB76* was detected in both primary and secondary veins and in flowers.

Furthermore, both Pro_{HAG2}*:GUS* and Pro_{HAG3}*:GUS* were detected in inflorescences, but only Pro_{HAG2}*:GUS* in flower organs (pistil, anther and receptacle tissues; Fig. 8e). By contrast, Pro_{HAG3}*:GUS* was detected in trichomes (Fig. 9d) and roots (Fig. 9c), where Pro_{HAG2}*:GUS* was absent. Mechanical stimuli induced *HAG2/MYB76* and *HAG3/MYB29* expression in inflorescences of flowering plants (Figs 8h, 9h).

In conclusion, Pro_{HAG2}*:GUS* and Pro_{HAG3}*:GUS* promoter activity was strongest in the vegetative and generative parts of the plants, at sites known for the accumulation of aliphatic glucosinolates. The data for *HAG2/MYB76* and *HAG3/MYB29* expression are consistent with AtGenExpress data from the Genevestigator microarray database (Zimmermann *et al*., 2004; http://www.genevestigator.ethz.ch).

Induction of *HAG2/MYB76* and *HAG3/MYB29* expression by elicitors and wounding

As glucosinolates are important components of plant defence, and are known to be induced by hormone treatment (Brader *et al*., 2001; Mikkelsen & Halkier, 2003; Cipollini *et al*., 2004; Devoto & Turner, 2005; Mewis *et al*., 2005; Sasaki-Sekimoto *et al*., 2005) and wounding (Schuster *et al*., 2006; Gigolashvili *et al*., 2007a, 2007b), we asked whether or not *HAG2/MYB76* and *HAG3/MYB29* are involved in one of these signalling pathways. Wild-type plants were treated with MeJA and SA or were wounded, and the response of *HAG2/MYB76* and *HAG3/MYB29* was monitored using real-time PCR.

Inflorescences induced by mechanical stimuli showed an induction of both regulators after only 1 min of wounding (Fig. 10a). *HAG2/MYB76* and *HAG3/MYB29* transcript levels reached their maximum within 5 min after wounding, and were already back to control levels after 30 min. This strongly indicates a transient induction of *HAG2/MYB76* and *HAG3/MYB29* expression after wounding.

We also analysed the responsiveness of *HAG2/MYB76* and *HAG3/MYB29* to major stress-signalling hormones such as MeJA and SA. Seedlings of wild-type *Arabidopsis* plants were

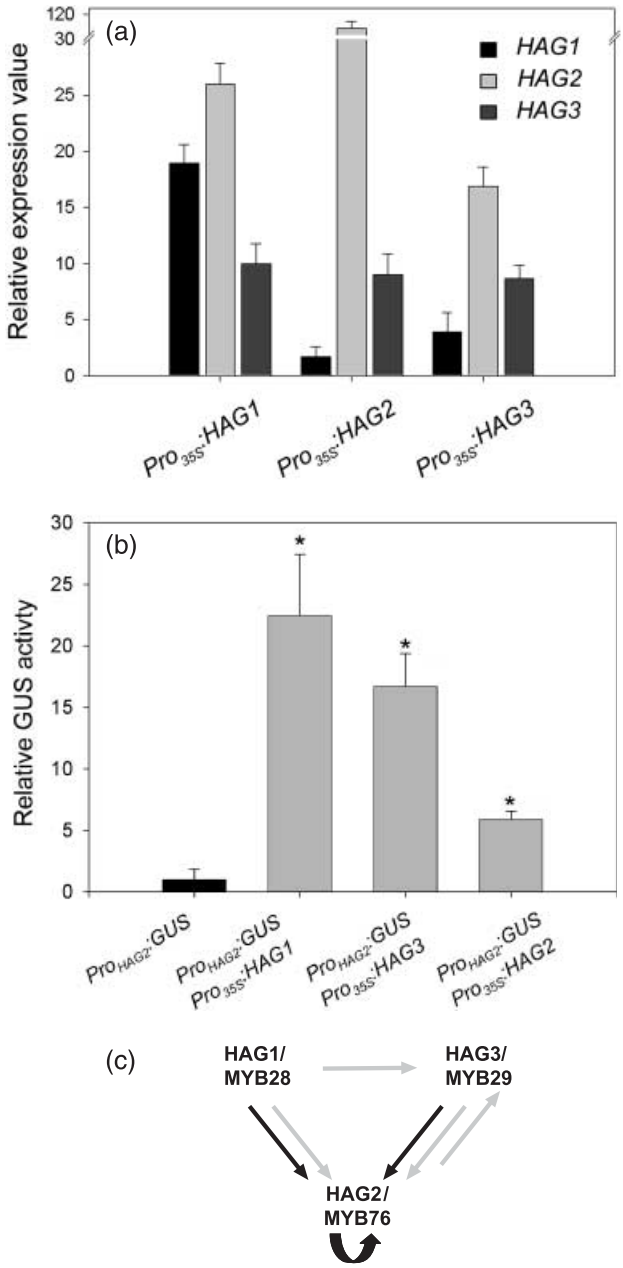

Fig. 6 Regulatory network of HAG1/MYB28, HAG2/MYB76 and HAG3/MYB29 in the control of aliphatic glucosinolate biosynthesis. (a) Steady-state transcript levels of *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29* in Pro_{35S}:*HAG1*, Pro_{35S}:*HAG2* and Pro_{35S}:*HAG3* overexpression lines. Relative gene expression values (real-time PCR data) are shown compared with Col-0 (wt = 1). Total RNA was prepared from rosette leaves of 5-wk-old *Arabidopsis thaliana* plants and, after first-strand biosynthesis, gene-specific primers for *HAG* genes were used. Each PCR assay was repeated three times. (b) Cotransformation assays pointing to the activation of Pro_{HAG2}:GUS (reporter) by *HAG3/MYB29*, *HAG2/MYB76* and *HAG1/MYB28* (effectors) (means of relative GUS activity ± SD, $n = 3$; Pro_{HAG2}:GUS = 1). *Nicotiana benthamiana* leaves were transformed with the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D containing either the reporter construct (*TargetPromoter:GUS:pGWB3i*) or the reporter (*TargetPromoter:GUS:pGWB3i*) and the effector (Pro_{35S}:*HAG2*, Pro_{35S}:*HAG3* or Pro_{35S}:*HAG1*). *, $P < 0.05$. (c) Schematic representation of all observed *HAG2/MYB76*, *HAG3/MYB29* and *HAG1/MYB28 trans*activations. Black arrows indicate *trans*activations measured in coexpression studies in *N. benthamiana* using reporter–effector constructs (b). Grey arrows indicate cross-activation of transcription factors in *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29* overexpression lines using real-time PCR analysis (a).

exposed in aqueous solutions containing the respective hormones and samples were taken for real-time PCR analysis (Fig. 10b,c). An induction of *HAG3/MYB29* could be observed within 5–15 min after application of MeJA, and the *HAG3/MYB29* transcript level returned to its original levels after 2 h. Treatment with SA caused an opposite effect, and led to a pronounced downregulation of *HAG3/MYB29* within 5 min (Fig. 10c). *HAG2/MYB76* did not respond to hormone treatment with either MeJA or SA (Fig. 10b,c).

Discussion

HAG3/MYB29 and HAG2/MYB76 are regulators of aliphatic glucosinolate biosynthesis

Recently, HIG1/MYB51 and HIG2/MYB122 have been identified as positive regulators of indolic glucosinolate biosynthesis (Gigolashvili *et al.*, 2007a). Together with ATR1/MYB34, they belong to subgroup 12 of R2R3-MYB transcription factors. HAG1/MYB28, HAG2/MYB76 and HAG3/MYB29 form a second subclade within this MYB subgroup, and HAG1/MYB28 was recently assigned as a key regulator of the biosynthesis of short- and long-chained aliphatic glucosinolates (Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007). We describe here the impact of the other two MYB proteins, HAG2/MYB76 and HAG3/MYB29, on the biosynthesis of aliphatic glucosinolates.

It is demonstrated that the level of *HAG3/MYB29* and *HAG2/MYB76* transcripts in overexpression lines corresponded nicely to an elevated accumulation of aliphatic glucosinolates (Figs 1, 2). Overexpressing lines contained up to fourfold higher levels of short-chained aliphatic glucosinolates (3MSOP, 4MSOB and 5MSOP). In some strong overexpressor lines (Pro_{35S}:*HAG3-6* and *-12*; Pro_{35S}:*HAG2-6* and *-23*), the content of long-chained aliphatic glucosinolates (8MSOO) is also increased. However, elevated content of long-chained aliphatic glucosinolates was not observed in other overexpression lines, and might be caused by secondary effects due to strong overexpression. It is feasible that a drastically increased pool of short-chained oxo-acids in chloroplasts, required for the generation of short-chained aliphatic glucosinolates, may spontaneously re-enter side-chain elongation cycles catalysed by the MAML enzyme, and may ultimately lead to the generation of long-chained aliphatic glucosinolates.

In the *hag3* knockout mutant, the level of short-chained aliphatic glucosinolates was significantly reduced, whereas the level of long-chained aliphatic glucosinolates remained

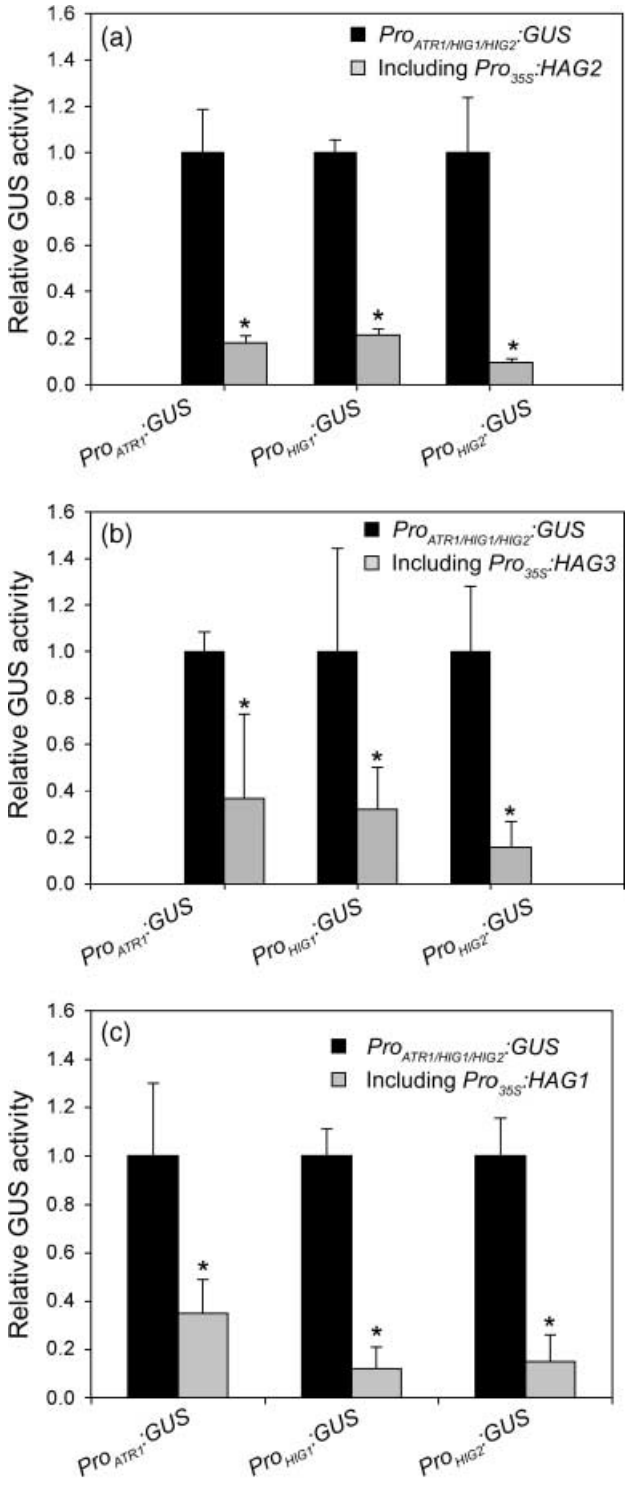

Fig. 7 Reciprocal negative feedback control of mRNA levels of indolic glucosinolate regulators by *HAG2/MYB76* (a); *HAG3/MYB29* (b); *HAG1/MYB28* (c). Cotransformation assays to determine the repression of promoters of genes involved in the control of indolic glucosinolate biosynthesis (*HIG1/MYB51*, *HIG2/MYB122 and ATR1/MYB34*) by the regulators of aliphatic glucosinolates, HAG2/MYB76 and HAG3/MYB29; means of relative GUS activity ± SD, $n = 3$; $Pro_{ATR1/HIG1/HIG2}$:*GUS* = 1). *Nicotiana benthamiana* leaves were transformed with the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D containing either the reporter construct (Promoter_of_indolic regulator:GUS:pGWB3i) as control or the reporter and one of the HAG effectors driven by the 35S CaMV promoter (Pro_{35S}:*HAG2:pGWB2* and Pro_{35S}:*HAG3:pGWB2*). *, $P < 0.05$.

unchanged (Table 1). This is an indication that HAG3/MYB29 is a regulator of short-chained but not of long-chained glucosinolates. These observations are somewhat in contrast to a previous report stating that there is no change in the content of aliphatic glucosinolates in *MYB29* knockdown plants carrying an insertion in the 5′-UTR region (Hirai *et al.*, 2007). In our hands, disruption of the *HAG3/MYB29* gene in exon 3 caused a significant reduction of short-chained aliphatic glucosinolates. However, *hag3* knockout mutant plants contained reduced but not completely diminished levels of aliphatic glucosinolates, probably caused by a compensatory function of HAG1/MYB28. In contrast to *hag3*, the *hag2* knockout mutant was not affected in glucosinolate accumulation, indicating that HAG2/MYB76 itself exerts only a limited control on aliphatic glucosinolate biosynthesis, except in response to wounding (Fig. 10a). In addition, *HAG2/MYB76* is expressed only weakly in plants in comparison with *HAG1/MYB28* and *HAG3/MYB29* (Fig. 1c), and the loss of HAG2/MYB76 could easily be compensated for by HAG1/MYB28 and HAG3/MYB29. As revealed by promoter–GUS expression studies, *HAG1/MYB28* (Gigolashvili *et al.*, 2007b) and *HAG3/MYB29* (Fig. 9) have overlapping expression sites and should therefore be able to complement the defect in *HAG2/MYB76*.

HAG2/MYB76 and HAG3/MYB29 have overlapping, but specific functions in the regulation of aliphatic glucosinolates

As revealed by analysis of the *hag2* mutant, HAG2/MYB76 appears to play only a minor role in the control of aliphatic glucosinolate biosynthesis. This view is also supported by the analysis of corresponding overexpression plants. *HAG2/MYB76*-overexpressing lines accumulated fewer aliphatic glucosinolates than *HAG3/MYB29* overexpression lines. In addition, compared with *HAG3/MYB29*, an expression level of *HAG2/MYB76* about one order of magnitude higher is required for a comparable accumulation of aliphatic glucosinolates (Figs 1b, 2b). However, such high *HAG2/MYB76* expression levels can be achieved on wounding (Fig. 10a). Finally, a dramatic overexpression of both *HAG3/MYB29* and *HAG1/MYB28* led to a pronounced retardation in growth and development, elongation of internodes, defective gravitropic responses and plant sterility (Fig. 5), an effect that was not observed for *HAG2/MYB76*-overexpression lines. The growth phenotype observed for plants strongly overexpressing *HAG1/MYB28* and *HAG3/MYB29* might be caused, in part, by an increased flow of methionine into

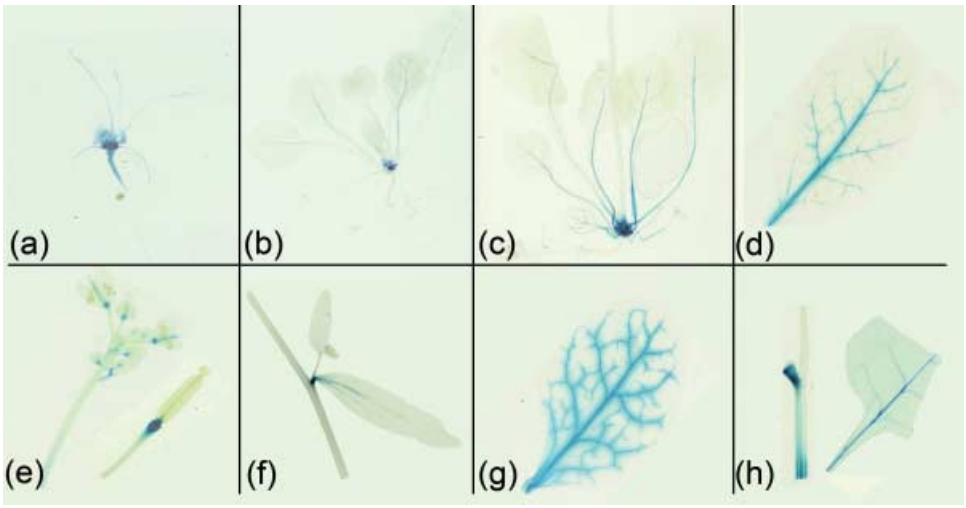

Fig. 8 Histochemical GUS staining in tissues of Pro_{HAG2}:*GUS* plants. (a) 7-d-old *Arabidopsis thaliana* seedling; (b) 14-d-old seedling; (c) 3-wk-old plant; (d) leaf of a 5-wk-old plant; (e) flowers and siliques; (f) GUS staining at base of cauline leaf; (g) adult leaf; (h) GUS induction at cut sites of inflorescences but no induction in leaves.

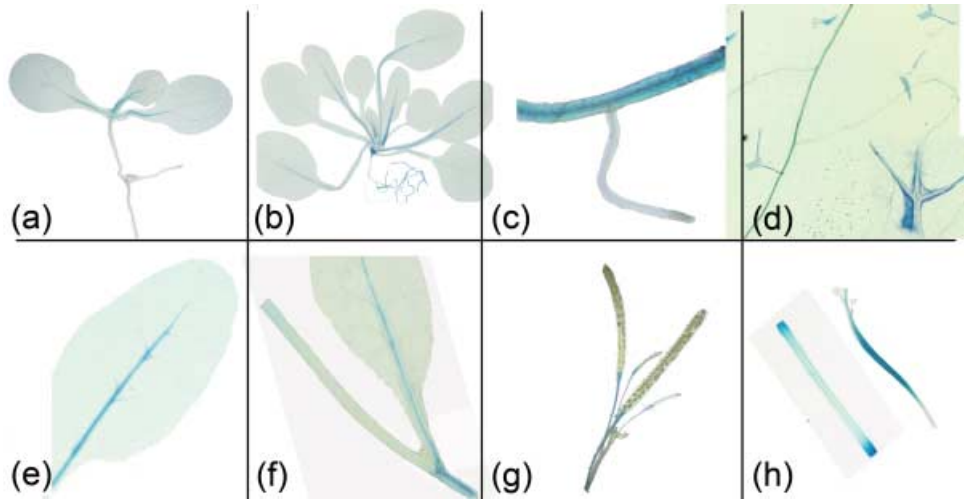

Fig. 9 Histochemical GUS staining in tissues of Pro_{HAG3}:*GUS* plants. (a) 7-d-old *Arabidopsis thaliana* seedling; (b) 14-d-old plant; (c) roots; (d) trichomes; (e) adult leaf; (f) inflorescences of a flowering plant with a cauline leaf; (g) siliques; (h) GUS induction at sites of mechanical stimuli in inflorescences.

aliphatic glucosinolates leading to a deficiency in methionine availability as precursor for ethylene biosynthesis, associated in turn with impaired gravitropic plant responses, as reported previously (Kramer *et al.*, 2003; Edelmann & Roth, 2006).

Both HAG2/MYB76 and HAG3/MYB29 are able specifically to activate the whole set of aliphatic glucosinolate biosynthetic pathway genes; however, they exhibit some distinct features (Figs 3, 4). MAML and CYP83A1, and also CYP79B2, involved in the production of indolic glucosinolates, were more strongly activated by *HAG2/MYB76* than by *HAG3/MYB29*, whereas the activation of CYP79F2 was more pronounced in *HAG3/MYB29*-overexpression lines. All promoters of glucosinolate biosynthetic genes tested in this study contain at least two or more putative MYB-binding sites (AthaMap, www.athamap.de). It should be kept in mind that HAG1/MYB28 serves as the strongest activator of aliphatic glucosinolate biosynthetic genes (Fig. 4; Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007).

The expression patterns of *HAG2/MYB76* and *HAG3/MYB29* were partially different from each other and from that of *HAG1/MYB28* (Figs 8, 9). *HAG2/MYB76* expression was present in the transition zone from root to foliar parts, flowers and secondary veins of leaves, where expression of *HAG3/MYB29* and *HAG1/MYB28* was absent, whereas *HAG3/MYB2a* was present in young siliques, trichomes and roots, where expression of *HAG2/MYB76* could not be detected. Thus the expression patterns of *HAG2/MYB76* and *HAG3/MYB29* nicely overlap with those of the aliphatic glucosinolate

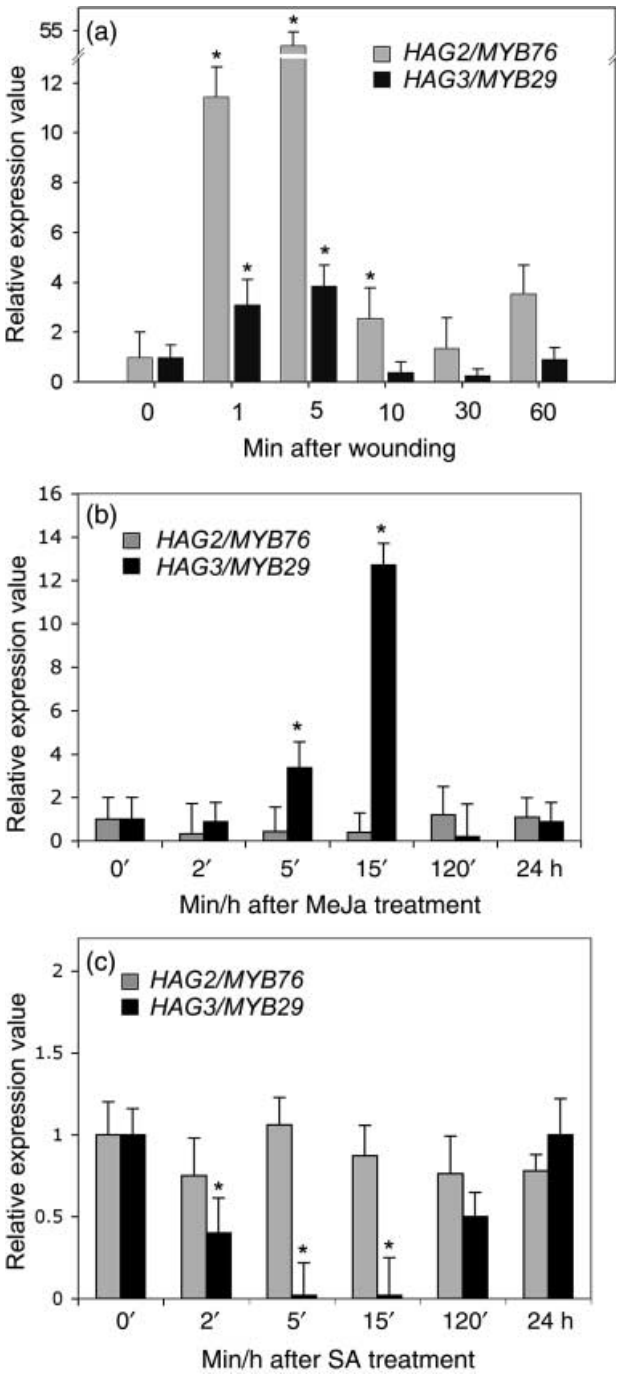

Fig. 10 Induction of *HAG2/MYB76* and *HAG3/MYB29* expression by wounding, methyl jasmonate (MeJA) and salicylic acid (SA). (a) Wounding experiments. The plant material (*Arabidopsis thaliana* inflorescences) were punctured, and, after times indicated, used for real-time RT–PCR experiments (means ± SD, $n = 3$). Relative expression values are given compared with nonwounded tissue (non-wounded = 1). For details see Materials and Methods. (b,c) Hormone experiments. 3-wk-old seedlings were exposed in aqueous solutions to 10 μM MeJA (b) or 10 μM SA (c), or kept in aqueous solutions without the addition of hormones. Samples were taken after 0, 2, 5, 10, 15 and 120 min and 24 h. Relative gene expression values are shown compared with noninduced plants (non-induced = 1). *, Significantly different (Student's test; $P < 0.05$) in comparison with noninduced tissue.

biosynthesis genes *MAM3*, *BCAT4* and *CYP79F1* (Reintanz *et al.*, 2001; Chen *et al.*, 2003; Schuster *et al.*, 2006; Textor *et al.*, 2007). This observation is consistent with the known sites of Met-derived aliphatic glucosinolate accumulation and the suggested role of *HAG3/MYB29* and *HAG2/MYB76* in this pathway.

HAG2/MYB76 and HAG3/MYB29: responses to wounding and MeJA treatment

Environmental stimuli such as herbivore attack, wounding or hormone treatment (e.g. with MeJa and SA) are known to have an impact on glucosinolate biosynthesis (Brader *et al.*, 2001; Kliebenstein *et al.*, 2002; Mikkelsen *et al.*, 2003; Mewis *et al.*, 2005). Expression of *IQD1*, *HIG1/MYB51* and *HAG1/MYB28* have been shown to respond very quickly to wounding (Levy *et al.*, 2005; Gigolashvili *et al.*, 2007a, 2007b). Here we demonstrate that the expression of both *HAG2/MYB76* and *HAG3/MYB29* respond to mechanical stimuli within 5 min, as has been shown for aliphatic glucosinolate biosynthetic genes such as *BCAT4* and *MAM1* (Schuster *et al.*, 2006), indicating that both MYB factors are involved in the induction of aliphatic glucosinolate production on biotic challenge linked to wounding. Notably, expression of *HAG2/MYB76* is induced more than 50-fold in response to wounding. It has been shown recently that treatment with MeJa for 3 h leads to a twofold increase in the expression level of *HAG3/MYB29* but not of *HAG1/MYB28* (Hirai *et al.*, 2007). Here we show that the expression of *HAG3/MYB29* responded positively to treatment with exogenous MeJa and negatively to SA (Fig. 10). Within 15 min, the *HAG3/MYB29* expression level increased more than 10-fold. By contrast, *HAG2/MYB76* expression was independent from MeJa and SA, whereas HAG1/MYB28 is triggered by glucose (Gigolashvili *et al.*, 2007b).

Negative regulation of indolic glucosinolate biosynthesis by regulators of the aliphatic glucosinolate biosynthetic pathway

Positive regulators of aliphatic glucosinolate accumulation (all three *HAG* genes) were shown to downregulate the expression of regulators of indolic glucosinolate accumulation (ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122; Fig. 7). This observation is consistent with decreased levels of indolic glucosinolates observed in lines *Pro$_{35S}$:HAG3-12* (Fig. 2a) and *Pro$_{35S}$:HAG1-11* (Gigolashvili *et al.*, 2007b), and with previously described examples of mutual negative regulation of Met- and Trp-derived glucosinolate pathways (Reintanz *et al.*, 2001; Chen *et al.*, 2003; Hemm *et al.*, 2003; Levy *et al.*, 2005; Gigolashvili *et al.*, 2007a). For example, knockout mutants of the *CYP79F1* and *CYP83A1* genes with impaired aliphatic glucosinolate biosynthesis accumulated indolic glucosinolates. Similarly, the *HIG1-1D* dominant mutant,

containing higher levels of indolic glucosinolates, contained significantly decreased levels of aliphatic glucosinolates (Gigolashvili *et al.*, 2007a). Also, the overexpression of *IQD1*, a positive regulator of indolic glucosinolate genes, caused a repression of *CYP79F1* and *CYP79F2* genes involved in aliphatic glucosinolate biosynthesis (Levy *et al.*, 2005).

These data suggest that the observed negative feedback regulation of the indolic glucosinolate biosynthesis might be linked to control of the sulfur pool in plants overproducing positive regulators of aliphatic glucosinolate biosynthesis (*HAG* genes) in response to environmental challenges. The repression of regulators of the indolic glucosinolate pathway (*HIG* genes, *ATR1/MYB34*) in plants overexpressing aliphatic glucosinolates may lead to a metabolic balance between both glucosinolate biosynthetic pathways, and may thereby contribute to sulfur homoeostasis.

Remarkably, *HAG* genes not only prevented the expression of regulators of the indolic glucosinolate pathway, but also activated CYP79B2 (Fig. 4), a key enzyme in the biosynthesis of indolic glucosinolates, apparently counteracting the repression of indolic glucosinolate production. However, one has to bear in mind that the product of CYP79B2, IAOx, is at a branching point also leading to the biosynthesis of auxin. We propose that the activation of CYP79B2 maintains auxin biosynthesis even in the presence of a repressed indolic glucosinolate pathway. A downregulation of CYP79B2 in *HAG* overexpression plants would finally lead to a low-auxin phenotype, as manifested in the severe growth phenotype of *cyp79B2* mutants (Zhao *et al.*, 2001).

Notably, increased accumulation of the indolic glucosinolate I3M was observed in lines strongly overexpressing *HAG2/MYB76* (Fig. 2b); also, a *HAG1/MYB28* overexpression suspension cell line contained elevated levels of indolic glucosinolates (Hirai *et al.*, 2007). We propose that a strong overexpression of *HAG* genes and the simultaneous activation of *CYP79B2* serve to maintain auxin homeostasis in the cell, but cause an increased accumulation of indolic glucosinolates as a secondary effect. It has been shown previously that 35S CaMV *CYP79B2* overexpression lines contain elevated levels of both auxin and I3M (Mikkelsen *et al.*, 2000; Zhao *et al.*, 2002).

HAG1/MYB28, *HAG3/MYB29* and *HAG2/MYB76* encompass a gene regulatory network

HAG1/MYB28 and *HAG3/MYB29* overexpression plants showed an increase in *HAG2/MYB76* transcript, and analysis of *HAG1/MYB28* and *HAG2/MYB76* overexpression plants showed an accumulation of the *HAG3/MYB29* transcript (Fig. 6a). The interactions between HAG1/MYB28, HAG3/MYB29 and HAG2/MYB76 with the promoter of the *HAG2/MYB76* gene were studied in tobacco plants to avoid interference by endogenous MYB transcription factors (Fig. 6b).

Based on the interaction scheme resulting from these data (Fig. 6c), *HAG1/MYB28* with the highest transcript level of all three *HAG* genes shows the least interactions of the three transcription factors, not being strongly activated by either of the other two. By contrast, both HAG3/MYB28 and HAG2/MYB76 show similar interaction patterns, both being activated by *HAG1/MYB28* and both inducing each other. *HAG2/MYB76*, which contributes only slightly to the glucosinolate profile of wild-type plants if expressed alone, is able to perceive signals from *HAG1/MYB28* and *HAG3/MYB28* or in response to wounding. Because transcription of the three genes is also induced by different stimuli, such as wounding and MeJa, we suggest that the three *HAG* genes can integrate several environmental cues by activating each other's transcription, probably leading to a range of different glucosinolate profiles. Despite these exciting insights into the *HAG* gene regulatory network, more work is needed to elucidate the exact function and dynamics of the proposed interactions.

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