University of Cologne, Institute for Botany

Analysis of the MYB28, MYB29 and MYB76 transcription factors involved in the biosynthesis of aliphatic glucosinolates in *Arabidopsis thaliana*

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“The truth is rarely pure and never simple”.

Oscar Wilde

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1. Introduction

1.1 Structures and biosynthesis of glucosinolates

Glucosinolates (GSL) are nitrogen- and sulfur-containing classes of about 200 naturally occurring thioglucosides that are characteristic of the *Cruciferae* and related families including the model plant *A. thaliana*. Glucosinolates share a chemical structure consisting of a *β-D*-glucopyranose residue linked via a sulphur atom to a (Z)-N-hydroximinosulfate ester and the glucosinolate-defining core structure called the glucone (R-group) which is derived from several amino acids (Fig. 1A). Glucosinolates are classified depending on the nature of amino acid residue into aliphatic glucosinolates derived from Ala, Leu, Ile, Met, or Val; aromatic glucosinolates derived from Phe or Tyr and indolic glucosinolates derived from Trp (Fig. 1B, C). However, the most abundant and typical structures are aliphatic, indolic and aromatic glucosinolates derived from Met, Trp and Phe, correspondingly (Dawson et al., 1993; Toroser et al., 1995; Field et al., 2004).

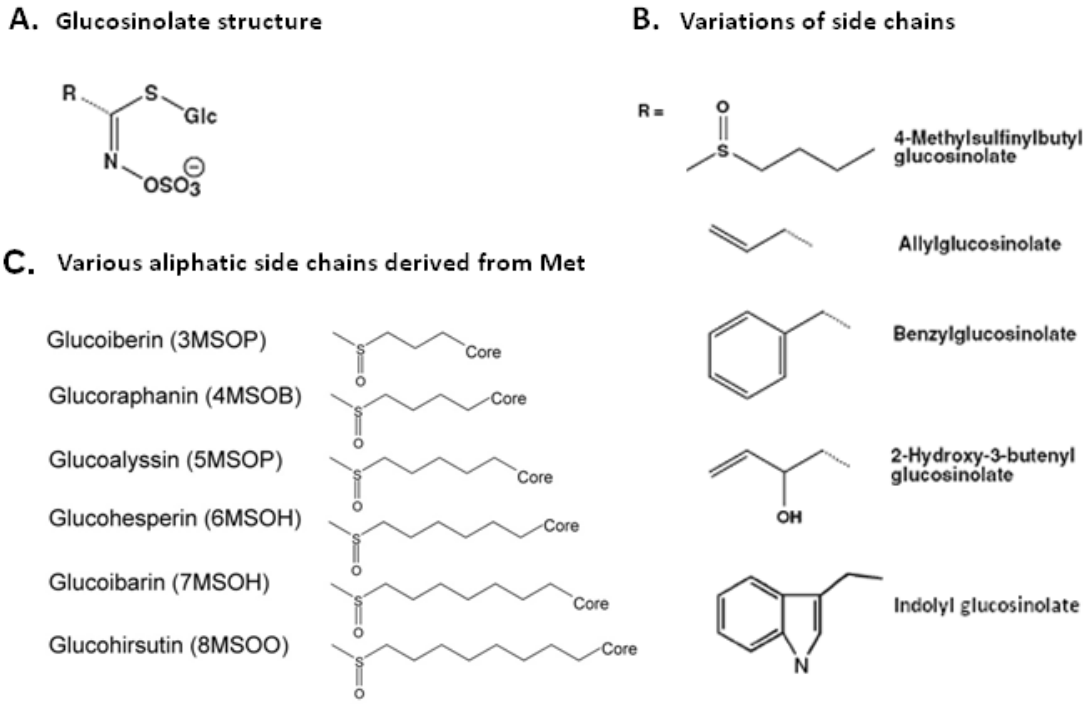

Figure 1. Chemical glucosinolate structures depend from the amino acid precursor: (A) Common structure of glucosinolates; (B) Examples of specific glucosinolates with typical variation in the structure of side chain; (C) Different side chain-elongated aliphatic methionine-derived glucosinolates.

Although glucosinolates represent a chemically diverse class of plant secondary compounds, biosynthesis of these compounds consist of three main stages: (i) condensation and side chain-elongation of amino acids, (ii) development of the core glucosinolate structure and (iii) secondary side-chain modifications of glucosinolates (Fig. 2). The first and second stages of glucosinolate core biosynthesis have extensively been studied in *A. thaliana* and great part of the enzymes in the biosynthetic pathways is now characterized (Grubb and Abel, 2006; Halkier and Gershenzon, 2006). Diversities in glucosinolate structure are mainly achieved using chain-elongated form of methionine and valine for glucosinolates biosynthesis and similar to the biosynthesis of leucine from valine and acetate (Mikkelsen and Halkier, 2003). The side-chain elongation and modification of glucosinolate structures that give a rise to the great variety of these compounds continue to be an important area for the continued studies.

Glucosinolate biosynthesis starts with the transamination of the amino acid by several branched-chain aminotrasferases (BCATs) to produce the corresponding α-keto acid. For instance, in biosynthesis of methionine-derived glucosinolates, methionine (Met) or a chain-elongated form of Met is deaminated by cytosolic BCAT4 (Schuster et al., 2006) and chloroplastidic BCAT3 enzymes (Knill et al., 2008). The BCAT4 catalyzes the initial step of Met chain elongation by converting Met to 4-methylthio-2-oxobutanoic acid (MTOB). The BCAT3 mainly catalyzes the conversion of 5-methylthiopentyl-2-oxo and 6-methylthiohexyl-2-oxo acids to their respective Met derivatives (homomethionine and dihomomethionine), possibly indicating an alternative biosynthetic flux of α-keto acids into chain-elongated amino acid intermediates within the chloroplast. After transamination, the resulting α-keto acids are subsequently metabolized in a condensation reaction with acetyl-CoA, catalyzing by MAM1 and MAML enzymes inside of chloroplasts to form a substituted 2-malate derivative (Textor et al., 2007), followed by isomerization to yield a 3-malate derivative. Next, the 3-malate-derivative is converted by oxidative decarboxylation to a homoketo acid with one additional carbon in the side chain than the starting compound. Thus, homoketo acids can pass through additional elongation cycles creating homoketo acids with increased side chain length up to nine methylene groups (Falk et al., 2004). Further synthesis of glucosinolate core structure occurs in the cytosol and begins with the conversion of precursor amino acids to aldoximes by cytochrome P450 monooxygenases of the CYP79 family. Therefore, at least two

different cell compartments and transport steps are required for the biosynthesis of chain-elongated Met-derived aliphatic glucosinolates: the import of MTOB from the cytosol into chloroplast, and the transport of Met derivatives from the chloroplast into the cytosol. Interestingly, the putative transport proteins as well as enzymes of isomerization and oxidative decarboxylation reactions have not been identified yet and thus remain attractive research topics (Fig. 2A).

Several studies have demonstrated that CYP79 monooxygenases are involved in the conversion of different amino acids to aldoximes (Hansen and Halkier, 2005). The CYP79B2 and CYP79B3 enzymes metabolize the formation of indole-3-acetaldoxime (IAOx) from tryptophan (Hull et al., 2000; Mikkelsen et al., 2000; Mikkelsen et al., 2003). A *cyp79B2/cyp79B3* double knockout completely lacks indole glucosinolates indicating that IAOx is the only precursor for the synthesis of indole glucosinolates from tryptophan. Moreover, IAOx is an important branching point for the biosynthesis of several other Trp-derived compounds like the plant hormone auxin (IAA) and the phytoalexin camalexin (Glawischnig et al., 2004). The monooxygenases CYP79F1 and CYP79F2 are involved in the conversion of chain-elongated Met amino acid precursors into aliphatic aldoximes. CYP79F1 and CYP79F2 have overlapping, but also distinct functions in the biosynthesis of Met-derived glucosinolates. Whereas CYP79F1 is able to metabolize mono- to hexahomomethionine resulting in both short- and long-chain aliphatic glucosinolates, the CYP79F2 seems to be exclusively converting long-chain elongated penta- and hexahomomethionines to corresponding aldoximes (Reintanz et al., 2001; Chen et al., 2003; Tantikanjana et al., 2004; Hansen and Halkier, 2005). The obtained aldoximes are further oxidized by cytochromes P450 of the CYP83 family producing *aci*-nitro-compounds. Two non-redundant CYP83A1 and CYP83B1 enzymes were characterized in *Arabidopsis* plants (Hemm et al., 2003; Naur et al., 2003). Biochemical characterization of CYP83A1 revealed its capacity to metabolize specifically Met-derived aldoximes whereas its homologue CYP83B1 has a higher affinity towards indolic and aromatic aldoximes derived from tryptophan, phenylalanine, and tyrosine (Bak and Feyereisen, 2001; Bak, 2001; Smolen and Bender, 2002). Produced by CYP83 enzymes, *aci*-nitro-compounds or nitrile oxides are strong electrophiles that may spontaneously react with cystein as the thiol donor or enzymatically metabolized by glutathione-S-transferases (GSTs) to form S-alkylthiohydroximate conjugates as evidenced by *in vivo* studies (Wetter, 1968). However, this part of glucosinolate biosynthesis remains speculative. The resulting S-

alkylthiohydroximates are then cleaved by a C-S lyase to yield thiohydroximates, pyruvate and ammonia. Metabolic analysis of C-S lyase knock-out mutant (SUPERROOT1-SUR1) revealed that *sur1* does not contain any aliphatic and aromatic glucosinolates. It is, therefore, suggested that C-S lyase is a single gene family which lack a side specificity in glucosinolate biosynthesis (Mikkelsen et al., 2004). Subsequently, thiohydroximates are used as substrates to produce desulfoglucosinolates via a glucosylation reaction catalyzed by UGT74B1 glucosyltransferase (Grubb et al., 2004; Bowles et al., 2005). Analysis of *ugt74b1* knockout mutant showed considerably decreased but not completely abolished level of indolic and aliphatic glucosinolates. Thus, it is speculated that other UGT activities are present in *Arabidopsis* plants. UGT74C1 has been suggested as a candidate gene to function in glucosinolate biosynthesis and seems to be more specific for Met-derived thiohydroximates (S. Abel, personal communication). At the last stage of biosynthesis, desulfoglucosinolates are sulphated by PAPS:desulfoglucosinolate transferases resulting in the formation of parent glucosinolate structures (Fig. 2B). Three *Arabidopsis* sulfurtransferases AtST5a, AtST5b and AtST5c have been identified and characterized in glucosinolate biosynthesis. The AtST5a preferably metabolizes tryptophan- and phenylalanine-derived desulfoglucosinolates whereas AtST5b and AtST5c mediate reactions with aliphatic Me-derived glucosinolates (Piotrowski et al., 2004; Klein et al., 2006).

Secondary side-chain modifications of glucosinolates are generally considered to represent the final stage in glucosinolate synthesis. Following the biosynthesis of parent glucosinolates or in some cases desulfoglucosinolates, the side-chain may undergo various modifications by mean of various oxidation, alkylation and/or esterification reactions (Fig. 2C). Remarkably, the natural variation of glucosinolates is achieved by several side-chain modifications of glucosinolates via action of two α-ketoglutarate-dependent dioxygenases (AOP2 and AOP3) that control the conversion of methylsulfinylalkyl to alkenyl- and hydroxyalkyl glucosinolates, respectively (Kliebenstein et al., 2001b; Kliebenstein et al., 2005b). Recently, a flavin-monooxygenase was shown to catalyze the conversion of methylthioalkyl glucosinolates into methylsulfinylalkyl glucosinolates (Hansen et al., 2007). Together, the combine action of glucosinolates structural genes (MAMs, CYP79s, and CYP83s) and side-chain modification enzymes (AOP, flavin-monooxygenase) give rise to such highly diverse chemical structures of glucosinolates.

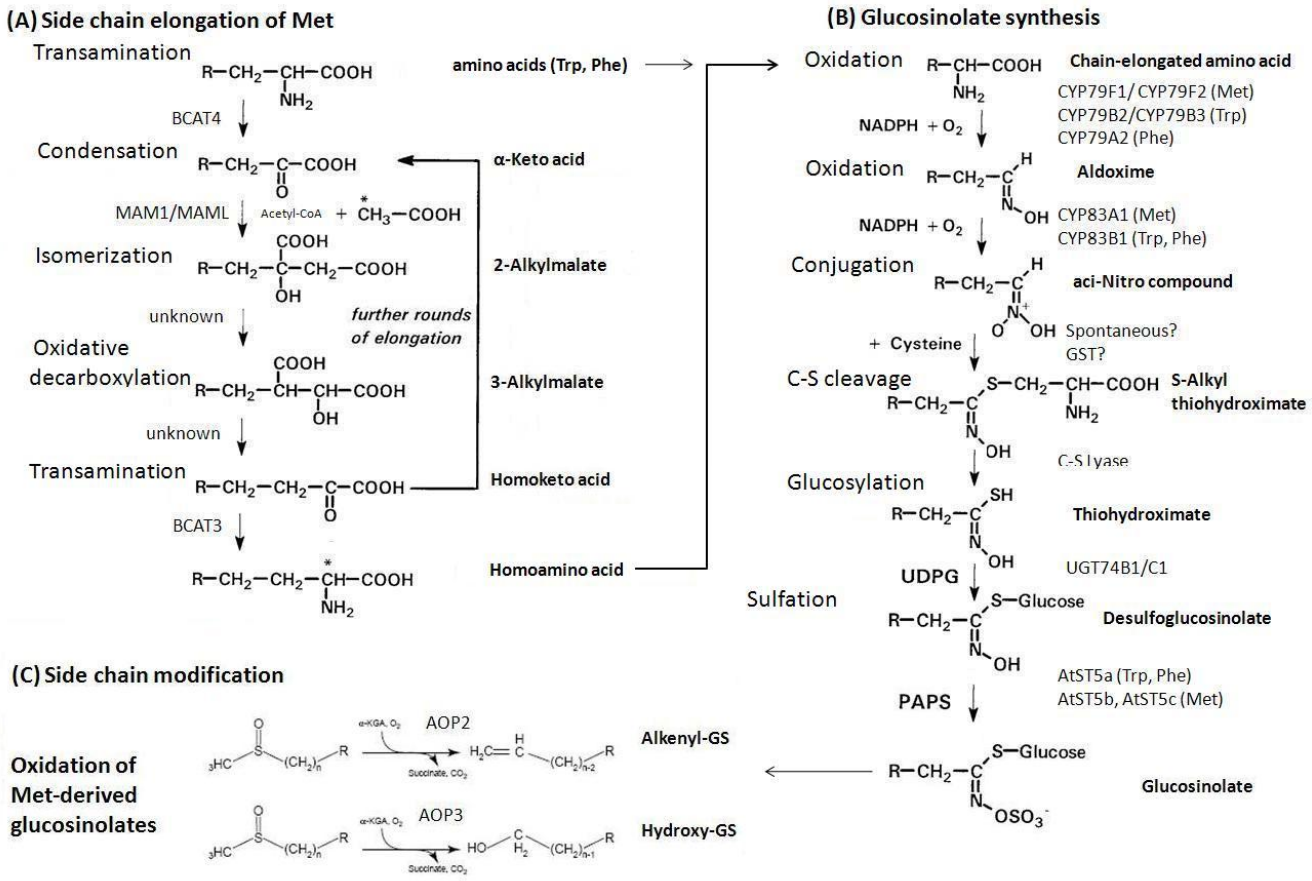

Figure 2. Stages of glucosinolate biosynthesis in *Arabidopsis thaliana* (A) Transamination, condensation and side elongation reactions of Met in aliphatic (Met-derived) glucosinolate biosynthesis converted by BCAT3 or BCAT4 (branched-chain aminotransferases), MAM1/MAML (methylthioalkylmalate synthases); (B) Biosynthesis of the glucosinolate core structure: CYP79 and CYP83 enzymes catalyzing the conversion of amino acids to aldoximes and *aci*-nitro compounds; *aci*-nitro compounds can react spontaneously with either cysteine or, perhaps catalyzed by glutathione S-transferase (GST), with glutathione; C-S lyase produces thiohydroximates which are glucosylated by UGT74B1/C1 to desulfo-glucosinolates; sulphation leads to the synthesis of primary glucosinolates by AtSTa, AtSTb and AtSTc sulfotransferases; α-ketoglutarate dioxygenases AOP2 and AOP3 involved in side chain modification of glucosinolates; R - variable amino acid side chain.

1.2 Biological role of glucosinolates

1.2.1 Glucosinolate degradation and its role for plants

Glucosinolates are present in all parts of the plant; however, the level of glucosinolates varies at different developmental stages in plant tissues and is affected by several biotic and abiotic factors such as growth conditions, wounding, fungal infection, insect damage and other forms of biotic stresses. Generally, high levels of glucosinolates are found in young leaves, shoots, silique walls and seeds (Fahey et al., 2001; Brown et al., 2003a). These hydrophilic compounds are normally stable and sequestered in vacuoles of most plant tissues. Therefore, the primary function of glucosinolates under non-stress conditions is still unclear. It has been proposed that glucosinolates serve as an internal storage of sulphur which can be mobilized by putative thioglucosidases in order to reuse sulphur in primary metabolism upon sulphur deficiency (Rausch and Wachter, 2005). Recent microarray studies have showed that the activation of sulphate acquisition and the repression of glucosinolate production may occur in parallel in response to sulphur limitation (Maruyama-Nakashita et al., 2003; Hirai et al., 2005). Nevertheless, the process of glucosinolate degradation indicates an important role of these compounds in plant-insect interactions and plant protection against biotic stresses via action of the binary glucosinolate–myrosinase system (see below), also called the 'mustard oil bomb' (Luthy and Matile, 1984). In plants of the order Brassicales, including the model plant *Arabidopsis thaliana*, the glucosinolate–myrosinase system serves as a major chemical defense mechanism against insects, bacterial and fungal pathogens (Tierens, 2001; Ratzka et al., 2002; Wittstock and Gershenzon, 2002; Kliebenstein et al., 2005a). Degradation of glucosinolates only occurs upon plant tissue damage (Fig. 3). Glucosinolates are metabolized by a specific β-thioglucosidase (TGG), also called myrosinase, which is localized in special myrosin cells (idioblasts), scattered throughout the plant tissues and spatially separated from each other (Xue et al., 1995; Koroleva, 2000; Andreasson, 2001). In *Arabidopsis* plants, two redundant TGG1 and TGG2 myrosinases have recently been identified. Metabolic analysis of the *tgg1/tgg2* double knockout mutant revealed the loss of myrosinase activity linked to the absence of damage-induced glucosinolate degradation of mainly aliphatic and

glucosinolates (Barth and Jander, 2006). Myrosinase activity results in the hydrolysis of the thioglucoside linkage leading to the formation of unstable thiohydroximate-O-sulfate aglycon. This unstable aglycon may spontaneously and nonenzymatically rearrange into different bioactive products such as isothiocyanates, thiocyanates, nitriles, oxazolidine-2-thiones or epithioalkanes (Fig. 3). Certain chemical conditions, co-factors (pH, availability of ferrous ions) and the presence of additional proteins (myrosinase epithiospecifier proteins-EPS) determine the final structure and composition of the degradation products (Shuttuck, 1993; Lambrix et al., 2001; Zabala et al., 2005). Glucosinolate degradation products possess biocidal activities because of their toxicity to a variety of pathogens and generalist herbivores (Fahey et al., 2001; Bednarek et al., 2005; Brader et al., 2006a). However, some other compounds as allylisothiocyanates and allylnitriles seem to be involved in plant–insect interactions as allelochemicals (Mueller, 2001; Wittstock et al., 2004).

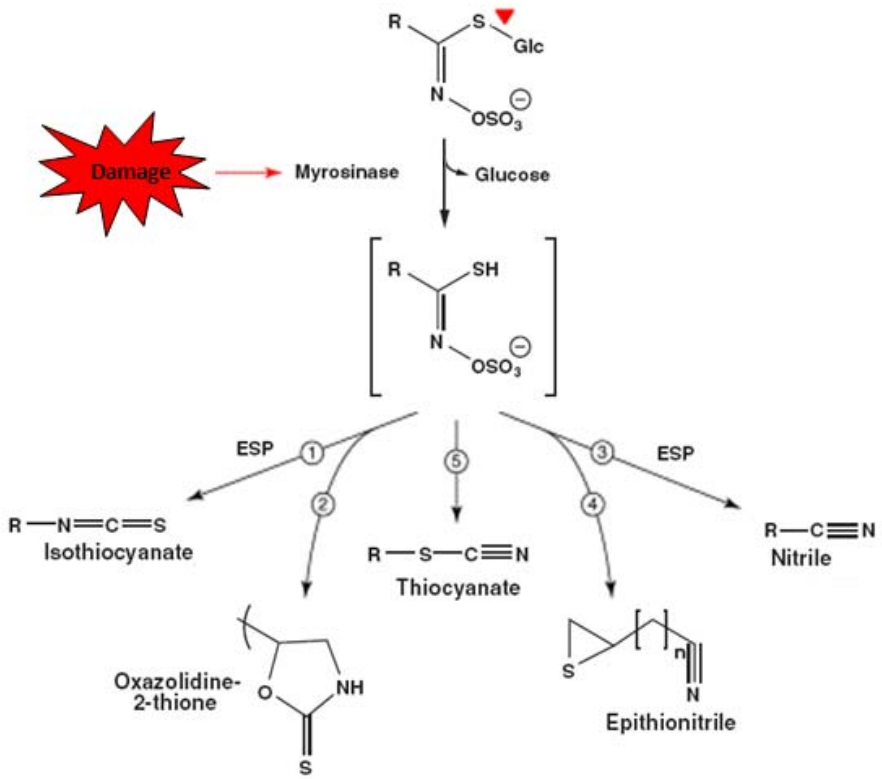

Figure 3. Scheme of glucosinolate degradation: a binary glucosinolate-myrosinase chemical defence system also known as 'mustard oil bomb'; brackets indicate an unstable intermediate – aglucone; EPS- epithiospecifier protein, R - variable amino acid side chain.

1.2.2 Agricultural, nutritional and anticancerogenic significance of glucosinolates

The wide range of biological active compounds generated by the degradation of glucosinolates via action of the glucosinolate-myrosinase system has biological and economical importance. Glucosinolate breakdown products are responsible for the biting taste of important condiments, such as horseradish and mustard, and they contribute to the characteristic flavors of many vegetables, including cabbage, broccoli and cauliflower (Mithen, 2001). The distinct taste and flavors of these foods are due primarily to isothiocyanates as glucosinolates hydrolysis products. Nevertheless, the presence of some glucosinolates in crop plants, such as oilseed rape (*Brassica napus*) and Brassica vegetables is undesirable due to the toxicological effects of their breakdown products (Fahey et al., 2001). Glucosinolates can cause problems in the feed industry because high glucosinolate plants cannot be used for feeding of farm animals (Griffiths et al., 1998). Therefore, some of the plant breeding strategies have focused on reducing the glucosinolate content of agricultural forages and rape seed plants (Sakac, 2006). In spite of this, glucosinolates found an extensive application in agriculture as “bio-fumigants” (Kirkegaard and Sarwar, 1998; Kirkegaard et al., 1998). “Bio-fumigation” is based on the same hydrolytic principle as the natural plant protection where plant material with high glucosinolate contents is incorporated into the soil to suppress soil pathogens, insects, nematodes and weeds (Zasada and Ferris, 2004; Chung et al., 2005; Vaughn et al., 2005). Thus, plants containing high amounts of glucosinolates also serve as a good remedial pre-crop for cereals (Smith et al., 2004; Vaughn et al., 2005).

Without doubt, the most important role of glucosinolates for humans is the suppression of cancerogenesis. This is caused by certain glucosinolate degradation products as isothiocyanates, nitriles, cyano-epithioalans and thiols (Hayes et al., 2008). Several studies have demonstrated that breakdown products of certain glucosinolates such as aliphatic and indole isothiocyanates have a higher anticancerogenic effect compared to thiocyanates and nitriles (Talalay and Fahey, 2001). The breakdown compounds generated by the glucosinolate-myrosinase system revealed strong effects on distinct levels of cancer development via activation

of several chemoprotective regulatory mechanisms. First of all, the degradation products of glucosinolates have blocking activities reducing the impact of environmental carcinogens in animal models (Traw et al., 2003). Isothiocyanates (ITC) are known to induce antioxidant genes and detoxification enzymes of phase II such as quinone reductase (QR), glutathione-S-transferase (GST) and glucuronosyl transferases (GT) through activation of NrF2 (NF-E2 related factor 2) and AhR (arylhydrocarbon receptor) (Hayes et al., 2008). Furthermore, degradation products of aliphatic glucosinolates have been shown to stimulate cell cycle arrest and apoptosis of cancer cells (Bonnesen et al., 2001).

However, the problem remains to interpret experimental results due to the variable composition and relatively low concentration of some glucosinolates in plant extracts. Epidemiological studies have illustrated the positive health aspects of these compounds resulting from the intake of Brassica vegetables to decrease the risk of cancer in the lung, stomach, colon and rectum (van Poppel et al., 1999). Thus, only a few glucosinolates with potential anticarcinogenic properties have been studied in more detail, especially from broccoli (London et al., 2000). For instance, the ITC sulforafan and sulforaphanin (homologue of aliphatic 4MSOB in *Arabidopsis*) can inhibit chemical cancerogenesis, protect against adenomatous polyposis and prevent UV-light-mediated skin cancerogenesis (Dinkova-Kostova et al., 2006; Shen et al., 2007). Consequently, it has been suggested that certain glucosinolates and their degradation products have potentially different influences on nutrition and processes of cancerogenesis. Therefore, there is a strong interest to regulate and optimize the concentration and composition of different glucosinolates as well as the level of individual glucosinolates in a tissue-specific manner to improve the nutritional value and pest resistance of crops. In order to regulate glucosinolates accumulation, several approaches have been proposed, mainly based on the manipulation of single genes encoding glucosinolate core pathway enzymes such as methylthioalkylmalat synthases and/or cytochrome P450 monooxygenases of the CYP79 family (Zang et al., 2008). However better results may be achieved using specific glucosinolates regulators which would allow to regulate multiple enzymes of glucosinolate biosynthetic pathways simultaneously (Flügge and Gigolashvili, 2006).

1.3 Regulation of glucosinolate biosynthesis

Much progress has recently been made in the understanding of synthesis and regulation of glucosinolates in *A. thaliana.* Analysis of glucosinolate profiles, biosynthesis, distribution and degradation in plants tissues and organs revealed a complex regulatory network controlling these processes in response to various environmental stimuli (Petersen et al., 2002; Brown et al., 2003a). Genetic studies of gain- and loss-of-function *Arabidopsis* mutants showed that glucosinolate biosynthesis is regulated at different levels and induced by several plant defence signaling pathways. Therefore, most of the studies are aimed to discover novel regulatory mechanisms of glucosinolate metabolism in order to unravel their functions in relation to the various signaling and metabolic networks.

In *A. thaliana* plants, natural variations in composition, distribution and degradation products of glucosinolates are genetically controlled by several quantitative trait loci (QTL) that encode glucosinolates core pathway and modification enzymes (Kliebenstein et al., 2001a; Kliebenstein et al., 2002a). For instance, methylthioalkylmalate synthases (MAM1-3) which control the side-chain length of different methionine-derived aliphatic glucosinolates and alkenyl/hydroxypropyl (AOP2, AOP3) dioxygenases producing alkenyl side-chains modification of glucosinolates were identified via QTL analysis and therefore both contributed to the natural variation of glucosinolate structures in *A. thaliana* (Wentzell et al., 2007). The epithiospecifer modifier loci coding EPS proteins were shown to be responsible for the production of nitriles and isothiocyanates upon glucosinolate hydrolysis (Lambrix et al., 2001; Zabala et al., 2005). Furthermore, it has been reported that stress-induced glucosinolate production is associated with the major plant defence hormone signaling pathways as methyl jasmonate (MeJA), salicylic acid (SA) and ethylene (ET) (Brader et al., 2001; Mewis et al., 2005). Analysis of mutants defective in SA, ET and MeJa hormone signaling and their responses to biotic stresses has revealed complex interactions between different signaling pathways in regulation of glucosinolate biosynthesis. Since exogenous treatment with MeJa/ACC led to accumulation of indolic and specific aliphatic glucosinolates (Brader et al., 2001; Mikkelsen et al., 2003; Wittstock et al., 2004) and SA treatment caused accumulation of some indolic glucosinolates and repression of aliphatic glucosinolate biosynthesis,

the crosstalk between signaling pathways might be important to regulate different levels and composition of glucosinolates in response to specific biotic stresses (Kliebenstein et al., 2002b; Mewis et al., 2005).

Several nuclear proteins have recently been identified as potential regulators of glucosinolate metabolism and were shown to be controlled by different signaling pathways upon environmental stimuli. The *MYB34/ATR1* together with *MYB51/HIG1* and *MYB122/HIG2* belonging to the subgroup XII of R2R3-type MYB transcription factors were shown to specifically and directly upregulate the transcription of tryptophan and indole glucosinolate biosynthetic genes. Moreover, *MYB34/ATR1*, *MYB51/HIG1* and *MYB122/HIG2* transcription factors are involved in the homeostasis between indole glucosinolates and auxin (IAA) biosynthesis (Celenza et al., 2005; Gigolashvili et al., 2007b). Interestingly, *MYB34/ATR1* and *MYB51/HIG1* participate in MeJa-mediated production of indole glucosinolates. However, a different MeJa-induced regulation of *MYB34/ATR1* and *MYB51/HIG1* is achieved via action of the *MYC2/JIN1* BHLH transcription factor. *MYC2/JIN1* has been shown to act as a positive regulator of MeJA-dependent *MYB34/ATR1* expression. Conversely, a negative effect of *MYC2/JIN1* on MeJA-dependent *MYB51/HIG1* expression was shown indicating a complex regulatory network in MeJa-mediated glucosinolate production (Dombrecht et al., 2007). Furthermore, *MYB51/HIG1* might be involved in the site-specific regulation of IAA (auxin) and/or indolic glucosinolate biosynthesis upon ethylene induction in the root meristem (Berger, 2007). Additionally, *MYB51/HIG1* expression is transiently induced by wounding demonstrating an important role in response to biotic stresses, which is similar to early responses of the *IQD1* glucosinolate regulator. *IQD1* is a nuclear-localized calmodulin-binding protein, which led to elevated levels of both aliphatic and indolic glucosinolates (Levy et al., 2005). Although *IQD1* has been reported to upregulate structural genes of the indolic glucosinolate biosynthetic pathway, aliphatic glucosinolate pathway genes such as *CYP79F1* and *CYP79F2* were repressed. This case is similar to the *AtDof1.1*/*OBP2* (DNA-binding-with-one-finger) regulator of glucosinolate biosynthesis. Over-expression of the *AtDof1.1* also led to a moderate increase in the levels of aliphatic and indolic glucosinolates (Skirycz et al., 2006). Notably, *AtDof1.1* has been shown to specifically induce the transcription of *CYP83B1* and does not seem to affect on *CYP79F1* and *CYP79F2* genes as in the case for *IQD1*.

Furthermore, *MYB51/HIG1*, *AtDof1.1* and *IQD1* expression was induced upon wounding and herbivore attack resulting in increased levels of either indolic and/or

aliphatic glucosinolates and reduced performance of generalist herbivores e. g., *Spodoptera exigua, Spodoptera littoralis* and cabbage looper *Trichoplusia ni*, respectively (Levy et al., 2005; Skirycz et al., 2006; Gigolashvili et al., 2007b). Interestingly, *MYB51/HIG1* is able to directly regulate transcription of indolic glucosinolate biosynthetic genes by interacting with their promoters in *trans*-activation assays (Gigolashvili et al., 2007b). Conversely, AtDof1.1 and IQD1 did not possess any *trans*-activation potential towards promoters of glucosinolate biosynthesis genes and additional factors seem to be required in order to regulate glucosinolate biosynthetic genes (Gigolashvili et al., 2007a).

Finally, *SLIM1*, an ethylene-insensitive3-like transcription factor, represents a link between the regulation of sulphate uptake and assimilation and glucosinolate biosynthesis (Maruyama-Nakashita et al., 2006). *SLIM1* function is required to stimulate sulphate acquisition and degradation of glucosinolates under sulphur deficiency conditions thereby activating the enzymes of glucosinolate degradation. Remarkably, *MYB34/ATR1* and several aliphatic and indolic glucosinolate biosynthetic genes such as *BCAT4, MAM1, MAML* and *CYP79B2/B3* are negatively regulated by *SLIM1* in Arabidopsis roots. However, *SLIM1* was not able to directly regulate glucosinolate biosynthesis as in case of *IQD1* or *AtDof1.1* and thus may also work in concert with other glucosinolate regulators. Undeniably, this complex regulatory network could not be solely regulated at the gene transcription level but there is also a crosstalk between different signaling components and primary metabolism.

The primary aim of this work is to discover and characterize novel regulators of glucosinolate biosynthesis mainly focusing on members of subgroup XII R2R3-MYB transcription factors. This work provides evidence for the function of MYB28, MYB29 and MYB76 transcription factors in the regulation of glucosinolate biosynthesis *in planta* and in response to environmental challenges. Specific and coordinated functions of these regulators as well as a crosstalk with previously described regulators of glucosinolate biosynthesis are described. MYB28, MYB29 and MYB76 are referred to as HAG1, HAG3 and HAG2 (HIGH ALIPHATIC GLUCOSINOLATE1, -3 and -2) and were shown to be involved in the specific regulation of aliphatic methionine-derived glucosinolate biosynthesis.

2. Materials and Methods

2.1 *Materials*

2.1.1 Chemicals, enzymes, antibiotics, media and buffers

All used chemicals were analytically pure according to the manufacturers and were obtained from Roche (Mannheim, Germany, www.roche.de), Fluka (Buchs, CH and www.sigmaaldrich.com), Merck (Darmstadt, Germany, www.merck.de) and Sigma (München, Germany, www.sigmaaldrich.com), Duchefa (Haarlem, Netherlands, www.duchefa.com). DNA oligos were designed using the Primer Express^{tm} (version 1.0) software and purchased from Metabion (Martinsried, Germany). Restriction enzymes were purchased from MBI Fermentas (St. Leon-Rot, Germany) and Promega (www.promega.com/de/). Proof readings Taq-polymerases were provided from Biorad (www.biorad.com), Stratagene (www.stratagen.com) and Qiagen (www.qiagen.de, Hilden, Germany). Reverse transcription Superscript kits were bought from Invitrogen (www.invitrogen.com) and Bioline (www.bioline.com).

Antibiotics

Antibiotic	**Dissolve**	**Stock conc. (mg/ml)**	**Final conc.(µg/ml) for E.coli and A.tumefacium**
Kanamycin	H_2O	50 mg/ml	50 µg/ml
Ampicillin	H_2O	100 mg/ml	50 µg/ml
Carbenicillin	50%EtOH/ 50%water	100 mg/ml	50 µg/ml, 100 µg/ml (*A. tumefaciens*)
Hygromycin	-	50 mg/ml	50 µg/ml
Chloramphenicol	EtOH	10mg/ml, 75mg/ml	10 µg/ml (*E. coli*); 75µg/ml (*A. tumefaciens*)
Rifampicin	DMSO	30 mg/ml	150 µg/ml (*A. tumefaciens* strain GV3101); 20 µg/ml (*A.fumefaciens* strain LBA4404.pBBR1MCS*vir*GN54D)
Gentamycin	H_2O	10mg/ml, 25mg/ml, 40mg/ml	10 µg/ml (*E. coli*); 25 µg/ml (*A. tumefaciens* strain GV3101) 40 µg/ml *(A.fumefaciens* strain LBA4404.pBBR1MCS5*vir*GN54D).
Tetracyclin	EtOH	5mg/ml	5µg/ml
Spectinomycin	H_2O	100mg/ml	100 µg/ml

Bacterial media:

All frequently used media were prepared with deionised distilled water and sterilized by autoclaving at 120°C for 20 min. To make a solid media, 1.5% of agar was added before autoclaving.

Luria-Bertani (LB) medium for the growth of E.coli, 1L

10 g/l	Tryptone
5 g/l	Yeast extract
5 - 10 g/l	NaCl
1.5 %	Agar for plating

SOC medium, 1L

2% (w/v)	Bacto tryptone
0.5% (w/v)	Yeast extracts
10 mM	NaCl
2.5 mM	KCl
10 mM	$MgCl_2$
10 mM	$MgSO_4*7H_2O$
20 mM	Glucose

YEB medium for the growth of A.tumefacium, 1L

5.0 g	Beef extract
1.0 g	Yeast extract
5.0 g	Peptone (Bacto)
5.0 g	Sucrose
0.5 g	$MgSO_4*7H_2O$
1.5%	Bacto agar for plating

Plants and cell culture growing media

1/2MS (Murashige and Skoog Basal) medium for plant growth

2.3 g/L MS (245, Duchefa, Haarlem, NL)

1% (w/v) sucrose

Set pH= 5.6 with KOH

AT medium for *Arabidopsis thaliana* cell culture

4.3 g/L MS basal salt media (Duchefa)

1 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D)

4 ml of a vitamin B5 mixture (Sigma)

30 g/L sucrose

400 mg/L

Set pH= 5.8 with KOH

2, 4-D (2, 4 – Dichlorophenoxyacetic acid, Duchefa D0911)

[1 mg/ml] 40 mg + 4 ml of KOH (dissolving), pH= 7.

Fill up to 40 ml with dd H_2O, filter-sterilized and store at -20°C

B5-Vitamine stock

100 mg	Nicotin acid, [1 mg/ml]
100 mg	Pyridoxin-HCl, [1 mg/ml]
1000 mg	Thiamin-HCl, [10 mg/ml]

Fill up to 100 ml of ddH_2O, filter-sterilized and store at -20°C

Buffers

50 x TAE buffer

2 M Tris-HCl, pH= 7.5

50 mM EDTA

1 x TE

10 mM Tris-HCl, pH= 8.0

1 mM EDTA

0,1% (v/v) DEPC water for RNA preparations

DEPC in water, left overnight with stirring at room temperature, and then autoclaved.

GUS staining buffer

Stock solutions for 100ml:

1M $NaPO_4$ (137,99), pH7	13.8 g
0.25M EDTA (372.24)	9.3 g
5mM K-Ferricyanid (329.26)	0.16 g
5mM K-Ferricyanid (422.41)	0.21 g
20mM X-Glu (498.7)	100 mg in 10ml of DMF (dimethylformamide)
10% Triton X-100 (v/v)	1 ml in 10 ml H_2O

Working solution (Premix II) for 100ml

1 M $NaPO_4$	10 ml (0.1 M)
0.25 M EDTA	4 ml (10 mM)
5 mM K-Ferricyanid (329.26)	10 ml (0.5 mM)
5 mM K-Ferricyanid (422.41)	10 ml (0.5 mM)
20 mM X-Glu	5 ml (1 mM)
10% Triton X-100 (v/v)	1 ml (0.1%)
H_2O	60 ml

Molecular biological commercial kits:

QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany)

MiniElute Gel Extraction Kit (50) (Qiagen GmbH, Hilden, Germany)

QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany)

MiniElute PCR Purification Kit (Qiagen GmbH, Hilden, Germany)

QIAgen plasmid Mini and Midi Kit (Qiagen GmbH, Hilden, Germany)

Quantum Prep Plasmid miniprep Kit (BioRad, Munchen, Germany)

BCA™ Protein Assay Kit (Pierce, Rockford, USA)

Big Dye Terminator v1.1 cycle Sequencing Kit (Applied Biosystems, Foster City, USA)

Reverse Transcriptase (Invitrogen GmbH, Karlsruhe, Germany)

2.1.2 Cloning Vectors

The Gateway vectors used for cloning are listed in the Table:

Vector	Company/Source	Cloning purpose
pENTR-D/TOPO	Invitrogen	generating a Gateway compatible entry clones for further delivery into an expression vector via directional cloning
pDONR201/207	Invitrogen	generating a Gateway compatible entry clones for further delivery into an expression vector via BP reaction
pGWB2	Dr. T. Nakagawa, Shimane University	generating expression clones under control of 35S CaMV promoter
pGWB3	Dr. T. Nakagawa, Shimane University	generating expression clones with C-terminal GUS marker gene
pGWB3i	modified from pGWB3 by B. Berger	generating expression clones with C-terminal GUS gene. GUS gene contains an intron to avoid prokaryotic bacterial gene expression
pGWB5	Dr. T. Nakagawa, Shimane University	generating expression clones with GFP marker gene

2.1.3 Bacterial organisms and plant material

2.1.3.1 Bacteria strains:

Organism	Strain	Purpose
Escherichia coli		
	DH5α	Plasmid amplification
	XL10-Gold	Plasmid amplification

	DB3.1	Propagation of plasmids containing *ccdB* gene
Agrobacterium tumefaciens		
	GV3101	Stable plant transformation
	LB A4404.pBBR1MCS *vir*GN54D and LB4404.pBBR1MCS-5.virGN54D	Leaf infiltration and cell culture transfection. Transient gene expression

2.1.3.2 Plant material:

Organism/Ecotype	Purpose
Arabidopsis thaliana (L.) *Heynh*. (*Arabidopsis*) plants	
Ecotype Columbia (Col-0)	Used as a source wild type (WT) DNA, mRNA and genotype background for creation of transgenic plants
Col-0 T-DNA insertion lines	Used to study a loss-of-function of a single protein. Salk lines were obtained from the Nottingham *Arabidopsis* Stock Center (NASC, Alonso *et al*., 2003).
Arabodopsis cell suspension culture	Used for transient gene expression assays
Tobacco plants	
Nicotiana benthamiana	Used for transient gene expression assays

2.2 *Methods*

2.2.1 Methods of manipulation with *E. coli* and *Agrobacteria*

2.2.1.1 Protocol for preparation of chemically competent E. coli DH5α cells

All media for preparation of chemically competent *E. coli* cells were prepared according to protocols listed below:

Ψ-broth media for 1 L

20 g Bacto tryptone
5 g Yeast extract
4 g $MgSO_4*7H_2O$ (0.4%)
Set pH at 7.6 with 1 M KOH; sterilize 20 min at 120°C

TfB1 for 100 ml

1.21 g $RbCl_2$ (100 mM)
0.99 g $MnCl_2*4H_2O$ (50 mM)
0.3 g KOAc (Potassiumacetate) (30 mM)
0.15 g $CaCl_2*2H_2O$ (10 mM)
15 ml glycerol 100% (15% v/v)
Set pH at 5.8 with 0.2M HOAc (acetic acid). Filtersterilise, store at 4°C.

TfB2 for 20 ml

0.0242 g $RbCl_2$ (10 mM)
0.22053 g $CaCl_2*2H_2O$ (75 mM)
3 ml glycerol 100% (15% v/v)
0.0419 g MOPS (10 mM)
Set pH at 7.0, Filtersterilise, store at 4°C

A single colony of *E. coli DH5α* strain was inoculated in 5-10 ml of *Ψ*-broth media and grown at 37°C overnight in 100 ml Erlenmeyer flask. The bacterial pre-culture was diluted with 400 ml of media in a large 2 L flask to have a good ratio between surface and volume and was grown until OD_{550}= 0.48 (2-2.5 h). Then bacterial suspension was poured in 50 ml falcon tubes, chilled on ice for 15 min and

centrifuged at 2000-2500 rpm for 10 min at 4°C. All further steps were performed in cold room and on ice. The bacterial pellet was resuspended in 1 ml of ice cooled TfB1 buffer with gentle shaking and the end volume was adjusted to 15ml in a falcon tube. After 2 h of incubation on ice, the bacterial suspension was centrifuged at 2000 rpm for 5 min at 4°C. The obtained bacterial pellet was resuspended in 2 ml of TfB2 buffer. Aliquots of 200 µl were placed in precooled Eppendorf tubes and directly frozen in liquid nitrogen.

2.2.1.2 Heat-shock transformation protocol of chemically competent E. coli cells

For transformation, 50-100 µl of competent cells were thawed on ice and mixed with 200 ng of the experimental DNA or 2-4 µl of ligation mixture. Tubes were swirled gently and incubated on ice for 30 min. After incubation, cells were heat-shocked in a 42°C water bath for 30-60 sec and then immediately transferred on ice for 2 min. Then, 800-850 µl of the SOC medium was added to the cells and incubated at 37°C for 1h with shaking at 225-250 rpm. For positive selection, 100-200 µl of transformation mixtures were plated on LB agar plates containing the appropriate antibiotic and incubated at 37°C.

2.2.1.3 Protocol for preparation of electro-competent Agrobacteria

Centrifuge tubes, falcon tubes, dd H_2O and 10% glycerin were pre-cooled on ice. Agrobacterial pre-culture was incubated overnight in 5 ml of LB or YEB media with vigorous shaking at 200-250 rpm and 28°C. Afterwards, pre-culture was mixed with 400ml of pre-warmed to RT of LB or YB media and grown at 28°C (10-12 h) until OD_{600}= 1. All further centrifugation steps were performed at 4000 rpm for 15 min and at 4°C. Flask was chilled on ice for 15-30 min and then centrifuged. The obtained pellet was resuspended in 200 ml of cold dd H_2O and centrifuged. Additionally, the pellet was washed with cold dd H_2O in a final volume of 50-100 ml and centrifuged again. Finally, the bacterial pellet was washed in 10 ml of 10% glycerol, centrifuged and subsequently resuspended in equal volume of 10% glycerol. Small volumes (50-100 µl) of cell aliquots were frozen in liquid nitrogen and stored at -80°C.

2.2.1.4 Transformation of electro-competent Agrobacteria

For one transformation, 50 µl of competent cells were placed on ice, mixed with 100-200ng of DNA vector (1-2 µl) and incubated for 2 min. After incubation, the mixture was placed into pre-cooled electoroporation chamber (2 mm gap).

Electroshock was performed at 25 µF, 400 Ω, 2.5 kV on Bio-Rad electroporator. One ml of YEB medium was immediately added to transformed cells and incubated at 28°C with shaking for 2 h. Finally, 50 µl and 150 µl of the bacterial culture was placed on selection plates with appropriate antibiotics and incubated at 28°C. Positive clones were analyzed using plasmid-specific primers by colony PCR in 2 days after incubation.

2.2.2 Plant Procedures

2.2.2.1 Seed sterilization protocol

2.2.2.1.1 Vapor-phase (gas) seeds sterilization

Required reagents:

Na-hypochlorite	12% (v/v)
Hydrochloric acid (HCl)	37% (v/v)

150-300 seeds were transferred into appropriate resealable containers, for example, microcentrifuge eppendorf tubes. A glass vessel for sterilization, typically a dessicator jar, was kept under fume hood. Tubes containing the seeds were placed on a rack and inside of a dessicator next to a beaker with 100 mL of sodium hypochlorite. Immediately prior to sealing the jar, 3 ml of concentrated HCl were added to the bleach. Seal jar and chlorine gas sterilization was allowed to proceed for 3 to 4 h up to overnight. After treatment, the chlorine gas was evaporated from the Eppendorf tubes for 1-2 hours under sterile bench.

2.2.2.1.2 Wet method

Required reagents:

1% NaOHCl

70% EtOH

Sterile dd H_20

0.1% Agarose

Seeds were transferred into 2 ml Eppendorf tubes and rinsed in 70% EtOH for 5 min. Subsequently, EtOH was removed by pipetting and then seeds were treated

with 1% NaOHCl for 5 to 15 min and centrifuged at 300rpm for 5-10min. All further manipulations were performed under the clean bench. Treated seeds were washed three times with sterile water, centrifuged and then mixed with 0.1% agarose in ratio 1:3 or 1:4 and plated on agar plates.

2.2.2.2 Plant growth conditions on soil and agar plates

Freshly collected seeds were plated equally on soil and then cold-treated at 4°C for 3 days in the dark. After stratification, seeds were covered by plastic lid to maintain high humidity during the first days. Surface sterilized seed were placed on half-strength Murashige and Skoog (MS) medium with agar and germinated in a culture chamber at 16/8 h of light/dark cycle, 75% humidity and 21°C. Afterwards, 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. For selection transgenic plants were grown on 1/2 MS medium containing 50 µg/ml of kanamycin or sprayed by BASTA on soil and, subsequently, treated as wild-type plants.

2.2.2.3 Arabidopsis stable transformation

Arabidopsis stable transformed plants were generated by *Agrobacterium*-mediated vacuum infiltration.

Infiltration medium for 500 ml:

MES/KOH, pH= 5.8	0.25 g
MS incl. modif. vitamins	1.1 g
Sucrose	25 g
6-Benzylaminopurin (BAP)	5 ng/ml
Tween 20	2 drops

Agrobacterium culture was inoculated in 5-10 ml of YEB media with antibiotics from a fresh plate and then grown for 24 to 48 hours depending on the strain. Afterwards the pre-culture was added to 300-400 ml of YEB medium with appropriate antibiotics in 4 L flask and grown until OD_{600} = 0.8-1.0. Subsequently, the suspension was centrifuged at 4000 rpm for 15 min and the obtained pellet was resuspended in

1/3 volume of infiltration medium (minimal volume of 300 ml). Agrobacterial suspension was poured into a beaker of an appropriate size and placed into the vacuum jar. Before transformation flowering *Arabidopsis* plants all siliques were removed and the plants were infiltrated by vacuum for 15-25 min. Transformed plants were covered by plastic lid and transferred into the greenhouse.

2.2.2.4 Cultivation of *Arabidopsis thaliana* cells

Arabidopsis thaliana Col-0 dark suspension culture was subcultured weekly and grown in 50 ml of *A. thaliana* (AT) medium (see 2.1.1). Dilution of the suspension cell culture was performed weekly in the ratio of 1:4 or 1:5 (10/15 ml of suspension culture and 40/35 ml of AT medium) with fresh medium. The cell culture was gently agitated at 150 rpm in the dark at 22°C.

2.2.2.5 Transformation of A. thaliana cell suspension culture

The protocol for transient transformation of cultured *A. thaliana* cells culture was modified from Koroleva et al. (2005) and described by Berger et al. (2007).

Hypervirulent Agrobacteria (LBA4404.pBBR1MCS-virGN54D or LBA4404.pBBR1MCS-virGN54D5) harbouring the reporter or effector construct and antisilencing 19K strains were grown for 24 h at 28°C in 3-5 ml of YEB medium containing antibiotics with shaking at 200 rpm. Bacterial cultures were centrifuged at 4000 rpm for 15 min and resuspended in 1 ml of AT medium. Before co-culture with *Agrobacteria*, *Arabidopsis* Col-0 suspension culture (3–7 days after previous subculture) was diluted 1:5 using fresh pre-warmed to RT medium. Hypervirulent agrobacterial strain and 19K antisilencing strain were mixed in a 1:1 ratio, and 50-60 µl of this suspension was added to 3 ml of cultured *A. thaliana* cells and grown for 3–5 days in the dark. After transfection, 1 ml of cell culture was examined for GUS activity measurements and the rest of the cells were stained with X-Gluc.

2.2.2.6 Agrobacterial infiltration of N. benthamiana leaves

1.5-2-month-old *Nicothiana benthamiana* plants were used for transient expression. Hypervirulent (LBA4404.pBBR1MCS virGN54D or LBA4404.pBBR1MCS virGN54D5) or GV3101 containing desirable constructs and antisilencing 19K

agrobacterial strains were taken from a fresh plate and grown overnight in 5 ml of YEB medium containing antibiotics. Then, agrobacterial cells were centrifuged at 4000 rpm for 15 min and the pellet was resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM 2-(*N*-morpholine)-ethanesulphonic acid, pH= 5.6) until OD_{600}= 0.7-0.8. Afterwards bacterial suspensions were mixed in equal molar ratio (1:1) with antisilencing 19K strain. Acetosyringon was added (0.15 mM, final concentration) and the suspension was incubated for 2–5 h at 30°C in a dark. The first tobacco leaves which have no round shape and a flat surface were infiltrated into abaxial side air space using a 1 ml syringe and after 3–5 days of infiltration were used for the analysis of GUS gene expression (GUS activity measurements or GUS staining).

2.2.2.7 Histochemical β-glucuronidase (GUS) activity analysis

GUS infiltration buffer (Premix II) was prepared as indicated in chapter 2.1.1. For staining the 5-bromo-4-chloro-3- indolyl-β-D-glucuronid acid (X-Gluc) was used as a substrate according to the modified protocol from Jefferson et al., 1987. The substrate X-Gluc was prepared as a stock at 20 mM in dimethylformamide (DMF), and 0.5 ml was added to the 9.5 ml of PremixII just before infiltration. Plant tissues were fixed in fixing solution (0.3% (v/v) formaldehyde, 10 mM MES, pH= 5.6, 0.3 M mannitol) for 30 min, then washed with 50 mM Na_2HPO_4, pH= 7 and, subsequently, vacuum-infiltrated for 15-20 min with GUS staining buffer and incubated overnight at 37°C. To get rid off plants pigments and to make the plant tissues transparent, 80% EtOH was used as a destaining solution. Samples were kept at 60°C overnight or boiled in microwave to accelerate the destaining process.

2.2.2.8 Plant hormone treatment and wounding

Arabidopsis seedlings (Col-O ecotype) were grown on half-strength MS media with 0.8% agar and 0.5% of sucrose for 10 days in a growth chamber at 22°C under long day conditions. After 10 days of growth, the medium was replaced by glucose-free liquid MS medium for 24 h, and then seedlings were treated with 3% glucose, 3% mannitol, methyljasmonate (MeJA, 10 μM), aminocyclopropane carboxylate (ACC, 10 μM) and salicylic acid (SA, 10 μM). Three independent sets of plants induced by plants elicitors (MeJA, ACC, SA, 3% glucose and 3% mannitol) were sampled for RNA isolation and analyzed by real-time RT-PCR.

For external mechanical stimuli, inflorescences and leaves of Col-O were wounded by simply cutting with a scalpel. Samples were collected after 1, 5 15, 30, 60 and 120 minutes after treatment and subjected to analysis by real-time RT-PCR. Injured parts of transgenic plants carrying *Promoter-GUS* constructs were collected and infiltrated for GUS staining after 3 to 5 min of wounding.

2.2.3 Microscopy and records

Analysis of fluorescence proteins were performed using a LEICA-DMRE fluorescence microscope with specific GFP, YFP and YFP-CFP filters. Nikon SMZ-U binocular stereoscope and Nikon Eclipse E800 microscope were used for recording GUS staining samples. Pictures were taken with high-resolution KY-F70 3-CCD JVC camera and recorded using the DISKUS software (www.hilgers.com). Afterwards all pictures and graphic materials were processed using the Adobe Photoshop SC2 program.

2.2.4 Extraction and HPLC/UPLC analysis of glucosinolates

Glucosinolates were extracted from 100 mg of homogenized freeze-dried rosette leaves or from 3 ml of *Arabidopsis* cell suspention culture by adding 1 ml of 80% (v/v) methanol with addition of 20 μl of 5 mM of benzyl glucosinolate as an internal standard. The supernatant was collected, and the plant material was additionally treated with 1mL of 80% (v/v) methanol. The extracts were combined and applied to DEAE Sephadex A-25 columns equilibrated with 0.5 M acetic acid/NaOH, pH= 5 and washed with 5 x 2 ml of water and 2 x 2 ml of 0.02 M acetic acid/NaOH, pH= 5. After the 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 in 6 x 1 ml of HPLC water. The eluate was lyophilized until dryness and resuspended in 300 µl of HPLC water. Samples were applied to HPLC analysis on an 1100 Series chromatograph (Hewlett-Packard, Waldbronn, Germany) or by an Acquity Ultra Performance LC system (Waters, Eschborn, Germany). For the HPCL analysis, 20 µl desulfoglucosinolates were applied to a Supelco C-18 column (Supelcosil LC-18, 5 µl 250 x 4.6 mm; Hewlett-Packard) and eluted by water using following 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).

For UPLC analysis, 5-7 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). Detection was performed at 229 nm and quantified based on response factor ((Müller et al., 2001; Brown et al., 2003b) and internal benzyl glucosinolate (www.glucosinolates.com) standard as previously described (Gigolashvili et al., 2007b).

2.2.5 Weight-gain assay with Spodoptera exigua (C. Müller, University Würzburg)

Eggs of the lepidopteran herbivore, *Spodoptera exigua* (Lepidoptera: Noctuidae), were obtained from Bayer Crop Science (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 transgenic overexpression 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.

2.3 Molecular Biology Techniques

2.3.1 Gateway® cloning technology

The entry clones were created using two different pENTR/D-TOPO and pDONR201/207 vector cloning systems; the pGWB vectors were used for generation of the expression clones. Detailed description of Gateway cloning technology is presented on the web site of Invitrogen (http://www.invitrogen.com/).

2.3.2 Primers design and cloning of artificial micro RNA

Target search and primers design of artificial plant micro RNA was performed using automated WMD2 web tool (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl?page=1). The pRS300 vector was used as a template for amplification of *Arabidopsis* artificial micro RNA precursors. Cloning and PCR amplification conditions were modified and adapted for pENTR/D-TOPO cloning system based on main protocol from Swab et.al., 2006.

PCR condition for amplification of amiRNA precursors using Pfu-Turbo (Stratagene)

[µl]	PCR components	PCR-conditions	[°C]
2	pRS300 (1:100 mini)	3min	95
1	10 pmol/µL Primer1	30sec	95
1	10 pmol/µL Primer2	30sec	54
1	dNTPs	40sec	72
4	Buffer	24x times to nr.2	
0.4	Pfu-Turbo	7min	72
30.6	PCR grade water	60min	8

Fusion PCR conditions for amplification of amiRNA using Pfu-Turbo (Stratagene)

[µl]	PCR components	PCR-conditions	[°C]
0.5	a, b, c products after gel extraction	3min	95
1	10 pmol/µL PrimerA topo	30sec	95
1	10 pmol/µL PrimerB	30sec	54
1	dNTPs	1min 30sec	72
4	Buffer	24x times to nr.2	
0.4	Pfu-Turbo	7min	72
30.6	PCR grade water	60min	8

2.3.3 DNA isolation

2.3.3.1 Plasmid isolation from E. coli cells (minipreps and midipreps)

Diatomaceous earth plasmid DNA miniprep protocol was used for small-scale DNA preparation.

Solution I (Cell Resuspension buffer)

Glucose	50 mM
Tris-HCl, pH= 8.0	25 mM
EDTA	10 mM
RNAse A (10 mg/ml)	20 µg/ml

Solution II (Alkaline Lysis Solution)

NaOH	0.2 M
SDS	1% (w/v)

Solution III (Neutralization Solution)

Guanidine-HCl	5.3 M
3M KOAc pH= 5.0	0.7 M

Binding matrix

Guanidine-HCl	5.3 M
1 M Tris pH= 8.0	20 mM
Diatomaceous earth	0.15 g/ml

Wash buffer

1 M Tris-NCl, pH=8.0	20 mM
0.5 M EDTA, pH=8.0	10 mM
5M NaCl	0.2 M
100% EtOH	50%

Elution buffer

Tris-HCl, pH=8.0	10 mM

A single colony was inoculated in 4-5 ml of LB medium with appropriate antibiotics and grown overnight at 37°C with shaking. Cells were harvested by centrifugation of 4 ml culture at 14000 rpm for 30 sec followed by resuspension in 200 µl of Solution I by vortexing. For lysis, 200µl of Solution II was added and mixed gently 10 times, and then, the cell lysate was incubated for 2-3 min at RT. For neutralization of the cell lysate, 200 µl of Solution III was added, inverted gently and incubated for 5 min on ice. Separation of cell debris from plasmid DNA was performed by centrifugation at 14000 rpm for 8-10 min. After centrifugation, the upper phase was transferred into filter columns, mixed properly by pipetting with 200 µl of Binding buffer and centrifuged. The pellet was washed twice with 500 µl of washing buffer and centrifuged at 14000 rpm for 30 sec. A second centrifugation step was performed for 2 min to remove all EtOH traces. To elute DNA, columns were transferred into a clean 1.5 ml Eppendorf tube and 100 µl of Elution buffer was applied (10 mM Tris pH= 8.0). Following centrifugation for 1 min, the mini-preps were stored at -20°C.

Large-scale plasmid (midi-prep) preparation was performed using the QIAGEN plasmid midi kit according to the manufacture's instruction (www.qiagen.com).

2.3.3.2 Genomic DNA isolation from plant material (fast prep)

Isolation of genome DNA was performed using fast method and DNAzol.

The fast protocol for DNA extraction was often used when DNA is required for regular amplifications.

Extraction buffer for fast genome DNA isolation:

20 ml 1M Tris-HCl (0.2M)

25 ml 1M NaCl

5 ml 0.05 M EDTA

5 ml 10% SDS

ad H_2O to 100ml

Frozen 50-100 mg of leaf material was pulverized and mixed with 400 µl of extraction buffer. After short vortexing for 5 sec, the probes were centrifuged at 13000 rpm for 1 min. The supernatant was transferred into the new eppendorf tube, mixed with 300 µl of isopropanol and centrifugated at 13000 rpm for 5 min. The

obtained pellet was air-dried and dissolved in 50 µl of TE buffer.

To obtain good quality and pure genome DNA, the isolation with DNAzol reagent was performed according to the manufacturer's instruction (Invtirogen, www.invitrogen.com).

2.3.4 Total RNA isolation from plant material, DNase I treatment and reverse transcription

Total RNA was extracted from rosette leaves of adult plants from the wild type and different mutant lines using TRIsure buffer (Bioline; http://www.biocompare.com) followed by the treatment with RNase-free DNase (Roth; http://www.carl-roth.de) to remove all genomic DNA contaminations. First-strand cDNA synthesis was performed at 42°C for 60 min in 50 µl of reaction mixture which contained 5 or 10 µg of total RNA, 20 pmol of oligo (dT), and 200 units of reverse transcriptase from the First-Strand cDNA Synthesis SSII Kit (Invitrogen Life Technologies) according to the manufacturer's instructions.

2.3.5 PCR - Polymerase Chain Reaction

All PCR reactions were performed on a MJ Research thermocycler (Munich, Germany).

2.3.5.1 PCR amplification using plasmid as a template

Depending on type of plasmid (low-copy or high-copy), it is recommended to dilute the DNA plasmid in a range from 1:25 to 1:100 with HPLC water or TE buffer.

1x reaction, 50 µL final volume

10x buffer (Qiagen, Stratagene, Biorad)	5 µL
50 mM MgCl2	0-2 µL
10 mM dNTPs	1 µL
10 pmol/µL primer A and B	0.5 µL each
Taq (Qiagen, Stratagene, Biorad)	1-2 units
DNA template	0.5-2 µL
HPLC to 50 µL	

Standard amplification PCR program

Step 1: initial denaturation	2.5-3 min at 94-98°C
Step 2: denaturation	30 sec at 94-98°C
Step 3: annealing	30 sec at 55°C, Tm-5°C
Step 4: extention	1 min per kb at 72°C
Step 5: polymerase deactivation	10 min at 72°C

23-32 cycles were performed from Step 2 to Step 4.

2.3.5.2 PCR amplification from genome DNA (gPCR) as a template

The gPCR frequently used for amplification of genome DNA fragments and identification of homozygous T-DNA insertion mutants.

[µl]	PCR components	PCR-conditions	[°C]
0,1-1 µl	genomic DNA	3.5-4min	95
1 µl	10 pmol/µl FW primer	30sec	95
1 µl	10 pmol/µl RV primer	30sec	55
2 µl	10 mM dNTPs	1min/kb	72
5µl	10x Buffer	32 cycles	
0,2µl	Qiagen-Taq	7min	72
38,8µl	PCR grade water	60min	8

2.3.5.3 Colony PCRs (cPCR for Agro and E. coli)

The colony PCR was used as a fast method to identify positive clones containing the desired insert. A single bacterial colony was picked up from a selective plate using a sterile toothpick and placed into one tube containing the PCR mix.

PCR condition for Agro-colony PCR with Qiagen Taq (1x reaction – 10µl end volume)

[µl]	PCR components	PCR-conditions	[°C]
1 µl	Single bacterial colony	5 min	95
0.25 µl	10 pmol/µl FW primer	30 sec	95
0.25 µl	10 pmol/µl RV primer	30 sec	55

0.1 µl	10 mM dNTPs	1 min/kb	72
1 µl	10x Buffer	32 cycles	
0.1 µl	Qiagen-Taq	7 min	72
7.3 µl	PCR grade water	60 min	8

PCR condition for *E.coli* colony PCR with homemade Taq (1x reaction -25µl end volume)

[µl]	PCR components	PCR-conditions	[°C]
1 µl	Single bacterial colony	5 min	95
1 µl	10 pmol/µl FW primer	30 sec	95
1 µl	10 pmol/µl RV primer	30 sec	55
1 µl	10 mM dNTPs	1 min/kb	72
2.5 µl	10x Buffer	32 cycles	
1.5 µl	50 mM $MgCl_2$	7 min	72
1 µl	homemade Taq	60 min	12
15 µl	PCR grade water		

2.3.6 Quantitave real time PCR (qRT-PCR)

Real-time PCR was performed using the Power SYBR Green master kit system (Applied Biosystems) according to the manufacturer's instructions in a GeneAmp® 5700 and 7300 Sequence Detection Systems (Applied Biosystems; http://www.appliedbiosystems.com). Relative quantification of expression levels was calculated using the comparative ΔCt method (manufacturer's instructions, Applied Biosystems) and normalized against the constitutively expressed actin-2 (At3g18780) gene. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is detected and used for measurements of the starting copy number of the target gene. The relative value for the expression level of each gene was calculated by the equation $Y = 2^{-\Delta\Delta Ct}$, where ΔCt is the difference between control and target products ($\Delta Ct = Ct_{(GENE)} - Ct_{(ACT)}$, and $\Delta\Delta Ct = \Delta Ct_{(mutant)} - \Delta Ct_{(wt)}$). The calculated relative expression values was standardized to the wild-type expression level (WT = 1).

2.3.7 DNA gel-electrophoresis

1-2% of agarose gel was used to electrophoretically separate DNA fragments. Agarose was mixed with electrophoresis buffer to the desired concentration, and then heated in a microwave until complete melting. Ethidium bromide was added to the gel (final concentration 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis.

2.3.8 DNA purification, gel elution and sequencing

Purification of DNA from PCR mixture was performed using QIAquick PCR Purification Kit (Qiagen GmbH, Germany) and from agarose gel the QIAquick Gel Extraction Kit was used (Qiagen GmbH, Germany) according to the manufacturer's instructions. Sequencing reactions were performed using a mixture of sequenase and fluorochrome-labeled terminators contained in the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The products were sequenced in an automated sequencer ABI PRISM™ 310 Genetic Analyzer, and the results were analyzed using the EditView and NiftyTelnet 1.1 SSHr3 programs (GCS database, release 2001, University of Cologne).

PCR sequencing reaction (1x, 10µl end volume)

PCR components	[µl]	PCR-conditions	[°C]
DNA	100-200 ng	20 sec	96
5x buffer	1µl	10 sec	96
Primer (10pmol/µL)	1µl	10 sec	55
PCR grade water	to 10µl	4 min	60
		30-35 cycles	

3. Results

3.1 Protein properties of the MYB28, MYB29 and MYB76 transcription factors and prediction of gene function using microarray database tools

The MYB28 (At5g61420), MYB29 (At5g07690) and MYB76 (At5g07700) are members of the large R2R3-MYB transcription factors family in *A. thaliana* and cluster into subgroup XII together with three other MYB factors MYB51/HIG1, MYB122/HIG2, and MYB34/ATR1 (Fig. 4). The predicted MYB28 protein has 367 amino acid residues in length, an isoelectric point of 5.71 and a molecular mass of about 41 kDa. The MYB76 and MYB29 proteins consist of 338 and 336 amino acids; the molecular weight of both proteins is about 38 kDa and the isoelectric points are 5.17 and 4.9, respectively. The MYB28 transcription factor possesses 58% and 57% identity in the amino acid level with MYB29 and MYB76, correspondingly. Several prediction programs like Pfam, SMART and PROSITE revealed that MYB28, MYB29 and MYB76 proteins contain two tandem repeats of 51 to 53 amino acids which termed as R2R3 Myb-type HTH DNA-binding domain. This repeat region is involved in DNA-binding and specifically recognize the YAAC(G/T)G sequence within the major groove of the DNA (www.expasy.org).

Previously, the MYB34/ATR1, MYB51/HIG1 and MYB122/HIG2 transcription factors were shown to be regulators of indolic glucosinolate biosynthesis and play an important role in the homeostasis between auxin and indolic glucosinolate biosynthetic pathways (Celenza et al., 2005; Gigolashvili et al., 2007b). The highly correlated expression of the *MYB28/HAG1*, *MYB76/HAG2* and *MYB29/HAG3* transcription factors with glucosinolate structural genes and their phylogenetic association with the *MYB34/ATR1, MYB51/HIG1* and *MYB122/HIG2* factors was considered as indication for the similarity in the biological functions.

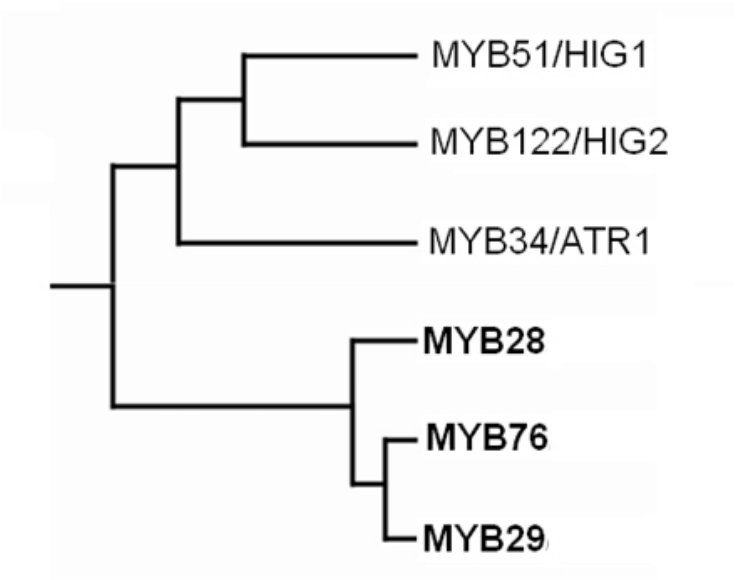

Figure 4. Phylogenetic tree of subgroup XII R2R3-MYB transcription factors (Stracke, 2001).

According to the gene expression microarray data MYB28, MYB76 and MYB29 transcription factors were highly associated and co-expressed with structural aliphatic glucosinolates biosynthetic genes, e.g. BCAT4, MAM1, CYP79F2, CYP83A1 etc. (Tab. 1). Therefore, a possible function for the MYB28, MYB29 and MYB76 transcription factors in the regulation of aliphatic glucosinolate biosynthesis was suggested based on the public microarray-based co-expression database (Toufighi et al., 2005).

Table 1. Microarray Gene Angler dataset reveals co-expression of the *MYB28, MYB29 and MYB76* transcription factors with glucosinolate biosynthesis and sulphate assimilation genes (http://bar.utoronto.ca).

AGI-ID	r-value	Annotation
At5g61420	**1.000**	**AtMYB28 transcription factor**
At4g13770	0.827	CYP83A1 (Cytochrome P450 monooxygenase family 83)
At5g23010	0.800	MAM1 (2-isopropylmalate synthase 3)
At1g16400	0.886	CYP79F2 (Cytochrome P450 monooxygenase family 79)
At2g31790	0.807	UDP-glucoronosyl/UDP-glucosyl transferase family protein

At3g19710	0.795	BCAT4 (branched-chain aminotransferase4)
At4g03060	0.784	AOP2 (alkenyl hydroxalkyl producing2)/ oxidoreductase
At4g39940	0.771	APK2 (APS-kinase 2);
At1g24100	0.693	UGT74B1 (UDP-glucosyl transferase 74B1);
At5g07690	0.671	AtMYB29_PMG2_HAG3 transcription factor
At1g62560	0.67	flavin-containing monooxygenase family protein
At1g18590	0.61	AtSOT16 (sulfotransferase family protein)
At5g23020	0.41	MAML/MAM3 (2-isopropylmalate synthase 2)
At2g14750	0.34	APK1 (APS-kinase 1)
At5g07690	**1.000**	**AtMYB29 transcription factor**
At1g62560	0.891	flavin-containing monooxygenase family protein
At1g18590	0.837	sulfotransferase family protein
At4g13770	0.834	CYP83A1_REF2 (Cytochrome P450 83A1);
At3g19710	0.813	BCAT4 (Branched-chain aminotransferase4)
At4g03060	0.793	AOP2 (Alkenyl hydroxalkyl producing 2);
At5g23010	0.751	MAM1 (2-isopropylmalate synthase 3);
At2g20610	0.745	SUR1_ALF1 (SUPERROOT 1);
At4g39940	0.731	AKN2 (APS-kinase 2);
At5g07700	**1.000**	**AtMYB76 transcription factor**
At4g03060	0.815	AOP2 (Alkenyl hydroxalkyl producing 2);
At5g07690	0.776	AtMYB29_HAG3 (myb domain protein 29);
At5g23010	0.762	IMS3_MAM1 (2-isopropylmalate synthase 3);
At1g62560	0.747	flavin-containing monooxygenase family protein
At4g13770	0.731	CYP83A1_REF2 (Cytochrome P450 83A1);

3.2 Subcellular localization of the MYB28, MYB29 and MYB76 transcription factors

The online prediction programmes like UniProtKB/Swiss-Prot (http://www.expasy.org/uniprot/) and LOCtree (Nair and Rost, 2005) web-programs were used to get information about the subcellular localization of MYB28, MYB29 and MYB76. Both prediction programs indicated a clear nuclear localization for MYB28,

MYB29 and MYB76 transcription factors. Amino acid residue sequences like LKKRL (LKKLR) and LKKLL were detected in MYB28, MYB29 and MYB76 proteins sequences and were supposed to act as SV40-type nuclear localization signals. To obtain experimental evidences for the subcellular localization of MYB28, MYB29 and MYB76, the full-length cDNAs were cloned into the GFP fusion vector pGWB5 conferring a C-terminal GFP under control of the *CaMV35S* promoter. For transient expression, cultured cells of *Arabidopsis* (Col-0) were transformed using the supervirulent Agrobacterium strain LBA4404.pBBR1MCS.virGN54D (Koroleva et al., 2005) carrying *Pro_{35S}:MYB28:GFP, Pro_{35S}:MYB29:GFP* and *Pro_{35S}:MYB76:GFP* constructs respectively. The obtained results clearly indicate that all three MYB factors are targeted to the nucleus, i.e. MYB28, MYB29 and MYB76 are nuclear-localised proteins, thereby supporting the hypothesis to act as transcriptional regulators (Fig. 5). The DNA specific DAPI staining was applied for nuclear staining and BCAT4 protein was used as a cytosolic control (Schuster et al., 2006).

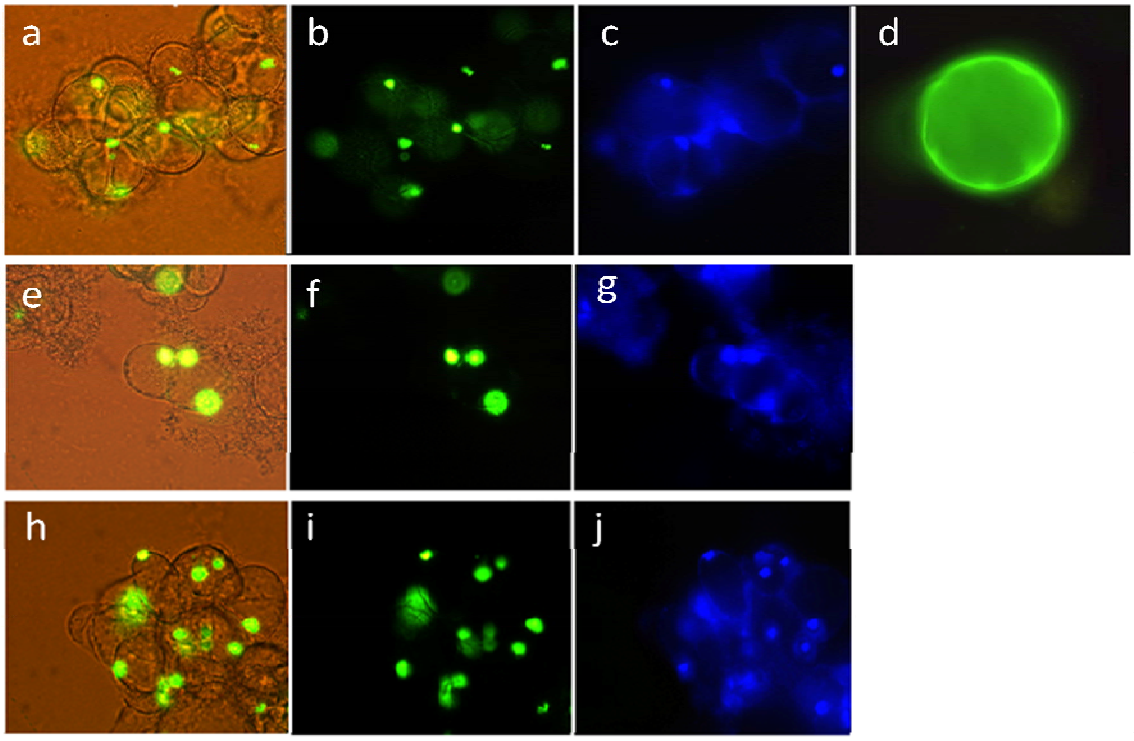

Figure 5. Subcellular localization of the MYB28-GFP *(a, b, c),* MYB76-GFP *(e, f, g),* MYB29-GFP *(h, I, j)* and BCAT4-GFP *(d)* translational fusion proteins in cultured *A. thaliana* cells: (a, e, h)-bright field with GFP; (b, d, f, i)- GFP filter only; (c, g, j)- DNA specific DAPI staining.

3.3 *Generation* of *Pro$_{35S}$:MYB28, Pro$_{35S}$:MYB29, Pro$_{35S}$:MYB76* gain-of-function mutants: expression of transgenes and growth phenotype

To study the role of MYB28, MYB29 and MYB76 proteins *in planta*, *A. thaliana* plants over-expressing these genes under the control of the *CaMV35S* promoter were generated using Gateway-compatible vectors and *A. tumefaciens*-mediated transformation. Several independent transgenic lines with stable gene expression from each transformation were selected by qRT-PCR. Subsequently, three representative over-expression lines for *MYB28* (*Pro$_{35S}$:MYB28-11, -12, -15*), *MYB76 MYB29* (*Pro$_{35S}$:MYB76-6, -12, 42*) and (*Pro$_{35S}$:MYB76-5, -6, -23*) with different steady-state mRNA levels were assayed to study metabolite content by HPLC/UPLC (Fig. 6).

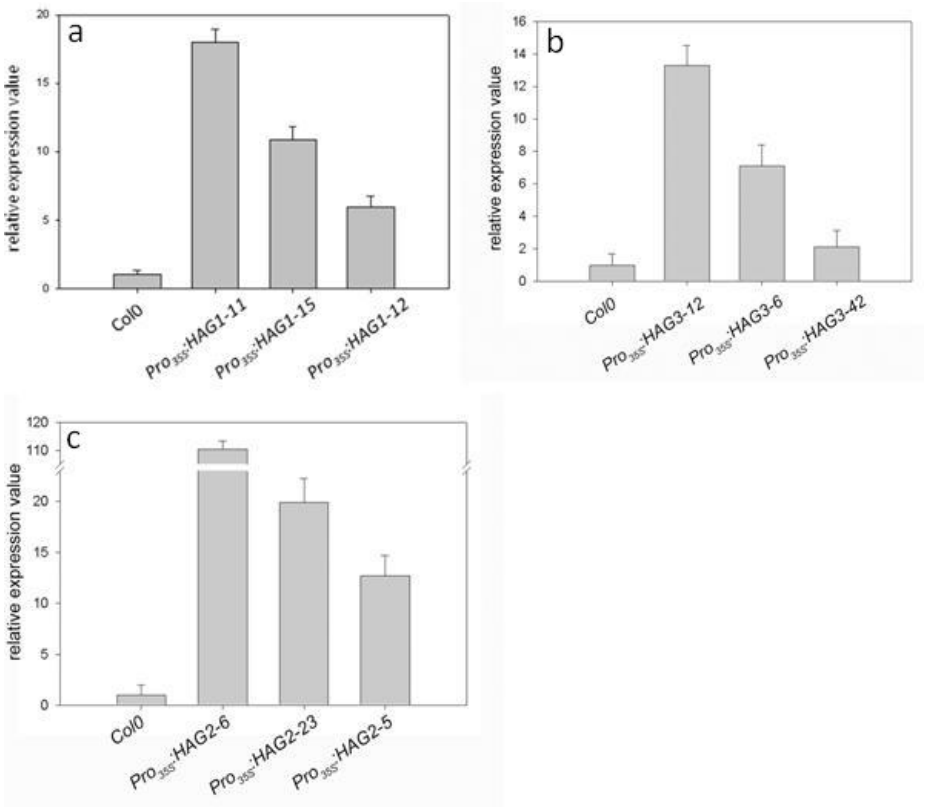

Figure 6. Relative gene expression levels of *MYB28*, *MYB29* and *MYB76* in rosette leaves of 4-week-old over-expression and wild-type Arabidopsis thaliana plants (WT=1). (a) *MYB28* transcript levels in *Pro$_{35S}$:MYB28* over-expression lines; (b) *MYB29* transcript levels in *Pro$_{35S}$:MYB29* over-expression lines; (c) *MYB76* transcript levels in *Pro$_{35S}$:MYB76* over-expression lines.

The transcript levels of these lines was determined by qRT-PCR using the fluorescent intercalating dye Power SYBR-Green in a GeneAmp® 5700 and 7300 Sequence Detection Systems (Applied Biosystems).

Further analysis of transgenic plants over-expressing the *MYB28* and *MYB29* genes revealed different growth phenotypes (Fig. 7). As evidenced by real-time RT-PCR, different levels of MYB28 transcript led to the development of moderate or strong growth phenotypes. The strongest *MYB28* over-expression lines (*Pro$_{35S}$:MYB28-1, -2)* showed a bushy-like phenotype: plants revealed a dramatic reduction in size and flowered earlier than the wild-type (Col-O) plants (Fig. 7B). Mutant plants with moderately increased levels of *MYB28* mRNA, e.g. *Pro$_{35S}$:MYB28-12* and *-15* lines were slightly retarded in growth compared to WT (Col-O) plants (Fig. 7A). Similarly, the phenotype of strong *MYB29* over-expression plants (Fig. 7B) reminded of the bushy growth phenotype of *MYB28* over-expression plants, whereas a moderate over-expression of *MYB29* caused only slight growth retardation in *Pro$_{35S}$:MYB29-6, -42* lines. Conversely, all *MYB76* over-expression lines possessed an unchanged growth phenotype (Fig. 7A).

3.4 The glucosinolate profiling of *MYB28, MYB29* and *MYB76* over-expression lines

The content of aliphatic and indolic glucosinolates was measured in over-expression lines with different steady-state transcript levels of *MYB28*, *MYB29* and *MYB76* genes (Fig. 6; Fig. 8). Freeze-dried rosette leaves of 4-week-old plants were used for the isolation of aliphatic and indolic glucosinolates (GSL). The levels of aliphatic glucosinolates such as 3MSOP (3-methylsulfinylpropyl-GSL), 4MSOB (4-methylsulfinylbutyl-GSL), 5MSOP (5-methylsulfinylpentyl-GSL), 4MTB (4-methylthiobutyl-GSL), 8MSOO (8-methylsulfinyloctyl-GSL) and the main indolic glucosinolate like I3M (indol-3-ylmethyl-GSL) were determined using HPLC analysis.

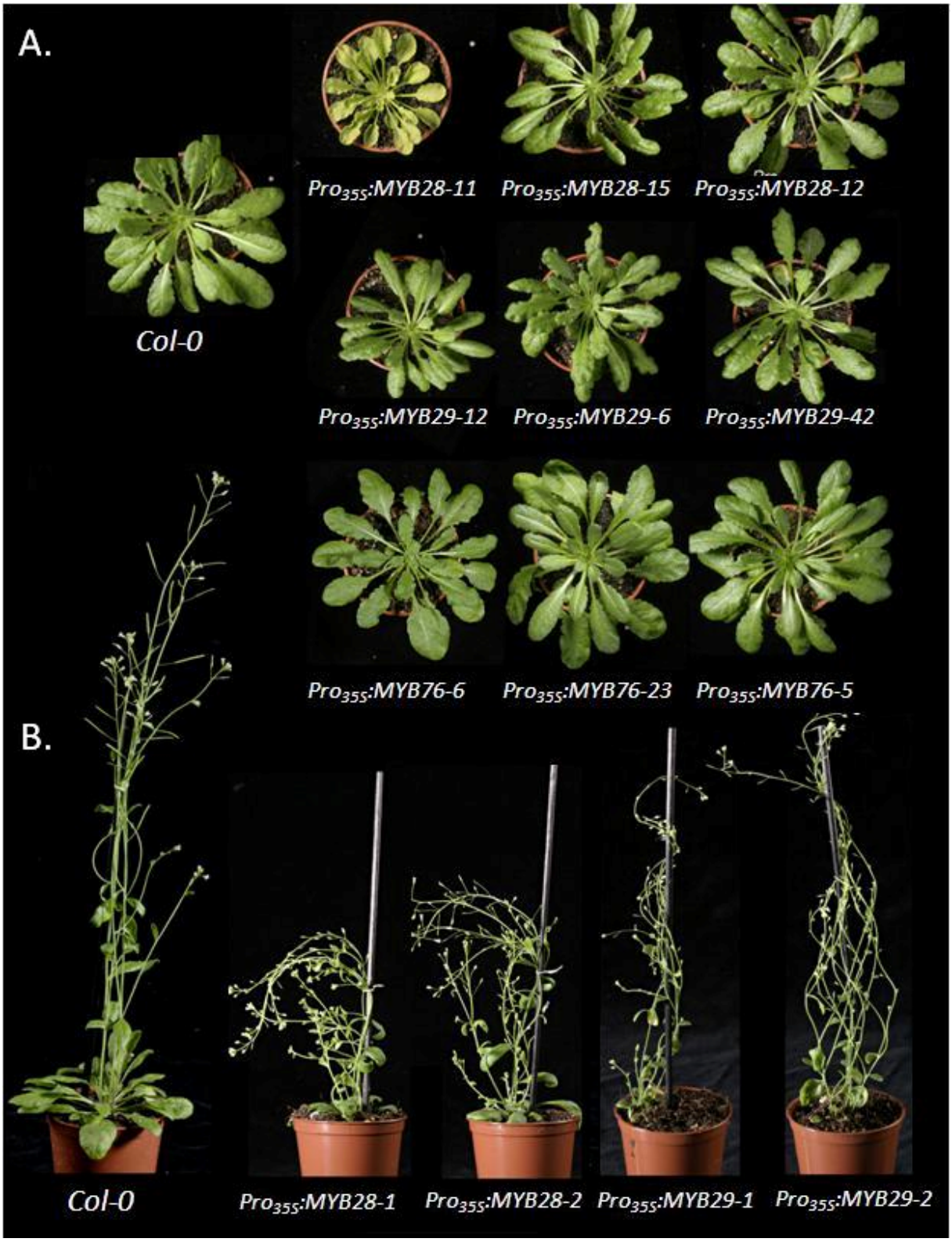

Figure 7. Growth phenotypes of (A) *Pro$_{35S}$:MYB28* (lines 11, 15, 12), *Pro$_{35S}$:MYB29* (lines 12, 6, 42), *Pro$_{35S}$:MYB76* (lines 6, 23, 5) over-expression plants; (B) strong *Pro$_{35S}$:MYB28* (line1, 2) and *Pro$_{35S}$:MYB29* (line1, 2) over-expression lines. All transgenic lines are in the Col-O wild-type background.

All transgenic MYB28, MYB29 and MYB76 over-expression lines possessed an altered Met-derived glucosinolate content compared to wild-type (Col-O) plants. Metabolic analysis of the *MYB28* over-expression lines (*Pro$_{35S}$:MYB28-11, -15* and *-12)* showed approximately two- to seven-fold higher levels of the main short-chain aliphatic glucosinolate 4MSOB while the levels of 3MSOP and 5MSOP glucosinolates were increased two- to three-fold in comparison with the wild-type (Col-O) plants. The content of long-chain aliphatic glucosinolates like 8MSOO remained unchanged or slightly decreased in *MYB28* over-expression lines. Obviously, the different content of aliphatic glucosinolates in *MYB28* over-expression lines correlated nicely with the corresponding *MYB28* transcript level. For example, the *Pro$_{35S}$:MYB28-11* line representing strong over-expression of the *MYB28* gene contained a higher amount of aliphatic glucosinolates than the moderate over-expression *Pro$_{35S}$:MYB28-12* and *Pro$_{35S}$:MYB28-15* lines (Fig. 6a; Fig. 8a).

Analysis of *MYB29* over-expression plants showed an increased content of both short- and long-chain aliphatic glucosinolates. The content of 4MSOB was increased two- to four-fold while the level of 3MSOP and 5MSOP was approximately two to six times higher compare to wild-type (Col-O) plants. The level of the long-chain aliphatic glucosinolate 8MSOO was three to four times higher than in the wild-type (Col-O) plants. Furthermore, the increased level of the long-chain aliphatic glucosinolate 8MSOO correlated nicely with the *MYB29* transcript level and 8MSOO mainly accumulated in the strong *Pro$_{35S}$:MYB29-6* and *Pro$_{35S}$:MYB29-12* over-expression lines. The line *Pro$_{35S}$:MYB29-42* with an only two-fold increase in the *MYB29* transcript level did not show any changes in 8MSOO content (Fig.6b; Fig. 8b).

Over-expression of *MYB76* showed an increase in levels of both short-chain and long-chain aliphatic glucosinolates in comparison with wild-type plants. However, a much higher transcript level of *MYB76* was required to obtain the increases in glucosinolate contents compared to *MYB28* and *MYB29* regulators. For example, line *Pro$_{35S}$:MYB76-6* showing 100-fold higher transcript levels showed an only three- to four-fold higher content of 4MSOB than the wild-type whereas 14-times increased transcript level of *MYB28* or *MYB29* caused the same effect (Fig. 6c; Fig. 8c). Likewise, the levels of the short-chain (3MSOP and 5MSOP) and long-chain (8MSOO) glucosinolates were increased only two- to three times in *MYB76* over-expression lines (Fig. 8c). The amount of main indolic glucosinolate such as I3M was

almost unchanged in over-expression lines with moderate or slight increases in steady-state transcript levels of *MYB28* (*Pro$_{35S}$:MYB28-15* and *-12* lines) and *MYB29* (*Pro$_{35S}$:MYB29-42* and *-6* lines). Moreover, the level of the main indolic glucosinolate I3M was even decreased in the strong *MYB28* and *MYB29* over-expression lines (Fig. 6a, b; Fig. 8a, b). Interestingly, the over-expression of *MYB76* resulted not only in the accumulation of aliphatic glucosinolates but also led to two- to fourfold increase in the level of the indolic glucosinolate I3M (Fig. 8c). Thereby, in contrast to *MYB28* and *MYB29* regulators, the over-expression of *MYB76* caused only moderate effects on the accumulation of short and long-chain aliphatic glucosinolates and resulted as well in accumulation of indole glucosinolates.

On the basis that over-expression of *MYB28, MYB29* and *MYB76* cause accumulation of aliphatic glucosinolates we rename these genes *HIGH ALIPHATIC GLUCOSINOLATE (HAG) 1, 3,* and *2*, that is, *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2*.

3.5 Creation of *MYB28/HAG1-RNAi* plants and isolation of *myb29/hag3* and *myb76/hag2* T-DNA insertion mutants

The loss of *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* functions *in planta* was elucidated via analysis of *MYB28/HAG1-RNAi* knock-down, *myb29/hag3* and *myb76/hag2* knock-out mutants. To study MYB28/HAG1 function *in planta*, a silencing construct was generated using the gateway-compatible *pJawohl17* vector (kindly provided by I. Somssich, MPI for Plant Breeding Research, Cologne, Germany). A specific target region for silencing of the *MYB28/HAG1* gene was designed and two complementary 5'-3' and 3'-5' fragments were amplified from wild-type cDNA using Gateway *att*B1- and *att*B2- extended primers (attB1-TTAATGGCTTCACTGAGCAGATTC; attB2-TGATGAGACTTCTTGGGAAACATC). The DNA fragment was cloned into the pDONR-207 vector (Invitrogen Life Technologies) and, subsequently, recombined from the entry clone into the *pJawohl17* destination vector using a LR reaction (Invitrogen Life Technologies).

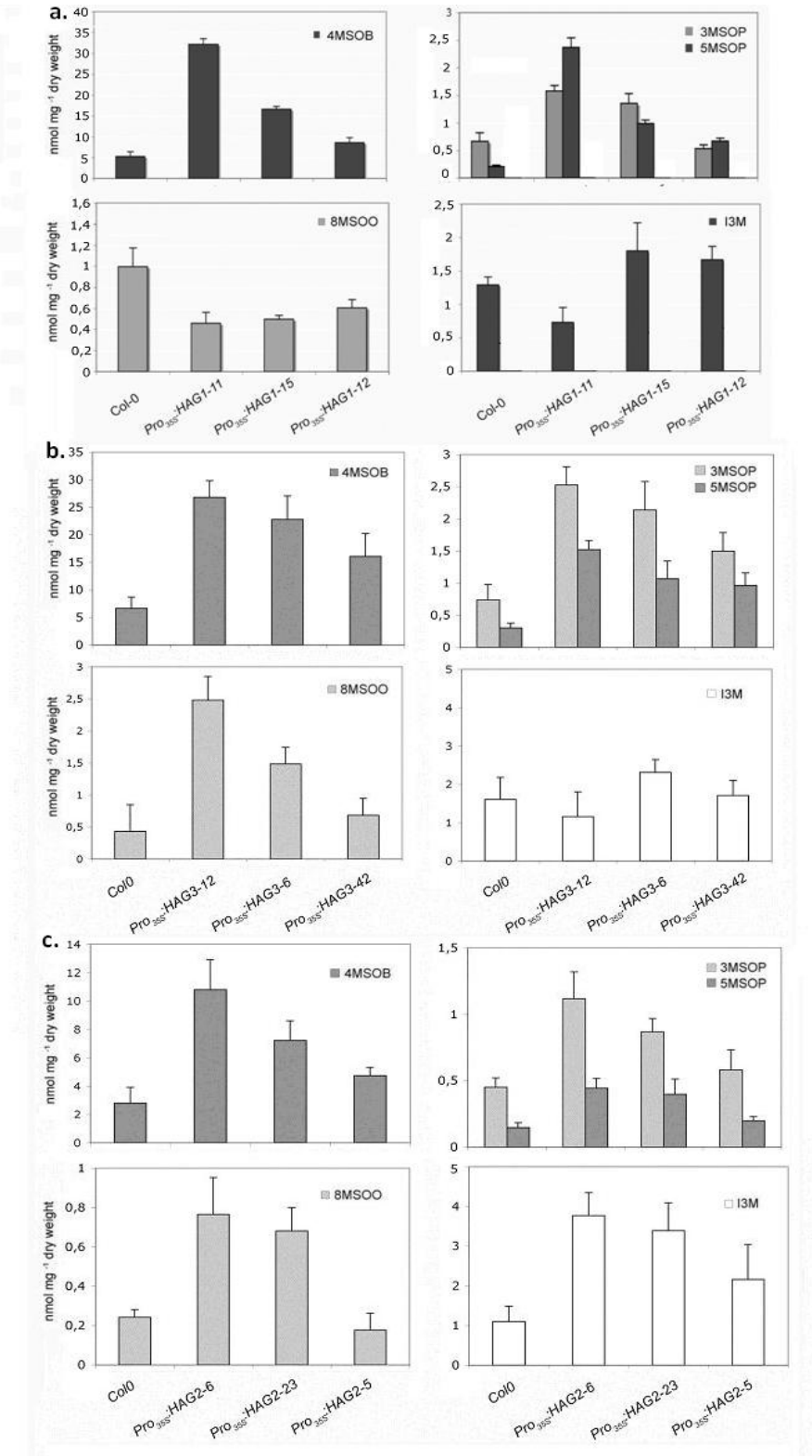

Figure 8. Glucosinolate contents in rosette leaves of 4-week-old *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* over-expression plants. (a) Glucosinolate contents in *Pro$_{35S}$:HAG1-11, -15* and *-12* over-expression plants, (b) Glucosinolate contents in *Pro$_{35S}$:HAG3-12, -6* and *-42* over-expression plants, (c) Glucosinolate contents in *Pro$_{35S}$:HAG2-6*, *-23* and *-5* over-expression plants; means ± SD, *n* = 5 (4MSOB, 4-methylsulfinylbutyl-GSL; 3MSOP, 3-methylsulfinylpropyl-GSL; 5MSOP, 5-methylsulfinylpentyl-GSL; 8MSOO, 8-methylsulfinyloctyl-GSL; I3M, indol-3-yl-methyl-GSL).

To monitor the *MYB28/HAG1* transcript level, several stable transformed lines were analysed by semi-quantitative RT-PCR analysis. Three RNAi lines (*HAG1-RNAi-10, -14*, and *-12)* with decreased levels of *MYB28/HAG1* transcripts were isolated and subsequently used for further analysis (Fig. 9A). Notably, the *MYB28/HAG1-RNAi* plants did not show any visible effects on plant morphology compared to wild-type (Col-O) plants.

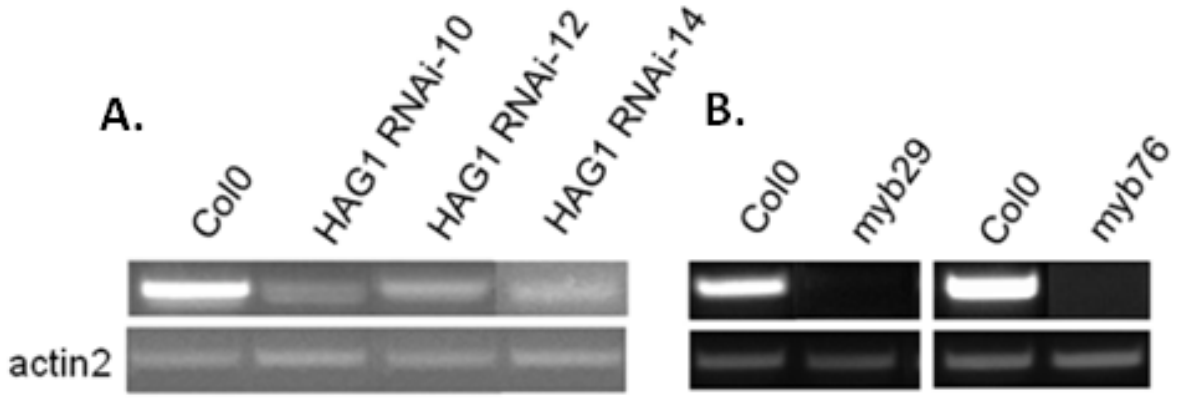

Figure 9. RT-PCR analysis of samples taken from wild-type (Col-0), *MYB28/HAG1-RNAi*, *myb29/hag3* and *myb76/hag2* knock-out plants.

The T-DNA insertion mutants of *MYB76/HAG2* (SALK line N55242 harbouring the insertion in the first exon) and *MYB29/HAG3* (GABI-KAT line GK-040H12 harbouring a T-DNA insertion in the third exon) were isolated by PCR using gDNA and the transcript level of disrupted genes was verified by semi-quantitative RT-PCR (analysis *of myb29* knock-outs was performed by M. Engqvist, AG Flügge). The *MYB76/HAG2* and *MYB29/HAG3* transcripts were not detectable in homozygous mutants indicating complete knock-outs of the corresponding genes (Fig. 9B). Similar to *MYB28/HAG1-RNAi* plants, single *myb29/hag3* and *myb76/hag2* knock-out mutants showed no visible effects on growth phenotype compared to wild-type plants (data not shown).

3.6 The glucosinolate content of *MYB28/HAG1-RNAi* plants, *myb29/hag3* and *myb76/hag2* knockout mutants

Metabolite analysis of *MYB28/HAG1-RNAi*, *myb76/hag2* and *myb29/hag3* loss-of-function mutants displayed a considerable reduction in the content of different aliphatic glucosinolates. However, the level of the indolic glucosinolate I3M remained unaffected in loss-of-function mutants. Analysis of *MYB28/HAG1-RNAi* plants showed that these lines contained lesser amounts of both long- and short-chain aliphatic glucosinolates, namely of 4MSOB, 3MSOP, 5MSOP and 8MSOO, compared to Col-O plants. The *myb29/hag3* mutant contained only reduced levels of short-chain aliphatic glucosinolates in leaves such as 3MSOP, 4MSOB and 5MSOB, while the level of long-chain aliphatic glucosinolate (8MSOO) was unaltered. Conversely, the *myb76/hag2* mutant did not reveal changes in short- and long-chain glucosinolate contents except for short-chain aliphatic 3MSOP and 4MSOB glucosinolates which were slightly decreased in *hag2* plants (Tab. 2).

Table 2. Glucosinolate contents in rosette leaves of 4-week-old *MYB28/HAG1-RNAi*, *myb76*/*hag2* and *myb29*/*hag3* knockout plants in comparison with Col-O plants (4MSOB, 4-methylsulfinylbutyl-GSL; 3MSOP, 3-methylsulfinylpropyl-GSL; 5MSOP, 5-methylsulfinylpentyl-GSL; 8MSOO, 8-methylsulfinyloctyl-GSL; I3M, indol-3-yl-methyl-GSL; *, $P < 0.05$).

Glucosinolate content	*MYB28/HAG1-RNAi*		*myb29/hag3*		*myb76/hag2*	
	WT±SD	RNAi±SD	WT±SD	*hag3*±SD	WT±SD	*hag2*±SD
3MSOP	0.66±0.15	0.02±0.01	0,74±0,24	0,59*±0,10	0,45±0,07	0,39*±0,09
4MSOB	5.20±1.4	0.39±0.14	6,97±2,03	5,08*±1,11	2,80±1,11	2,19±0,89
5MSOP	0.21±0.11	0.02±0.095	0,30±0,07	0,19*±0,06	0,15±0,04	0,10±0,06
8MSOO	0.82±0.31	0.10±0.04	0,43±0,05	0,42±0,04	0,24±0,04	0,23±0,07
I3M	1.29±0.2	1.26±0.18	1,60±0,34	1,68±0,39	1,11±0,39	1,32±0,26

3.7 Transcription profiling of *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* gain- and loss-of-function mutants

Since metabolite analyses of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* gain-of-function and loss-of-function mutants revealed increased and decreased levels of aliphatic glucosinolates, the question arisen whether the transcription of aliphatic glucosinolate structural biosynthetic genes is under the control of MYB/HAG transcription factors. To monitor the steady-state mRNA levels of aliphatic glucosinolate biosynthetic genes, the gain- and loss-of-function *MYB/HAG* mutants were assayed by quantitative RT-PCR. The transcript profiles of the strongest *MYB28/HAG1*, *MYB76/HAG2* and *MYB29/HAG3* over-expression lines (*Pro$_{35S}$:HAG1-11, Pro$_{35S}$:HAG2-6* and *Pro$_{35S}$:HAG3-12*), knockdown *MYB28/HAG1* RNAi, knock-out *myb28/hag3* and *myb76/hag2* plants were analysed in detail. As potential targets for *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2*, the aliphatic glucosinolate biosynthetic genes starting from *MAM1, MAML (MAM3)* to the last enzymes *AtSt5b, AtST5c* and the indolic glucosinolate biosynthetic gene *TSB1* as a negative control were considered.

According to the real-time RT-PCR data, elevated transcript levels of aliphatic glucosinolate biosynthetic genes like *MAM1, MAML (MAM3), CYP79F1, CYP79F2, CYP81A1, AtSt5b* and *AtSt5c* were detected in *Arabidopsis* mutants over-expressing the *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* transcription factors compared to wild-type (Col-O) plants (Fig. 10a). Moreover, the increase in the mRNA steady-state levels of aliphatic glucosinolate biosynthetic genes corresponded nicely to the expression levels of *MYB/HAG* regulators and is consistent with increased contents of aliphatic glucosinolates. Indeed, the highest transcript levels of aliphatic glucosinolate biosynthetic genes were detected in *Pro$_{35S}$:HAG1-11* and *Pro$_{35S}$:HAG3-12* over-expression lines, that also possessed the highest amounts of aliphatic glucosinolates. In contrast, the *Pro$_{35S}$:HAG2-6* over-expression line revealed an about five- to eight-fold upregulation of the potential target genes and accumulated less aliphatic glucosinolates than *MYB28/HAG1* and *MYB29/HAG3* over-expression lines (Fig. 6c; Fig. 10a).

Transcript analysis of *MYB28/HAG1-RNAi* knock-down plants, *myb76/hag2* and *myb29/hag3* knockout mutants revealed considerable decreases in the transcript

levels of all aliphatic glucosinolate biosynthetic genes compared to wild-type (Col-O) plants (Fig. 10b, c) and correlated with decreased contents of aliphatic glucosinolates in these lines as well (Tab.2). Remarkably, the decrease in *MYB28/HAG1* mRNA level caused downregulation of all aliphatic glucosinolate biosynthetic genes in the respective RNAi lines (Fig. 9A, 10b). As an example, the most strongly affected *HAG1-RNAi-10* line contained lesser amounts of aliphatic glucosinolates due to the considerably low transcript level of glucosinolate biosynthetic genes. This is in contrast to the *HAG1-RNAi-12* and *HAG1-RNAi-14* lines that showed only a partial decrease in the *MYB28/HAG1* transcript level (Fig. 10b).Although the effect was not so pronounced as in case of the *HAG1-RNAi-10* line, mutants defective in MYB29/HAG3 and MYB76/HAG2 function also showed a decrease in the transcript level of aliphatic glucosinolate biosynthetic genes (Fig. 10c).

Notably, the transcript level of the indole glucosinolate *TSB1* gene remained unchanged in over-expression lines with moderately increased levels of *MYB/HAG* regulators. Conversely, the transcript level of *TSB1* gene was slightly increased in *MYB28*/*HAG1-RNAi* plants, *myb29/hag3* and *myb76/hag2* knock-out mutants (Fig. 10b, c).

3.8 MYB28/HAG1, MYB29/HAG3 and MYB76/HAG2 *trans*-activate glucosinolate biosynthetic and sulphate assimilation genes

Transient *trans*-activation assay was used as a tool to assess activation potential of the MYB/HAG transcription factors on glucosinolate biosynthetic genes. For this, reporter constructs containing promoters of aliphatic glucosinolate biosynthetic genes as *MAM1*, *MAML, CYP79F1, CYP79F2, CYP83A1, C-S lyase* and indolic glucosinolate biosynthetic gene *ASA1* as a negative control were cloned into the pGWB3i vector containing the *uidA* (*GUS*) reporter gene. As effector constructs, the *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* were cloned into the pGWB2 vector under control of the *CaMV35S* promoter.

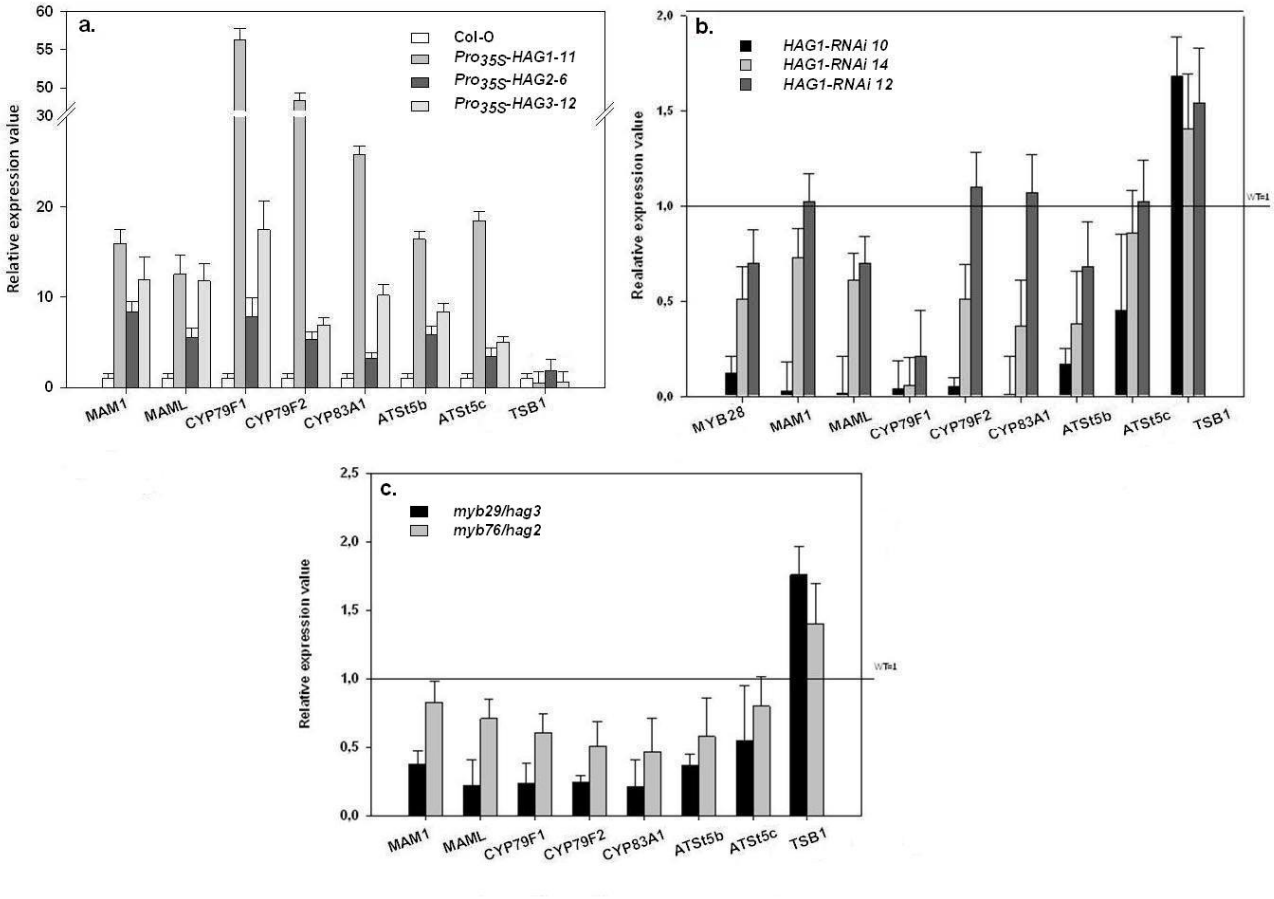

Figure 10. Transcript levels of glucosinolate biosynthetic genes in rosette leaves of 4-week-old *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* gain-of-function mutants (a) and *MYB28/HAG1-RNAi* (b), *myb29/hag3* and myb76/*hag2* knock-out plants (c). Relative gene expression values are shown in comparison with the wild-type (Col-O) = 1.

Simultaneously, reporter and effector constructs were co-expressed in cultured *A. thaliana* cells. The *trans*-activation potential of transcription factors towards promoters of aliphatic and indolic glucosinolate biosynthetic genes was estimated by measurements of GUS activity and determined histochemically, using X-Gluc staining (Berger et al., 2007).

The transient expression of the reporter construct fused to promoters of candidate target genes without an effector construct revealed only very faint GUS activity. However, *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* were able to activate *in trans* all promoters of aliphatic glucosinolate biosynthetic pathway genes such as *MAM1, MAML, CYP79F1, CYP79F2, CYP83A1* and C-S lyase (Fig. 11).

Furthermore, their *trans*-activation capacities were different towards some biosynthetic genes such as *MAM1, MAML, CYP79F1* and *CYP79F2*. The results indicated that MYB28/HAG1 and MYB29/HAG3 had a higher *trans*-activation potential towards the promoter of *MAM1* gene than MYB76/HAG2 (Fig. 11). On the contrary, the promoter fragment of *MAML* was less upregulated than *MAM1* and differently affected by all *MYB/HAG* transcription factors. Our results clearly show that the promoter of *MAML* was stronger activated *in trans* mainly by the action of MYB28/HAG1, whereas the MYB29/HAG3 and MYB76/HAG2 factors showed less capacity to activate the *MAML* promoter (Fig. 11).

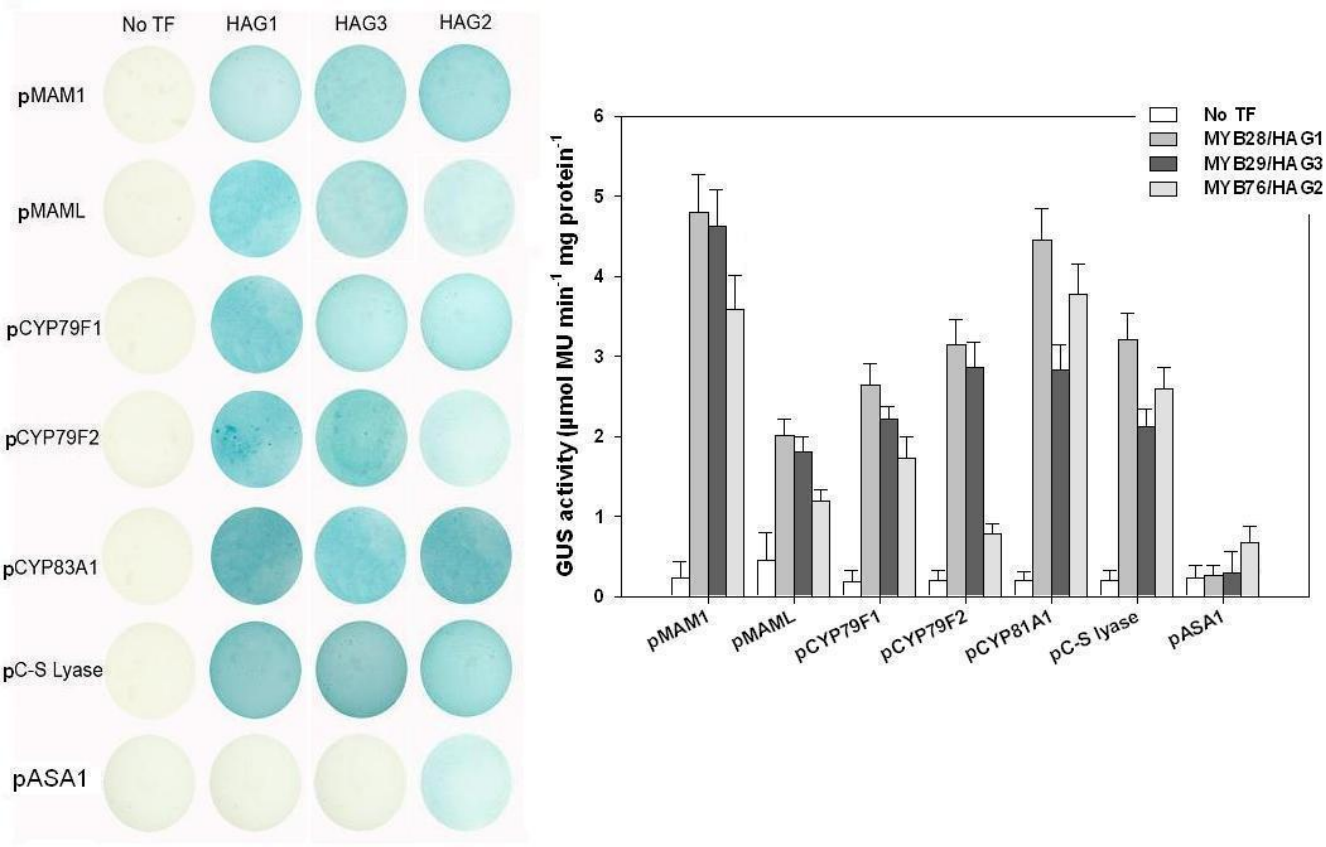

Figure 11. *Trans*-activation assays to determine the target gene specificity of *MYB28/HAG1*, *MYB29/HAG3,* and *MYB76/HAG2* towards target promoters of glucosinolate biosynthetic pathway genes: *MAM1, MAML, CYP79F1, CYP79F2, CYP83A1, C-S lyase, ASA1*. Histochemical GUS staining of cultured cells. Quantitative evaluation of GUS activity was performed 2–3 d after transformation. White bars represent expression of only the *Target Promoter:GUS* constructs, grey bars represent the expression of *Target Promoter:GUS* constructs co-transformed with the effectors (means of GUS activity in µmol MU min^{-1} and mg protein ±SD, n=5).

The *CYP79F1* gene was equally upregulated by MYB28/HAG1 and MYB29/HAG3 but to a lesser extent by MYB76/HAG2. Conversely, the *CYP79F2* gene was stronger *trans*-activated by the MYB28/HAG1 and slightly less by MYB29/HAG3. On the whole, MYB28/HAG1 revealed a stronger capacity to activate the promoters of *MAML* and *CYP79F2* genes which are involved in the conversion of long-chain aliphatic glucosinolates. Therefore, MYB28/HAG1 has higher impact on the regulation of long-chain Met-derived glucosinolates than MYB29/HAG3 and MYB76/HAG2. *Trans*-activation experiments of MYB28/HAG1 and MYB29/HAG3 regulators towards promoters of indolic glucosinolate biosynthetic genes revealed no activation of the *ASA1* gene (Fig. 11).

Along with the activation of GSL biosynthetic genes, all *MYB/HAG* transcription factors are involved in the regulation of sulphate assimilation genes like ATP sulphurylases and APS kinases (collaboration with a group of Dr. S. Kopriva, JIC, UK). To assess the role of MYB/HAG regulators towards sulphate assimilation genes, four promoters of APT sulphurylases and APS kinases, respectively, were cloned into the pGWB3i vector and used as reporter constructs in co-transformation assays with *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* as effectors. Results of *trans*-activation experiments revealed that the over-expression of MYB/HAG regulators led to the upregulation of some ATP sulphurylases (ATPS) and APS kinases (APK) (Fig. 12). From the four individual ATPS, *ATPS1* and *ATPS3* were specifically activated by MYB/HAG regulators. Conversely, the *ATPS2* isoform was shown to be slightly downregulated and *ATPS4* was not affected by *MYB/HAG* over-expression (data not shown). Further analysis of APK isoformes revealed that all three *MYB/HAG* genes were able to upregulate *APK1* and *APK2* but not the other two isoforms (data not shown). Thus, the results of these *trans*-activation experiments clearly indicate that MYB/HAG transcription factors are involved in the regulation of sulphate assimilation genes in order to increase the phosphoadenosine phosphosulfate (PAPS) content which is necessary for the sulphation of desulphoglucosinolates (Piotrowski et al., 2004).

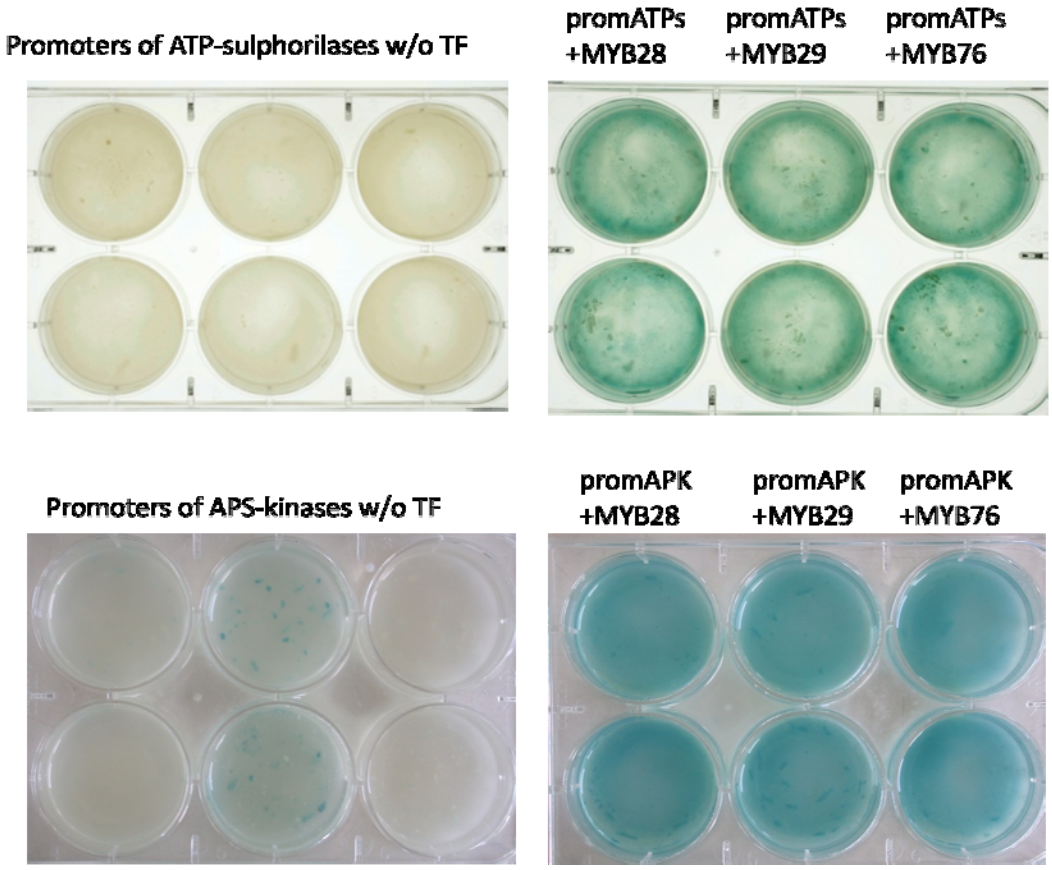

Figure 12. *Trans*-activation of ATP sulphurylases and APS kinases promoters by MYB/HAG factors in cultured *Arabidopsis* cells.

3.9 Tissue specific expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* genes

To study the tissue-specific expression of *MYB/HAG* transcription factors, promoter regions of *MYB28/HAG1* (-1995 to +157 bp) fused with 3'UTR region (about 380 bp), *MYB29/HAG3* (-1368 to +81 bp) and *MYB76/HAG2* (-1726 to +275 bp) were used to create a translational fusion construct with the *uidA* (*GUS*) reporter gene. Stable *Arabidopsis* transformants were obtained using *A. tumefaciens*-mediated transformation and selection on MS plates with kanamycin. Subsequently, several stable transformed *Arabidopsis* transgenic lines expressing Pro_{HAG1}*:GUS,* Pro_{HAG2}*:GUS* and Pro_{HAG3}*:GUS* constructs were analyzed for tissue-specific gene expression. Detailed analysis of the Pro_{HAG}*:GUS* plants was carried out at different

ontogenetic stages and revealed not only similar but also specific expression patterns for the *MYB28, MYB29* and *MYB76* transcription factors (Fig 13 - I, II, III).

GUS expression driven by *MYB/HAG* promoters was not detected in early stages of development in cotyledons, hypocotyls and roots of 3- to 7-day-old seedlings (Fig. 13). Faint GUS staining appeared in stems, petioles and the main veins of true leaves in all 10- to 14-day-old seedlings (Fig. 13 – I a, II a, III a). However, in case of *MYB76/HAG2,* strong GUS expression was also observed in the transition zone between roots and the foliar part of seedlings. Subsequently, three-week-old seedlings showed increase in GUS expression which was mainly detected in lateral roots for plants expressing the Pro_{HAG1}*:GUS* construct (Fig. 13 - I c). Furthermore, promoter-GUS activity was detected in expanding leaves of all Pro_{HAG}*:GUS* transgenic plants and its expression reached a maximum level in adult leaves of non-flowering plants. At this stage, *MYB29/HAG3* was barely expressed in the primary vein while for *MYB28/HAG1* and *MYB76/HAG2*, GUS staining was observed in both primary and secondary veins of leaf (Fig. 13 - I b; II b; III b). During senescence, GUS expression was considerably decreased in leaves and appeared in inflorescences upon transition from the vegetative to the generative stages. In inflorescences GUS staining was mainly observed in stems and only Pro_{HAG1}*:GUS* and Pro_{HAG2}*:GUS* plants also showed GUS activity in flowers (Fig. 13 – I d, II c). Moreover, Pro_{HAG1}*:GUS* and Pro_{HAG3}*:GUS* expression was also detected in trichomes (Fig. 13 - I e; III e) and roots, whereas Pro_{HAG2}*:GUS* was absent in these tissues. At the latest stages of development, GUS expression was very faint and almost completely disappeared in leaves, but still remained detectable in inflorescences (Fig. 13 - I f). Remarkably, mechanical stimuli like wounding induced *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* expression mainly in inflorescences of flowering plants (Fig. 13 - I g; II d; III d). The *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* expression data are consistent with AtGenExpress data from the Genevestigator microarray database (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch/). In addition, it is worth noting that all *MYB/HAG* transcription factors were mainly expressed at sites of non-uniform distribution of aliphatic glucosinolates in leaves (Shroff et al., 2008) and, therefore, they might play an important role in the regulation of aliphatic glucosinolate biosynthesis in response to various biotic stresses.

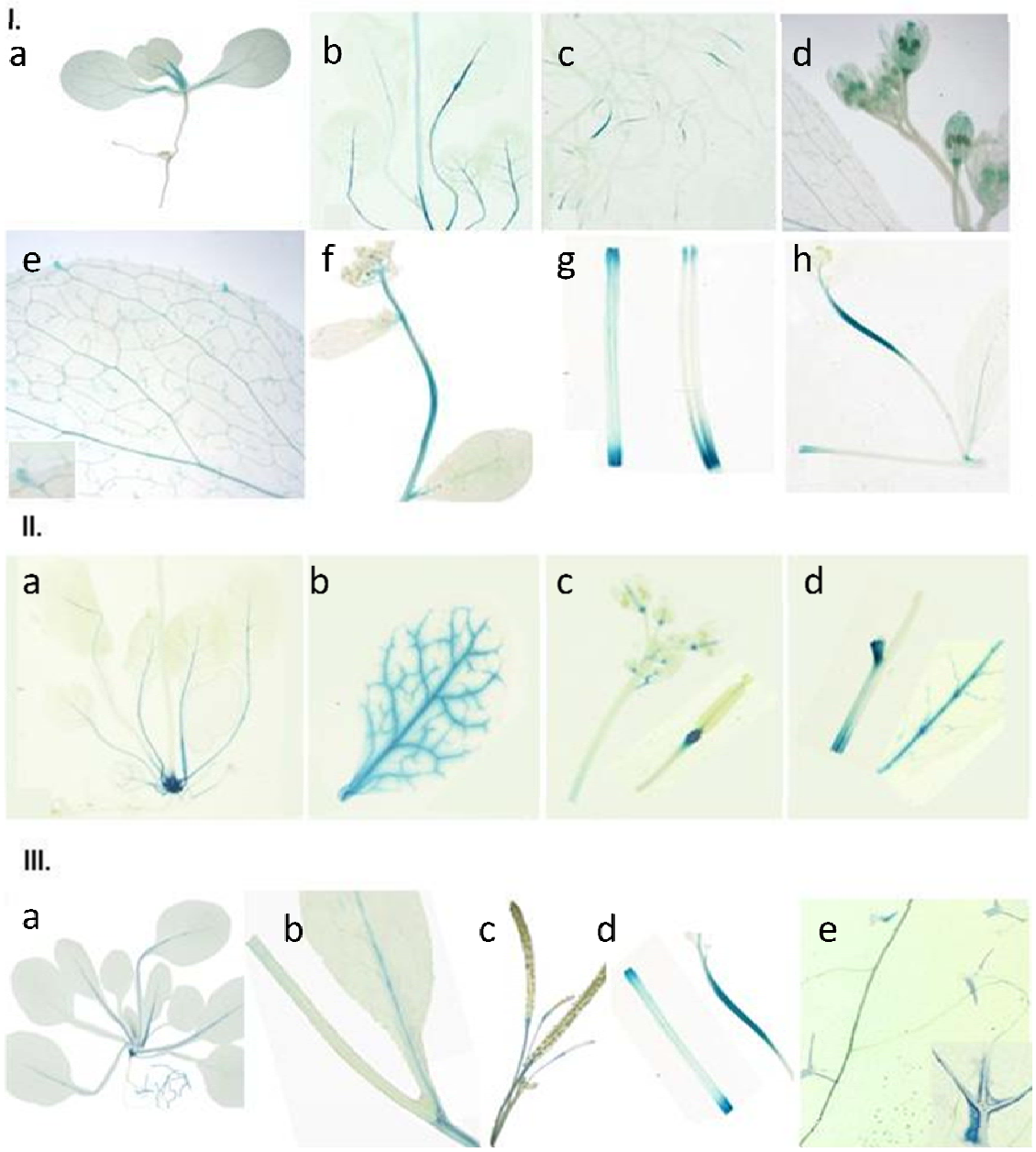

Figure 13. Histochemical GUS staining in tissues: I - *Pro*$_{MYB28/HAG1}$*:GUS* plants - (a) 14-day-old seedling, (b) three-week-old plant, (c) roots of three-to-four-week old plants, (d) flowers, (e) leaf of adult plant, (f) Inflorescences of flowering plant, (g) GUS induction at cut sites of inflorescences, (h) GUS induction at touch sites of inflorescences. II - *Pro*$_{MYB76/HAG2}$*:GUS* plants - (a)14-d-old seedling, (b) adult leaf, (c) flowers and siliques, (d) GUS induction at cut sites of inflorescences. III - *Pro*$_{MYB29/HAG3}$*:GUS* plants - (a) 14-d-old plants, (b) inflorescences of a flowering plant with a cauline leaf, (c) siliques, (d) GUS induction at cut sites of inflorescences, (e) trichomes.

3.10 Regulation of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription by plant elicitors

Glucosinolates are very well known plant secondary compounds that defend plants against both herbivores and pathogens (Koenraad et al., 2001; Raybould and Moyesà, 2001). There are several known plant signal molecules like methyl jasmonate (MeJa), salicylic acid (SA) and the ethylene precursor aminocyclopropane carboxylate (ACC) that have been suggested to be involved in the regulation of glucosinolate biosynthesis upon biotic stresses (Mewis et al., 2005). Sugars are known as other important signaling molecules that induce transcription of pathogen responsible genes (Xiao et al., 2000; Rolland et al., 2002). To study further the upstream regulation of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* by different signaling hormone pathways, wild-type (Col-O) plants were treated with methyl jasmonate (0.10 µM MeJa), salicylic acid (0.10 µM SA), aminocyclopropane carboxylate (0.10 µM ACC) and glucose (3% Gluc). Subsequently, transcript levels of *MYB28/HAG1*, *MYB76/HAG2* and *MYB29/HAG3* were monitored using real-time RT-PCR.

According to the qRT-PCR data, the transcript level of the *MYB28/HAG1* gene was almost six fold increased after 2 h of glucose treatment compared to non-treated plants and returned to its original level after 24 h of induction. Treatment with 3% mannitol, used as an osmotic negative control, did not reveal any changes in transcript level of the *MYB28/HAG1* gene, as well as ACC and MeJa treatments (Fig. 14A, B, D). Similar results were obtained for Pro_{HAG1}*:GUS* plants: glucose treatment led to increased GUS activity in leaves, petioles and roots of Pro_{HAG1}*:GUS* plants but not MeJa, SA, ACC or mannitol applications (Fig. 14A). Thus, glucose was identified as an important signaling molecule which induces *MYB28/HAG1* gene expression, whereas expression of the *MYB29/HAG3* and *MYB76/HAG2* genes was not affected by glucose or mannitol. SA treatment caused a negative effect on expression of all *MYB/HAG* genes and resulted in the considerable downregulation of *MYB28/HAG1, MYB29/HAG3* and to an only slight downregulation of *MYB76/HAG2* (Fig. 14C). Induction of *MYB29/HAG3* gene expression was observed within 5-15 min of MeJa treatment (Fig. 14D). Remarkably, *MYB76/HAG2* expression was not strongly

affected by MeJA, SA, ACC or glucose treatments, probably due to its accessory role in the regulation of aliphatic glucosinolates.

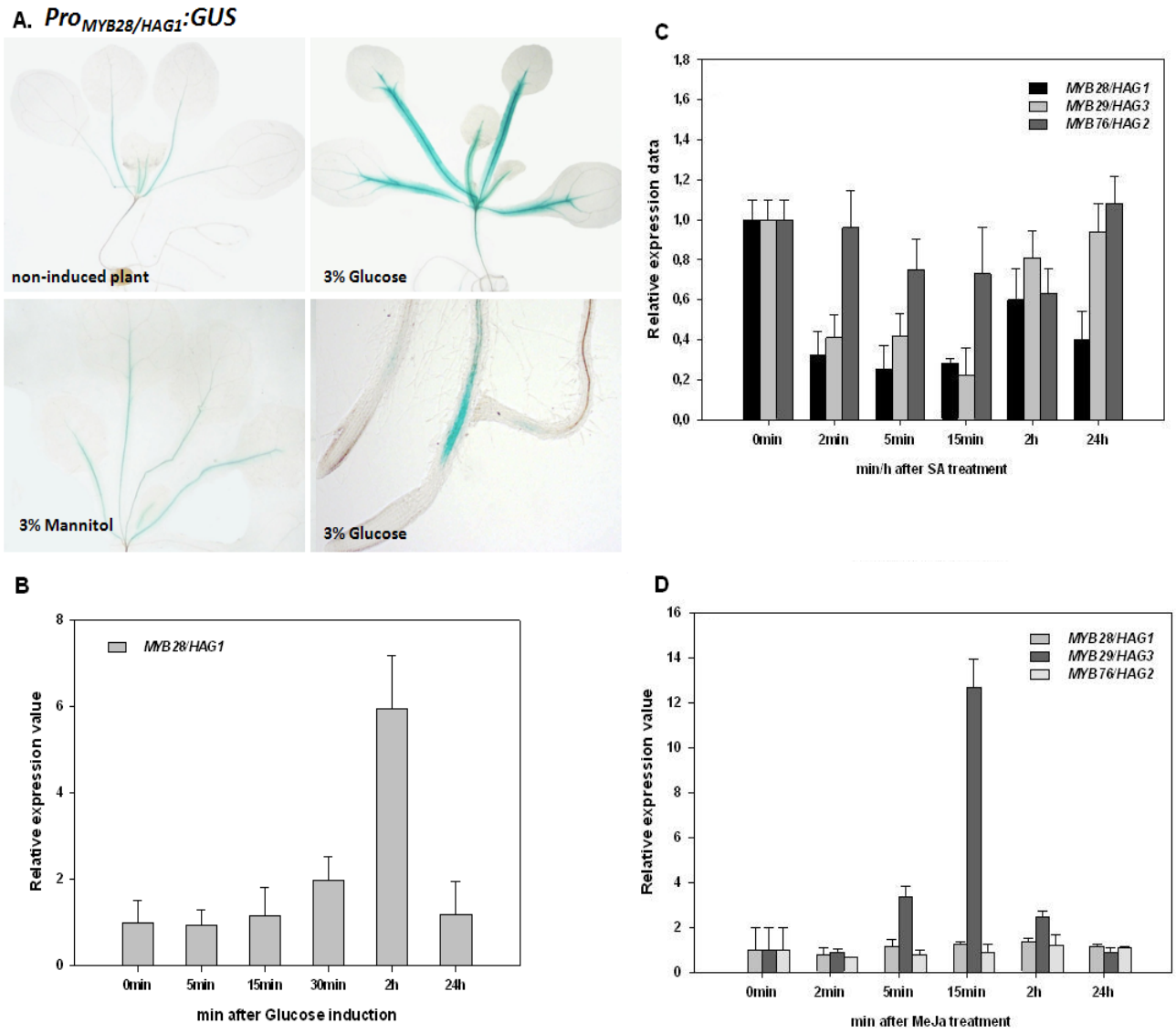

Figure 14. Response of *MYB28/HAG1*, *MYB76/HAG2* and *MYB29/HAG3* genes to glucose (A, B), SA (C) and MeJA (D) hormone treatments.

3.11 Expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* genes induced by mechanical damages or wounding

It has been reported previously that the activation of SA and MeJa signal transduction pathways are often induced by wounding (Chassot et al., 2008). Analysis of Pro_{HAG}*:GUS* plants showed that the expression of *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* was upregulated by mechanical damages in inflorescences which reminded of the previously reported mechano-sensitive reaction of MYB51/HIG1 and IQD1 regulators (Levy et al., 2005; Gigolashvili et al., 2007b). Therefore, to analyze the time-dependent induction of the *MYB/HAG* regulators by mechanical stimuli, rosette leaves and inflorescences of wild-type (Col-O) plants were subjected to mechanical treatment and harvested after 1, 5, 10, 30 and 60 min of wounding. Analysis of the wounded plants showed a rapid induction of all MYB/HAG genes already after 1 min of wounding. Transcript levels of *MYB28/HAG1* and *MYB29/HAG3* regulators were increased three- to four-fold within 1-5 min of wounding and dropped within 30 min to the level of unwounded plants (Fig. 15). Notably, *MYB76/HAG2* expression was induced more than 50-fold upon mechanical stimuli. Thus, real-time RT-PCR data indicates that gene expression of *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* factors was transiently induced upon wounding which observation is consistent with data obtained for Pro_{HAGs}*:GUS* plants (Fig. 13).

3.12 Interactions between the *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors.

It appears that MYB/HAG transcription factors have partially redundant functions in the regulation of aliphatic glucosinolates. To answer the question about specificity and redundancy between *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors, the analysis of *MYB/HAG* transcript levels in gain-of-function mutants and *trans*-activation assays toward *MYB/HAG* promoters were performed.

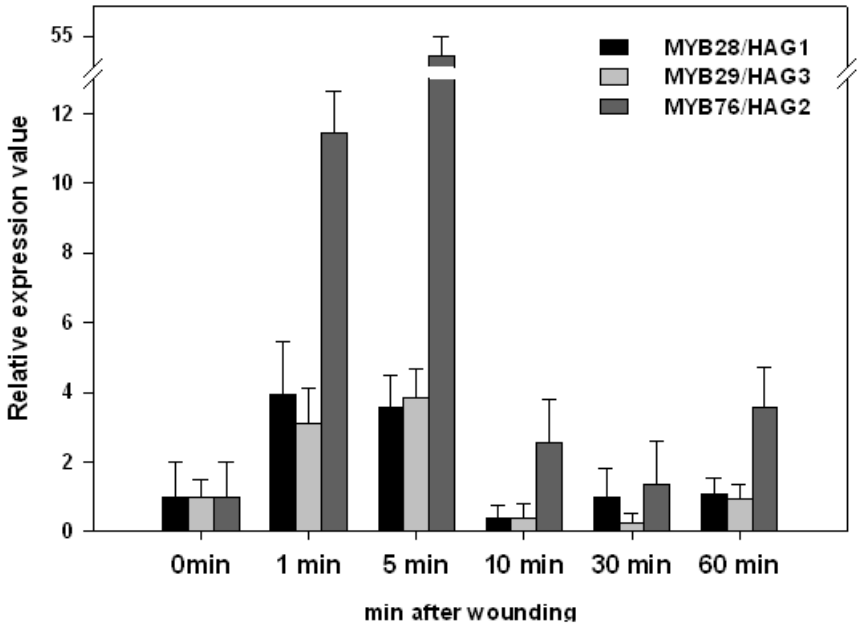

Figure 15. Induction of *MYB28/HAG1*, *MYB29/HAG3*, *MYB76/HAG2* gene expression by wounding; relative expression values are given compared to non-wounded plants (= 1).

According to the real-time RT-PCR data, transcript levels of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors were differently upregulated and affected by themselves in gain-of-function mutants as shown on Figure 16A. The transcript level of *MYB28/HAG1* was only slightly increased in the background of *MYB29/HAG3* and *MYB76/HAG2* over-expression plants. Conversely, *MYB28/HAG1* over-expression led to elevated transcript levels of *MYB29/HAG3* and *MYB76/HAG2* genes. An increase of *MYB29/HAG3* transcripts caused an accumulation of *MYB76/HAG2* mRNA and only a slight effect on *MYB28/HAG1* expression was detected. In turn, over-expression of the *MYB76/HAG2* resulted in upregulation of *MYB29/HAG3* but not of *MYB28/HAG1* (Fig. 16a). Therefore, real-time RT-PCR results certainly indicate a positive reciprocal feedback regulation between *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* transcription factors.

In order to further explore the specific regulation between MYB/HAG regulators, transient co-transformation assays using Pro_{HAG1}*:GUS,* Pro_{HAG2}*:GUS,* Pro_{HAG3}*:GUS* as reporter constructs and Pro_{35S}*:HAG1,* Pro_{35S}*:HAG2* and Pro_{35S}*:HAG3* as effectors

were performed (M. Engqvist, AG Flügge). According to the *trans*-activation data, the promoter activity of *MYB28/HAG1* was not affected by *MYB76/HAG2* or *MYB29/HAG3 in trans* and, therefore, it seems to be less dependent on the function of the other two *MYB/HAG* genes.

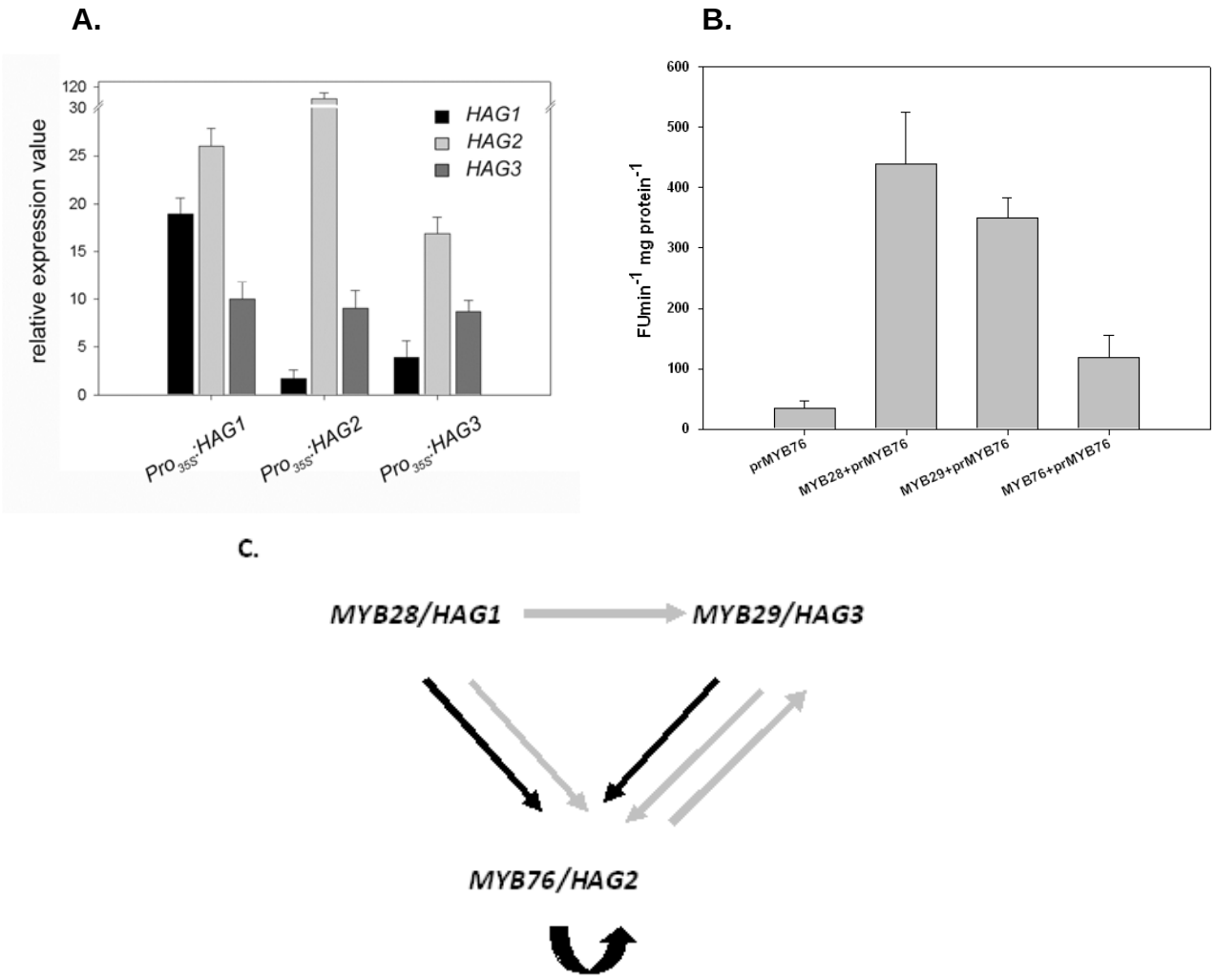

Figure 16. Coordinated action and interplay between *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* transcription factors in the regulation of aliphatic glucosinolate biosynthesis. (A) Steady-state transcript levels of *MYB28/HAG1*, *MYB2/HAG39* and *MYB76/HAG2* in *Pro$_{35S}$:HAG1*, *Pro$_{35S}$:HAG2* and *Pro$_{35S}$:HAG3* over-expression lines. Relative gene expression values of the real-time RT-PCR data are shown compared with WT (Col) ±1. (B) Co-transformation assays pointing to the activation of *Pro$_{HAG2}$:GUS* (reporter) by *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* (effectors) (means of relative GUS activity ±SD, n=3; *Pro$_{HAG2}$:GUS*=1). (C) Schematic representation of all observed *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* trans-activations. Black arrows indicate trans-activations measured in co-expression studies in *N. benthamiana* using reporter–effector constructs. Grey arrows indicate cross-activation of transcription factors in *MYB28/HAG1*, *MYB76/HAG2* and *MYB29/HAG3* over-expression lines using real-time RT-PCR analysis.

Conversely, promoters of the *MYB29/HAG3* and *MYB76/HAG2* genes were considerably induced by over-expression of *MYB28/HAG1* which data nicely corresponded to the real-time RT-PCR data. In addition, *MYB29/HAG3* was activated by both *MYB28/HAG1* and *MYB76/HAG2* (Fig. 16a). Although results from the *MYB76/HAG2* over-expression study indicated only its minor contribution in control of aliphatic glucosinolates biosynthesis, *MYB76/HAG2* was shown to be activated by *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* itself and was able to upregulate *MYB29/HAG3* expression, thereby contributing to aliphatic glucosinolate biosynthesis in concert with *MYB28/HAG1* and *MYB29/HAG3* (Fig. 16 b, c).

3.13 MYB/HAG factors repress the transcription of indolic glucosinolate regulator genes and interact with WRKY25 and SLIM1 transcription factors

It has been shown previously that several mutations which block or restrict biosynthesis of one class of glucosinolates resulted in a compensatory increase in the other class. Therefore, it is proposed that a homeostatic control of glucosinolate biosynthesis could be achieved by reciprocal negative feedback regulation between branches using glucosinolate intermediates or end products as inhibitors (Grubb and Abel, 2006). For example, an increased accumulation of indole glucosinolates in *MYB51/HIG1*, *MYB34/ATR1* and *MYB122/HIG2* over-expression plants caused a repression of aliphatic glucosinolate biosynthetic genes and a decrease in the level of aliphatic glucosinolates (Gigolashvili et al., 2007b). Conversely, a decreased content of the main indolic glucosinolate I3M was observed in strong *MYB28/HAG1* and *MYB29/HAG3* over-expression plants, probably due to the repression of indolic glucosinolate biosynthetic genes.

To study the crosstalk between indolic and aliphatic glucosinolate biosynthetic pathways at the level of interaction between indolic (*MYB51/HIG1, MYB122/HIG2, MYB34/ATR1*) and aliphatic (*MYB28/HAG1, MYB29/HAG3, MYB76/HAG2*) transcription factors, *trans*-activation assays were performed using Pro_{HIG1}*:GUS*,

Pro$_{HIG2}$:GUS and *Pro$_{ATR1}$:GUS* reporter constructs which were co-expressed with *Pro$_{35S}$:HAG1*, *Pro$_{35S}$:HAG2* and *Pro$_{35S}$:HAG3* effector constructs in tobacco (*Nicothiana benthamiana*) leaves (M. Engqvist, AG Flügge). As shown on Figure 17, the over-expression of *MYB/HAG* genes caused considerable downregulation of all regulators of the indolic glucosinolate pathway like MYB51/HIG1, MYB122/HIG2 and MYB34/ATR1. Therefore, the *trans*-activation data clearly indicate a negative feedback regulation existing between indolic (*MYB/HIG*) and aliphatic (*MYB/HAG*) regulators.

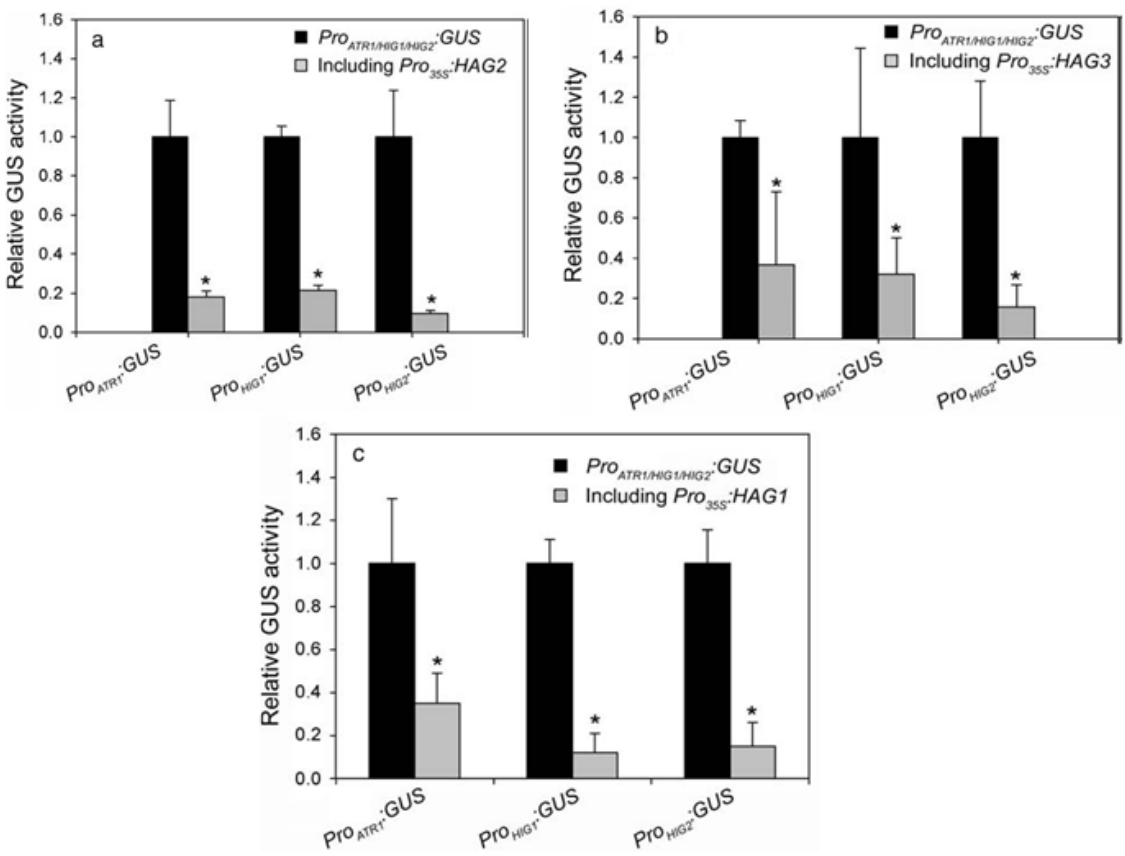

Figure 17. Co-transformation assays to determine the promoters repression of genes involved in the control of indolic glucosinolate biosynthesis (*HIG1/MYB51*, *HIG2/MYB122 and ATR1/MYB34*) by the regulators of aliphatic glucosinolates, MYB28/HAG1, MYB76/HAG2 and MYB29/HAG3; means of relative GUS activity ±SD, n=3; *Pro$_{ATR1/HIG1/HIG2}$:GUS* = 1).*, P < 0.05.

As it has been demonstrated by several studies that glucosinolate biosynthesis is regulated at multiple levels by environmental factors, developmental programs and gene transcription comprising a complex regulatory network (Grubb and Abel, 2006;

Yan and Chen, 2007). Further analysis also revealed that MYB/HAG transcription factors are involved in the interplay with WRKY25 and SLIM1 (sulphur limitation1) transcription factors. The interactions between WRKY25 and MYB/HAG transcription factors were observed in co-transformation assay using *Pro_{35S}:WRKY25* as effector and promoters of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* as reporter constructs in the tobacco system. As shown on Figure 18, there is a specific promoter activation of *MYB29/HAG3*, whereas promoters of *MYB28/HAG1* and *MYB76/HAG3* were not affected by WRKY25.

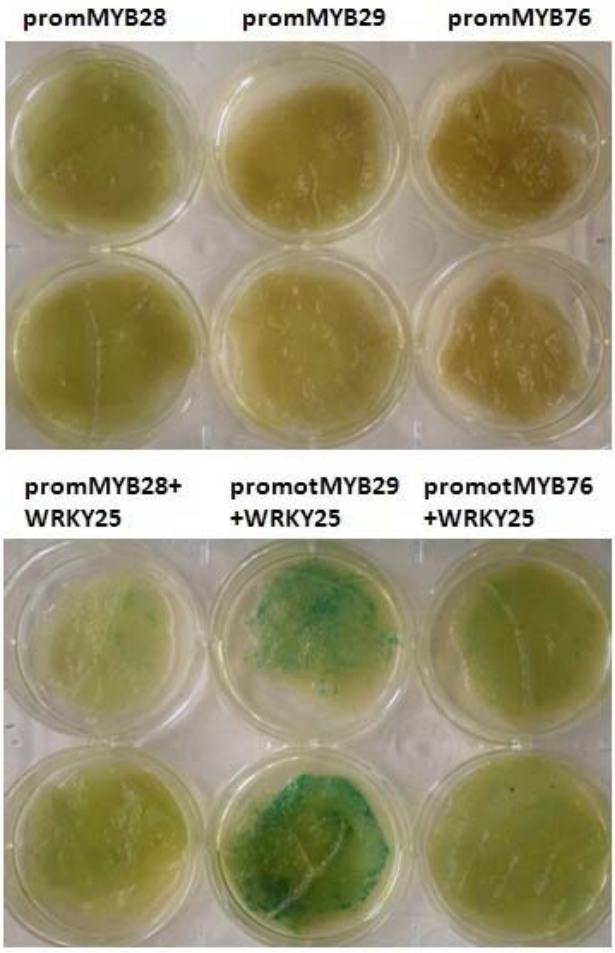

Figure 18. *Trans*-activation of *MYB/HAG* promoters by WRKY25 transcription factor in *N. benthamiana* leaves.

To study how SLIM1 affects *MYB/HAG* expression, real-time RT-PCR analysis was performed using cultured *Arabidopsis* (Col-O) cells transiently over-expressing

SLIM1. The results conclusively show that *SLIM1* over-expression caused a considerable repression of *MYB28/HAG1* and *MYB29/HAG3* regulators (Fig. 19).

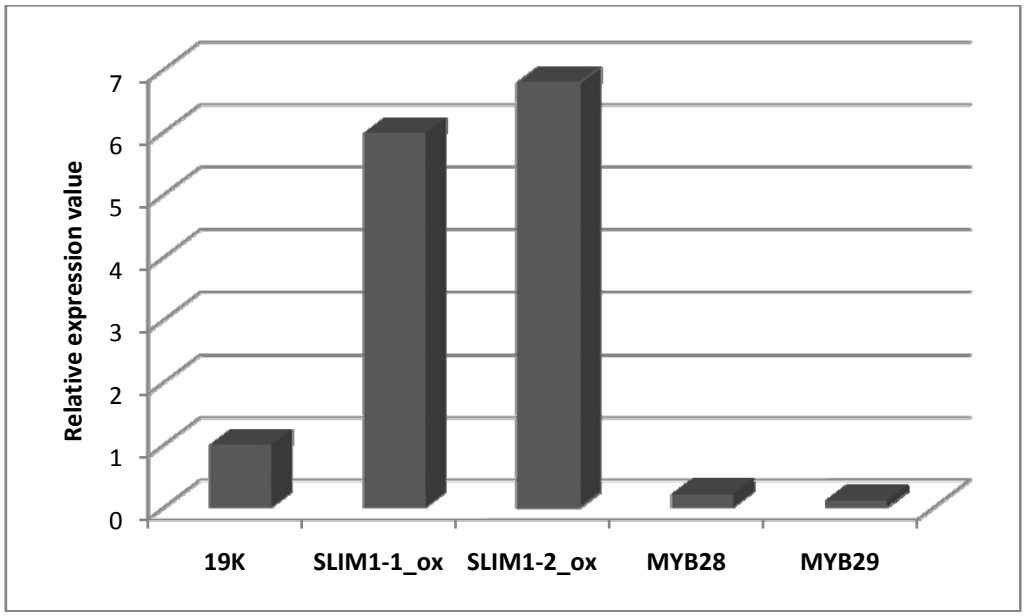

Figure 19. Repression of the *MYB28/HAG1* and *MYB29/HAG3* genes expression by SLIM1 transcription factor in cultured *Arabidopsis* cells.

3.14 Over-expression of *MYB28/HAG1* leads to an increased resistance against a generalist herbivore

Although the primary functions of glucosinolates are still unknown, their degradation products, which accumulate upon mechanical-induced tissue damage, are contributing to the plant defence arsenal against pathogens and generalist herbivores. To analyze if an increase of the *MY28/HAG1* expression correlates with enhanced plant resistance against the generalist lepidopteran herbivore *Spodoptera exigua* (Lepidoptera: Noctuidae), weight gain assays were carried out in collaboration with the University Würzburg (Dr. C. Müller). For this, 5-day-old larvae of *Spodoptera exigua* were kept on artificial diet and then used in feeding experiments on both wild-type and *MYB28/HAG1* over-expression plants (*Pro$_{35S}$:HAG1-15)*. The levels of total sugars and proteins were determined in Col-O and *MYB28/HAG1* over-expression plants before subjecting them for feeding of *Spodoptera* and no considerable

differences were detected between these plants (data not shown). The weight-gain experiments certainly show that growth and development of *Spodoptera* larvae on *MYB28/HAG1* over-expression plants were considerably reduced and showed more than 70% less fresh weight in comparison with larvae on wild-type plants within five days (Fig. 20). Thus, over-expression of *MYB28/HAG1* considerably reduced herbivore performance due to an accumulation of aliphatic glucosinolates.

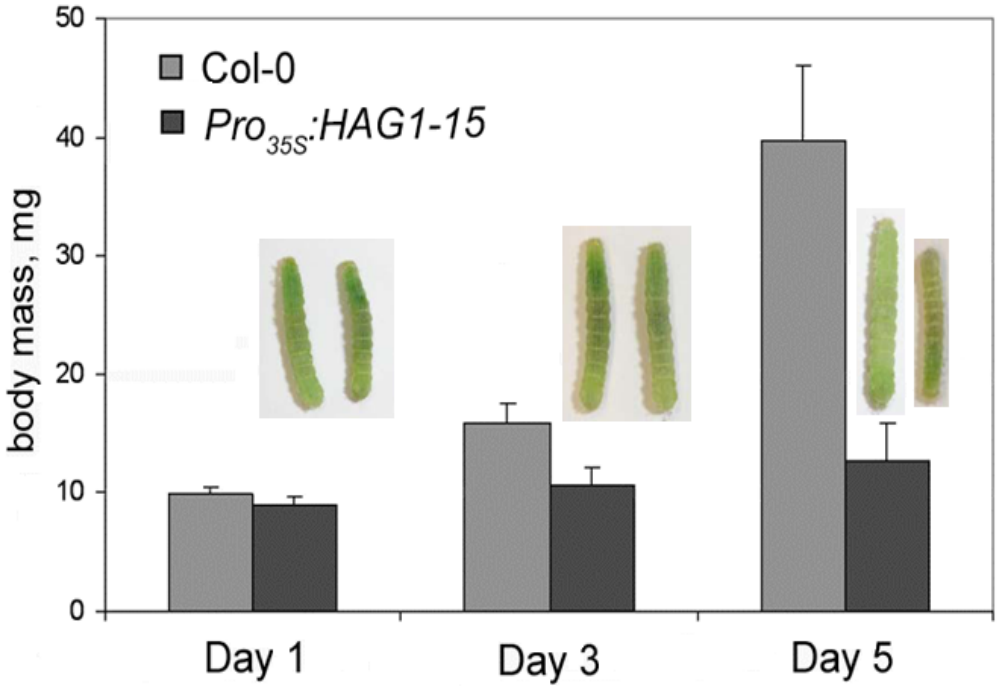

Figure 20. *MYB28/HAG1* over-expression reduces insect performance. Weight-gain assays of *Spodoptera exigua* larvae on 5-week-old *A. thaliana* plants. Larvae were kept on an artificial diet for 5 days and second-instar larvae were transferred to plants. Values are the mean fresh weight (and SE) of 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).

4. Discussion

4.1 *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* are positive regulators of aliphatic glucosinolate biosynthesis

Identification and characterization of new regulators of glucosinolate biosynthesis represent the most advanced approach to exploit the potential of glucosinolates in biotechnology, agriculture and medicine. *MYB34/ATR1, MYB51/HIG1, MYB122/HIG2, SLIM1, IQD1 and AtDof1.1* have recently been analyzed for their ability to regulate transcription of glucosinolate biosynthetic genes (Celenza et al., 2005; Levy et al., 2005; Maruyama-Nakashita et al., 2006; Skirycz et al., 2006; Gigolashvili et al., 2007b). Here, it is shown that the nuclear-localized R2R3-type *MYB28/HAG1, MYB76/HAG2* and *MYB29/HAG3* transcription factors are novel regulators of glucosinolate biosynthesis. The highly correlated co-expression of *MYB28/HAG1, MYB76/HAG2* and *MYB29/HAG3* with aliphatic glucosinolate pathway genes (*BCAT4, MAM1, CYP79F1* etc.) and phylogenetic association with *MYB34/ATR1, MYB51/HIG1 and MYB122/HIG2* as regulators of indolic glucosinolate biosynthesis suggest their function in the control of aliphatic glucosinolate biosynthesis (Fig. 4; Tab. 1).

The ectopic over-expression of *MYB28/HAG1, MYB76/HAG2* and *MYB29/HAG3* led to accumulation of up to four-fold higher levels of all aliphatic glucosinolates in gain-of-function mutants compared to the wild-type (Col-O). Furthermore, an increase in glucosinolate accumulation corresponded to the level of *MYB28/HAG1, MYB76/HAG2* and *MYB29/HAG3* transcripts in various over-expression lines. For example, the strong *Pro_{35S}:MYB28/HAG1-11, Pro_{35S}:MYB76/HAG2-6* and *Pro_{35S}:MYB29/HAG3-12* over-expression lines also contained a higher amount of aliphatic glucosinolates (Fig. 6; Fig. 8). Notably, plants over-expressing *MYB28/HAG1* contained considerably higher total levels of aliphatic GSL, especially 4MSOB, compared to *MYB29/HAG3* and *MYB76/HAG2* gain-of-function mutants. However, changes in the level of long-chain glucosinolates in *MYB28/HAG1* over-expression lines could not be detected. Conversely, in strong

Pro$_{35S}$:MYB29/HAG3-12 and *Pro$_{35S}$:MYB76/HAG2-6* over-expression lines, the content of long-chain 8MSOO glucosinolate was three to four times higher compared to wild-type plants (Fig. 8). Since an accumulation of the long-chain aliphatic glucosinolates was not detected in all *MYB/HAG* over-expression mutants, it seems rather to be a secondary effect due to the strong over expression of *MYB29/HAG3* and *MYB76/HAG2* causing an accumulation of short-chain oxo-acids in chloroplasts. These oxo-acids may spontaneously re-enter the side-chain elongation cycles, catalyzed by the *MAM3* (*MAML*) enzyme resulting in the generation of long-chain aliphatic glucosinolates.

Interestingly, strong over-expression *MYB28/HAG1* and *MYB29/HAG3* lines (*Pro$_{35S}$:HAG1-11* and *Pro$_{35S}$:HAG3-12,* Fig. 8) also showed a decrease in the level of main Trp-derived indolic glucosinolate I3M. This observation may indicate a negative crosstalk between the aliphatic and indolic glucosinolate biosynthetic pathways. It has been previously demonstrated that the double *CYP79F1* and *CYP79F2* knockout mutant completely lacking aliphatic glucosinolates revealed an increase in the levels of indolic glucosinolates (Tantikanjana et al., 2004). Similarly, the double knock-out of *CYP79B2* and *CYP79B3* genes, involved in the biosynthesis of indolic glucosinolates, is completely devoid of indolic glucosinolates and contained elevated levels of aliphatic glucosinolates (Celenza, 2001). The competition between aliphatic and indolic cytochrome P450 mono-oxygenases for electrons provided by limited NADPH supply could be a reason for the interdependence of aliphatic and indolic glucosinolate biosynthesis as it had been proposed by Grubb and Abel (2006).

Analysis of *MYB28/HAG1-RNAi* plants, *myb29/hag3* and *myb76/hag2* null mutants revealed differences in *MYB/HAG* functions to regulate biosynthesis of the chain-elongated aliphatic glucosinolates. The most considerable decrease in the level of total aliphatic glucosinolates was observed in *MYB28/HAG1-RNAi* plants and in the *myb29/hag3* mutant as a result of a considerable downregulation of aliphatic glucosinolate biosynthetic genes (Tab. 2; Fig. 10b, c). The levels of both short- and long-chain glucosinolates were decreased in *MYB28/HAG1-RNAi* plants but not completely abolished probably due to either the residual activity of *MYB28/HAG1* or functional complementation by *MYB29/HAG3* and *MYB76/HAG2*. Still *MYB76/HAG2* and *MYB29/HAG3* were obviously unable to fully compensate the function of *MYB28/HAG1* in RNAi lines. Analysis of *myb29/hag3* null mutants revealed that the

level of short-chain aliphatic glucosinolates was considerably reduced, whereas the content of long-chain aliphatic glucosinolates remained unchanged (Tab. 2). Moreover, *myb29/hag3* knockout plants were not completely lacking aliphatic glucosinolates because *MYB28/HAG1* and *MYB76/HAG2* were still functional in this mutant. Additionally, *myb76/hag2* knock-out mutant did not reveal considerable effects on glucosinolate accumulation and the loss of *MYB76/HAG2* function could easily be complemented by *MYB28/HAG1* and/or *MYB29/HAG3*. Therefore it is suggested that *MYB76/HAG2* plays an only minor role in the accumulation of aliphatic glucosinolates.

4.2 MYB28/HAG1, MYB29/HAG3 and MYB76/HAG2 specifically activate aliphatic glucosinolate biosynthetic and sulphate assimilation genes

Analysis of gain-of-function mutants demonstrate that ectopic over-expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* caused an accumulation of aliphatic (Met-derived) glucosinolates by means of specific upregulation of aliphatic GSL biosynthetic genes, e.g. *MAM1, MAML, CYP79F1, CYP79F2, CYP83A1, AtST5b* and *AtST5c*, without directly affecting genes of the indolic glucosinolate pathway, e.g. *TSB1* and *ASA1* (Fig. 10a; Fig. 11). As revealed in *trans*-activation experiments, *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* are able to activate the promoter of target genes starting from the first enzymes of chain-elongation reactions (*MAM1* and *MAML*) up to the last enzymes of glucosinolate biosynthesis (AtSt5b, c). Furthermore, differences in the regulation of *MAM1, MAML, CYP79F1* and *CYP79F2* that are involved in the production of Met-derived GSL with different chain-length by all *MYB/HAG* factors were observed in both gain-of-function mutants and in *trans*-activation experiments. According to these observations, *MAM1* and *MAML* genes, contributing to the side-chain elongation of aliphatic GSL, were stronger upregulated by *MYB28/HAG1* and *MYB29/HAG3,* whereas *MYB76/HAG2* showed a lesser activation capacity towards the *MAML* promoter (Fig. 11). On the whole, the *MAML* gene catalyzing mainly elongation reactions of long-chained Met-derived GSL was less activated by all MYB/HAG transcription factors compared to

MAM1, which is specifically involved to the biosynthesis of short-chain GSL (Fig. 10; Fig. 11). Further analysis revealed that *CYP79F1* and *CYPF2* genes, responsible for the oxidation of short- and long-chain GSL, were strongly up-regulated by *MYB28/HAG1* and less by *MYB29/HAG3* and *MYB76/HAG2*. Consequently, *MYB28/HAG1* has a higher capacity to activate transcription of aliphatic GSL biosynthetic genes compared to *MYB29/HAG3* and *MYB76/HAG2* (Fig. 10a; Fig. 11). Altogether, the great variety of glucosinolate profiles of *Arabidopsis* plants could be achieved by differential regulation of aliphatic GSL pathway genes by MYB/HAG.

Interestingly, MYB/HAG regulators are also able to specifically activate sulphate assimilation genes like ATP sulfurylases and APS kinases (Fig. 12). It has previously been shown that ATP sulfurylases catalyze the first step of sulphate assimilation yielding adenosine phosphosulphate (APS). Subsequently, APS is used for the biosynthesis of cysteine, methionine (Met) and phosphoadenosine phosphosulfate (PAPS). PAPS serve as universal sulphur donor and is synthesised via phosphorylation of adenosine phosphosulphate (APS) by APS kinases in *Arabidopsis*. Both Met and PAPS are important for GSL biosynthesis, because Met is the only precursor for the biosynthesis of aliphatic Met-derived GSL, whereas PAPS is required for the sulphation of desulfoglucosinolates as the last step of GSL biosynthesis (Glendening and Poulton, 1988). Together, MYB/HAG transcription factors are also suggested to be involved in the regulation of homeostasis between sulphate assimilation and GSL biosynthesis. Therefore, the complex regulatory mechanism for sulphur partitioning between primary and secondary metabolisms in *A. thaliana* is proposed to be controlled by MYB/HAG factors (Fig. 21).

4.3 *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* are expressed at the sites of aliphatic glucosinolate accumulation

Glucosinolates distribution within a plant may be crucial in understanding the actions of the glucosinolate defence machinery against herbivores and pathogens. It has previously been reported that indolic and aliphatic glucosinolates, representing two major groups of glucosinolates, were differently distributed in various organs of *Arabidopsis thaliana* plants. For instance, indolic glucosinolates were mainly

accumulating in vegetative organs and only reduced amounts could be detected in inflorescences and flowers. Conversely, aliphatic glucosinolates accumulate throughout the plant with highest concentrations in generative organs rather than in vegetative tissues. High levels of aliphatic glucosinolates were found in young rosette leaves and then decreased during maturation and flowering, resulting predominantly in an increase of indolic glucosinolates (Brown et al., 2003a; Halkier and Gershenzon, 2006).

Expression analysis of $Pro_{MYB28/HAG1}$*:GUS,* $Pro_{MYB76/HAG2}$*:GUS* and $Pro_{MYB29/HAG3}$*:GUS* lines together with data from the Genevestigator database (Zimmermann et al., 2004) revealed that all *MYB/HAG* regulators have similar expression patterns and were mainly expressed in inflorescences, flowers, petioles and rosette leaves, which corresponded to the main sites of accumulation of aliphatic Me-derived GSL (Fig. 13). In addition, the expression pattern of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* genes tightly overlaps with the previously reported tissue-specific expression of the main aliphatic glucosinolate biosynthetic genes, such as *MAM3, BCAT4, CYP79F1* and *CYP79F2,* that are found in higher amounts in vascular tissues of young and mature rosette leaves and in stems (Chen et al., 2003; Schuster et al., 2006; Textor et al., 2007). Furthermore, the expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* genes was decreased in rosette leaves and appeared in flowers and stems upon transition from the vegetative to the generative phase of development (Fig. 13). This observation is consistent with ontogenetic changes in the distribution and accumulation of aliphatic GSL in leaves which declines during maturation and flowering stages (Brown et al., 2003).

Interestingly, a non-uniform distribution of aliphatic glucosinolates was detected around the midveins and the periphery of *A. thaliana* rosette leaves (Shroff et al., 2008) and correlates well with the expression patterns of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* regulators (Fig. 13 - I b, II b, III b). Finally, expression of *MYB28/HAG1* and *MYB29/HAG3* was found in trichomes (Fig. 13 – I e, II e), indicating that *MYB28/HAG1* and *MYB29/HAG3* may be involved in early response reactions against herbivore or pathogen attacks, in order to enhance glucosinolate production. In addition, induction of *MYB/HAG* expression by mechanical stimuli is supporting their role in plant defence responses (Fig. 13 - I g, h, II d, III d).

Evidence is provided that the expression patterns of *MYB/HAG* factors overlapping with the expression of glucosinolate biosynthetic genes also cover the main sites of aliphatic (Met-derived) glucosinolate accumulation and correlate with the dynamic changes in the levels of aliphatic glucosinolates during ontogenesis. Furthermore, the expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors also overlap with the tissue-specific expression of other glucosinolate regulators (*MYB51/HIG1, MYB34/ATR1, IQD1, AtDof1.1* and *SLIM1*). It has been reported previously (Levi et al., 2005, Maruyama-Nakashita et al., 2006; Skirycz et al., 2006) that *IQD1, Dof1.1* and *SLIM1* regulators are not able to directly regulate transcription of GSL biosynthetic genes and might need interacting partners or factors. Therefore, MYB/HAG factors may functionally act in concert with each other and are involved in the interplay with other GSL biosynthesis regulators thus building up a complex regulatory network.

4.4 *MYB28/HAG1, MYB29/HAG3 and MYB76/HAG2* regulators reveal not only similar, but also specific features in the regulation of aliphatic glucosinolate biosynthesis

Metabolite and transcript analyses of *Arabidopsis* gain- and loss-of-function mutants showed that *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* have similar functions in the regulation of aliphatic glucosinolates biosynthesis. Nevertheless, there is also evidence indicating specific functions of MYB/HAG factors in the control of aliphatic glucosinolate biosynthesis (Fig. 8; Fig. 10; Fig. 11). For example, a similar growth phenotype was observed in strong *MYB28/HAG1* and *MYB29/HAG3* gain-of-function mutants showing a drastic retarded growth and development, an elongation of internodes, a defective gravitropic response and plant sterility (Fig. 7 B). This was not the case for *MYB76/HAG2* over-expression lines. It seems that the ectopic *MYB28/HAG1* and *MYB29/HAG3* over-expression under control of the *CaMV35S* promoter led to a dramatically increased flow of methionine into the biosynthesis of aliphatic (Met-derived) glucosinolates. The deficiency in the level of methionine, which also serves as a precursor for ethylene biosynthesis, may cause a decrease in

the level of this hormone. Therefore, low ethylene could be the reason for such impaired gravitropic plant response, as reported by Edelman et al., 2006.

Notably, the metabolite analysis of gain- and loss-of-function mutants, *trans*-activation and real-time RT-PCR data indicated that the *MYB28/HAG1* transcription factor serves as the strongest regulator of aliphatic glucosinolate biosynthetic genes among the MYB/HAG factors and is, therefore, suggested to be a key regulator of this pathway (Fig. 8a; Fig. 10a; Fig. 11). Furthermore, *MYB28/HAG1* function is required for biosynthesis of both short- and long-chain Met-derived GSL.

MYB29/HAG3 is a second regulator of aliphatic GSL biosynthesis. A defect in the *MYB29/HAG3* gene caused predominantly a decrease in the content of short-chain Met-derived glucosinolates (Tab. 2) and *MYB29/HAG3* seems to be associated with the control of short-chain glucosinolates. Although the disruption of *MYB29/HAG3* gene function resulted in a less pronounced effect on the glucosinolate profile compared to *MYB28/HAG1*, an accessory role for *MYB29/HAG3* in the regulation of aliphatic glucosinolates biosynthesis (without MeJa induction) is suggested.

Analysis of the *myb76/hag2* null mutant showed that *MYB76/HAG2* was not significantly affecting the transcript level of aliphatic GSL biosynthetic genes and glucosinolate composition. Notably, lines over-expressing *MYB76/HAG2* contained the lower amounts of total glucosinolates compared to *MYB28/HAG1* or *MYB29/HAG3* over-expressors and required a considerably high *MYB76/HAG2* transcript level (Fig. 8c; Fig. 10c; Tab. 2). Furthermore, Genevestigator digital northern database data (www.genevestigator.ethz.ch) and real-time RT-PCR analysis (Gigolashvili et al., 2008) indicated that the *MYB76/HAG2* expression was generally much lower compared to the *MYB28/HAG1* or *MYB29/HAG3* expression in Col-O plants. Thus, *MYB76/HAG2* is not able to contribute significantly to glucosinolate biosynthesis indicating its minor role under normal conditions. However, its contribution to the biosynthesis of aliphatic GSL along with its low expression level in wild-type plants and a weak co-expression value with the main aliphatic GSL biosynthetic genes (*BCAT4, MAM1, MAML, CYP79F1/F2*) may indicate that *MYB76/HAG2* is involved in the regulation of aliphatic GSL biosynthesis only in concert and/or interaction with other transcription factors, e.g. *MYB28/HAG1* or *MYB29/HAG3*.

In addition, different sites of expression were demonstrated for the *MYB/HAG* regulators as revealed by the analysis of $Pro_{MYB28/HAG1}$*:GUS,* $Pro_{MYB76/HAG2}$*:GUS*

and *Pro*$_{MYB29/HAG3}$*:GUS* plants. For instance, the *MYB76/HAG2* expression appeared to be very strong in the transition zone from root to shoot, flowers and secondary veins of leaves where expression of *MYB28/HAG1* and *MYB29/HAG3* was hardly detected. In contrast, *MYB28/HAG1* and *MYB29/HAG3* were expressed in young siliques, leaf trichomes and roots, where no expression of *MYB76/HAG2* was found. Furthermore, similar expression pattern of *MYB76/HAG2* with *MYB28/HAG1* and *MYB29/HAG3* indicates that *MYB76/HAG2* will be able at least partially to complement the deficiency in *MYB28/HAG1* or *MYB29/HAG3* activity.

4.5 *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* are induced by wounding and plant hormones (MeJa, glucose)

Different environmental stimuli like herbivore attack, wounding and stress hormones are involved in the upstream control of several glucosinolate regulators (Yan and Chen, 2007). Several microarray data revealed strong responses of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* to wounding or pathogen attack (http://www.atted.bio.titech.ac.jp/; Zimmermann et al., 2004). Indeed, analysis of *Pro*$_{HAG}$*:GUS* plants revealed that the expression of *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* is induced by mechanical stimuli. This observation is similar to responses previously reported for indolic GSL regulators like IQD1, AtDof1.1, MYB51/HIG1 and aliphatic GSL biosynthesis genes like *BCAT4, MAM1* and *MAML*. Interestingly, the expression of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* was induced within 1-5 min after wounding and returned back to its original level in hours, indicating a very fast response upon mechanical stress in a transient manner (Fig. 15). Notably, high expression level of *MYB76/HAG2* is required to induce biosynthesis of aliphatic glucosinolates that can, however, be achieved upon wounding (Fig. 6c; Fig. 8c; Fig. 15). It is, therefore, obvious that *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* play an important role in the promotion of increased glucosinolate production, generating immediate and early responses upon biotic challenges.

Several plant hormone signaling pathways are also involved in the specific regulation of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* gene expression.

Genevestigator microarray data (https://www.genevestigator.ethz.ch/) analysis of *Pro$_{HAG}$:GUS* plants and real-time RT-PCR results clearly indicated a strong response of *MYB28/HAG1* to glucose treatment (Fig. 14A, B). It has been shown previously that wounding or pathogen infection can modulate the carbohydrate status of plant tissues thereby changing the expression of pathogen-response genes. For instance, sugars were able to regulate the expression of the wound-inducible proteinase inhibitor II, lipoxygenase genes and pathogen-related PR1 and PR5 genes in a hexokinase-dependent or -independent manner (Johnson and Ryan, 1990; Sadka et al., 1994; Herbers et al., 1996; Xiao et al., 2000). Remarkably, a strong *in silico* response of *MYB28/HAG1* to glucose treatment was previously shown for *Arabidopsis* seedlings (Li et al., 2006). This glucose-dependent expression of *MYB28/HAG1* could be explained by the presence *cis* - glucose regulatory elements (e.g. AAACCCTAA, GTTAGGTT, and RCCGAC, Li et al., 2006) in the *MYB28/HAG1* promoter region. Thorough analysis of the *MYB28/HAG1* promoter revealed the presence of several tandems of glucose up-regulatory elements, especially in the 3'-UTR gene region. Together, *MYB28/HAG1* function seems to be essential to maintain a basal level of aliphatic glucosinolate biosynthesis by integrating signals from carbohydrate availability. In other words, *MYB28/HAG1* could be considered as a "housekeeping" regulator.

Further observations indicated a positive response of *MYB29/HAG3* to treatment with exogenous MeJa (Fig. 14D). Therefore, *MYB29/HAG3* is suggested to be an important player in the MeJa-mediated production of glucosinolates in *A. thaliana* plants. Treatment with SA led to considerable downregulation of *MYB28/HAG1* and *MYB29/HAG3* transcription factors (Fig. 14C) indicating a competition between these signaling pathways (Cipollini et al., 2004). In contrast, the *MYB76/HAG2* regulatory function is independent from MeJa, glucose, SA, and, thus a minor role for *MYB76/HAG3* in the biosynthesis of aliphatic glucosinolate under non-stressed conditions is suggested. Notably, a treatment with ACC did not reveal any effects on *MYB/HAG* transcription.

4.6 Negative regulation of indolic glucosinolate biosynthesis by MYB/HAG transcription factors

Interdependent control between different branches of glucosinolate biosynthesis has been suggested by several studies where blocking or restriction of the one class of glucosinolates causes a compensatory increase in the other class (Reintanz et al., 2001; Hemm et al., 2003; Grubb and Abel, 2006; Yan and Chen, 2007). However, no clear evidence of a crosstalk at the level of GSL regulators has been provided so far.

The results of *trans*-activation experiments conclusively show that *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG3* were able to repress the transcription of *MYB34/ATR1*, *MYB51/HIG1* and *MYB122/HIG2*, i.e. regulators of indolic glucosinolate biosynthesis, indicating a negative crosstalk between the aliphatic and indolic regulators of subgroup XII (Fig. 17). These observations corresponded to metabolic data of strong *MYB/HAG* over-expression lines (*Pro$_{35S}$:HAG1-11* and *Pro$_{35S}$:HAG3-6*) which is showed a higher content of aliphatic (Met-derived) glucosinolates but decreased levels of the main indolic glucosinolate I3M (Fig. 8a). Interestingly, over-expression of the positive regulators of indole glucosinolates like MYB51/HIG1, MYB34/ATR1 and IQD1 resulted in the repression of enzymes involved in biosynthesis of aliphatic glucosinolates (*CYP79F1*, *CYP79F2* and/or *CYP83A1*) in accordance with low levels of aliphatic glucosinolates (Levy et al., 2005; Gigolashvili et al., 2007b). It seems that a reciprocal negative feedback regulation is important for the balance between various types of glucosinolates. Also sulphur availability, as proposed by several microarray studies, seems to be an important element directing this balance (Hirai et al., 2003; Nikiforova et al., 2003; Hirai, 2005). Indeed, it has been shown that sulphate availability effects the expression of glucosinolate regulators like *MYB34/ATR1*, *MYB28/HAG1*, *MYB29/HAG3* and biosynthetic genes like *MAM1, CYP79F1*, *CYP79B2* etc in *Arabidopsis* plants (Maruyama-Nakashita et al., 2006; Hirai et al., 2007). Also specific isoforms of sulphate assimilation genes like ATP-sulfurilases and APS-kinases were regulated by *MYB/HAG* factors (Fig. 12; Fig. 21). However, the regulatory mechanisms controlling the homeostasis between glucosinolate biosynthesis and sulphur metabolism are unknown yet.

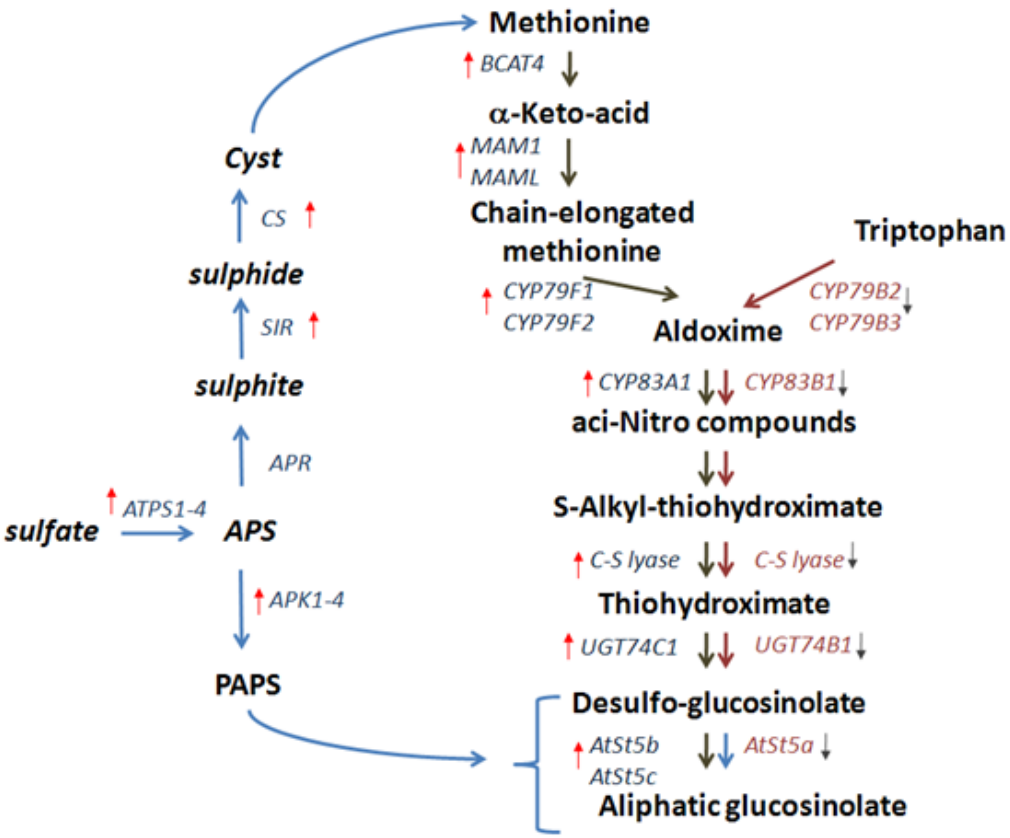

Figure 21. Scheme of a crosstalk between sulphate, aliphatic and indolic glucosinolate biosynthetic pathways (arrows indicate ↑ - upregulation and ↓ - downregulation of GSL and sulphur biosynthetic genes in strong *MYB/HAG* over-expression lines).

4.7 *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* comprise a complex gene regulatory network in glucosinolate metabolism

Similarities in *MYB/HAG* functions and co-expression of these three genes suggested that these regulators may act together to enhance biosynthesis of aliphatic GSL upon different environmental stimuli. A positive reciprocal feedback regulation between *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG3* transcription factors was observed in gain-of-function mutants using real-time RT-PCR analysis (Fig. 16a).

Subsequently, these observations were confirmed in *trans*-activation experiments indicating a direct and positive interdependent regulation between *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG3* (Fig. 16 a, b). According to the

interaction data (Fig. 16c), the promoter of *MYB28/HAG1* was not affected by over-expression of one of the other two *MYB/HAG* regulators. On the other hand, *MYB29/HAG3* and *MYB76/HAG2* showed similar interaction patterns, both were induced by *MYB28/HAG1* and both were activating each other (Fig. 16a, b; Fig. 22). Moreover, *MYB76/HAG2* was shown to be activated by itself *in trans* (Fig. 16b), suggesting a specific role for *MYB76/HAG2* in which it rather integrates signals from *MYB28/HAG1* and *MYB29/HAG3* regulators and seems to function as an enhancer in concert with the other MYB/HAG regulators to control biosynthesis of aliphatic GSL. Consequently, *MYB28/HAG1* seems to be a main player in the regulation of this network working in concert with *MYB29/HAG3* and *MYB76/HAG2* leading to production of a wide range of aliphatic GSL in high amounts upon different biotic challenges.

Furthermore, experimental data indicated that MYB/HAG transcription factors are involved in the interplay with other regulators (Fig. 17; Fig. 18; Fig. 19). According to the microarray data (http://www.atted.bio.titech.ac.jp/; Toufighi et al., 2005) *MYB/HIG* and *MYB/HAG* regulators were highly co-expressed with *WRKY25* and *WRKY33* genes which are known as key regulatory components of defence-related genes in response to microbial infection. The main function of *WRKY25* and *WRKY33* is suggested in the MPK4 signaling pathway that represses SA-mediated and activates MeJa/ACC-mediated defence responses to microbial pathogens (Zheng et al., 2006; Zheng et al., 2007). The promoter analysis of *MYB/HAG* and *MYB/HIG* regulators indicated a presence of the *cis*-acting element (C/T**TGA**CC/T) known as W-box, which is specifically recognized by WRKY proteins. Results of *trans*-activation experiments indicated that *WRKY25* was able to specifically activate the promoter of *MYB29/HAG3*. In addition, it has been shown that *MYB29/HAG3* is induced by MeJa and repressed by SA treatments. Therefore, it is suggested that *WRKY25* acts in concert with *MYB29/HAG3* to activate MeJa-mediated production of aliphatic glucosinolates upon pathogen infection. Moreover, based on the high co-expression of *WRKY25* and its homologue *WRKY33* with *MYB51/HIG1*, one may also expect an interplay between these regulators in MeJa-induced production of indolic glucosinolates. However, further studies are required to elucidate the role of *WRKY25* and *WRKY33* in the interplay with MeJa-induced regulators of aliphatic and indolic glucosinolate pathways (Fig. 22).

The crosstalk between sulphur metabolism and glucosinolate biosynthesis was studied with respect to *MYB28/HAG1* and *SLIM1* (sulphur limitation1*)*. *SLIM1*

controls the activation of sulphate acquisition and glucosinolates degradation upon sulphur deficient conditions (Maruyama-Nakashita et al., 2006). In addition, MYB/HAG regulators are involved in the regulation of sulphate assimilation genes indicating a possible interaction with *SLIM1* under low sulphate conditions. According to the real-time RT-PCR data, the expression of *MYB28/HAG1* is considerably repressed in cultured *Arabidopsis* cells over-expressing *SLIM1* (Fig. 19). This observation corresponded to previously reported data that *MYB34/ATR1* and several indolic and aliphatic glucosinolate biosynthetic genes were downregulated by *SLIM1* in *Arabidopsis* roots indicating its negative effect on glucosinolate biosynthesis upon sulphur deficiency. In brief, *SLIM1* expression is induced under low sulphur conditions leading to the downregulation of *MYB/HIG* and *MYB/HAG* regulators and a reduced biosynthesis of GSL. On the other hand, in order to increase the sulphur pool for primary metabolism, SLIM1 was suggested to activate glucosinolate breakdown processes. On the whole, the interaction of MYB/HAG regulators with SLIM1 (Fig. 19) and sulphate assimilation (Fig. 12) genes revealed a novel regulatory mechanism to control aliphatic GSL biosynthesis in response to changes in the nutritional status.

However, further studies are required to unravel how *MYB28/HAG1*, *MYB29/HAG3* and *MYB76/HAG2* are regulated and/or interacting with signaling components of other biosynthetic pathways, encompassing a complex regulatory network in production of aliphatic GSL under different environmental conditions (Fig. 21; Fig. 22).

4.8 Functional model of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors in plants protection against herbivores or pathogens

The function of *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcription factors can be schematically represented using the following model: (i) input signals (biotic stress or wounding) induce the production of *MYB/HAG* transcription factors; (ii) signal processing, i.e. activation of *MYB/HAG* by themselves, followed by

transcriptional activation of target GSL pathway genes by MYB/HAG factors; (iii) output, i.e. increase in the content of glucosinolates and changes the in plant resistance to herbivores and pathogens (Fig. 23).

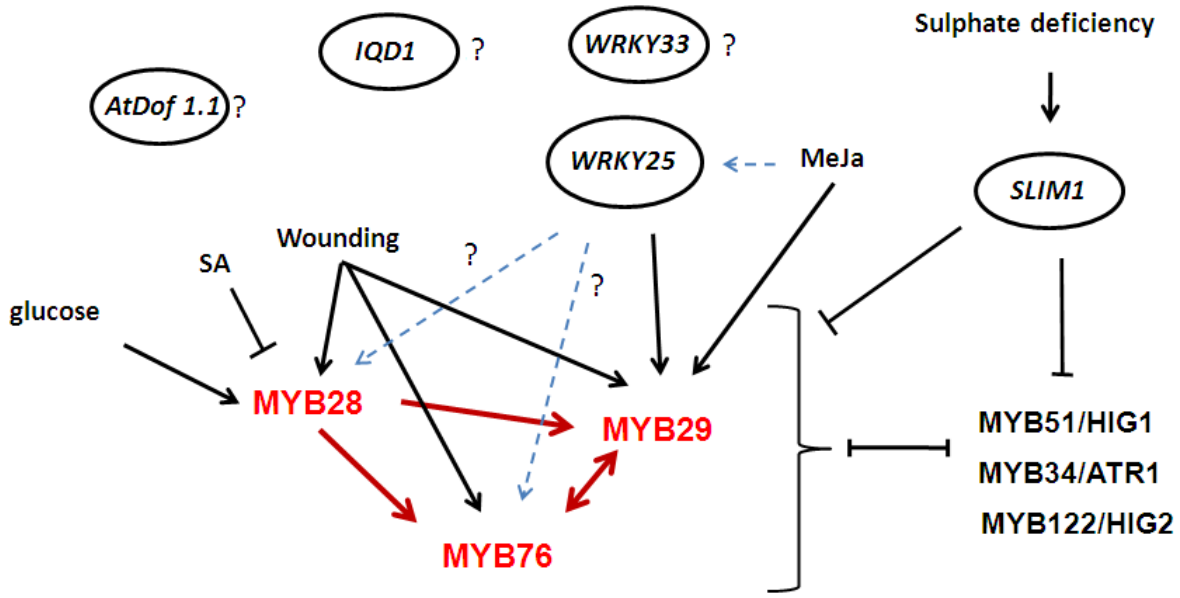

Figure 22. Crosstalk between transcription regulators controlling aliphatic GSL biosynthesis, different signaling pathways and other regulators (↑ - activation; ⊥ - repression). Question marks possible interaction candidates.

Different pathogen-related signaling pathways (wounding, MeJa, SA and glucose) are involved in regulation of MYB/HAG transcription factors. Expression of *MYB28/HAG1* and *MYB29/HAG3* in leaf trichomes and very rapid induction of all *MYB/HAG* factors within 1-5 min after wounding revealed their functions in early defence-responses to biotic challenge (Clauss et al., 2006).

It has recently been reported that plants respond to insect and herbivore attacks by accumulating higher amounts of glucosinolates (Brader et al., 2006b; Bruce et al., 2008). As some glucosinolates are constitutively present and production of others can be induced in response to specific pathogens or herbivores, the interdependent regulation among *MYB/HAG* factors may allow to enhance incoming signals and increasing the production of specific GSL. Therefore, an elevated level of aliphatic GSL may reduce the performance of generalist herbivores and/or pathogens as revealed by weight-gain experiments; *MYB28/HAG1* over-expression plants

accumulated higher contents of aliphatic glucosinolates and reduced weight-gain of generalist herbivore *Spodoptera exigua* larvae feeding on these plants (Fig. 20). In addition, the role of *MYB29/HAG3* regulator is probably linked to an increase of plant resistance upon microbial infection together with *WRKY25*. Thus, evidence is presented that functions of *MYB/HAG* regulators are important for the biosynthesis of aliphatic GSL and plant resistance against generalist herbivores and pathogens.

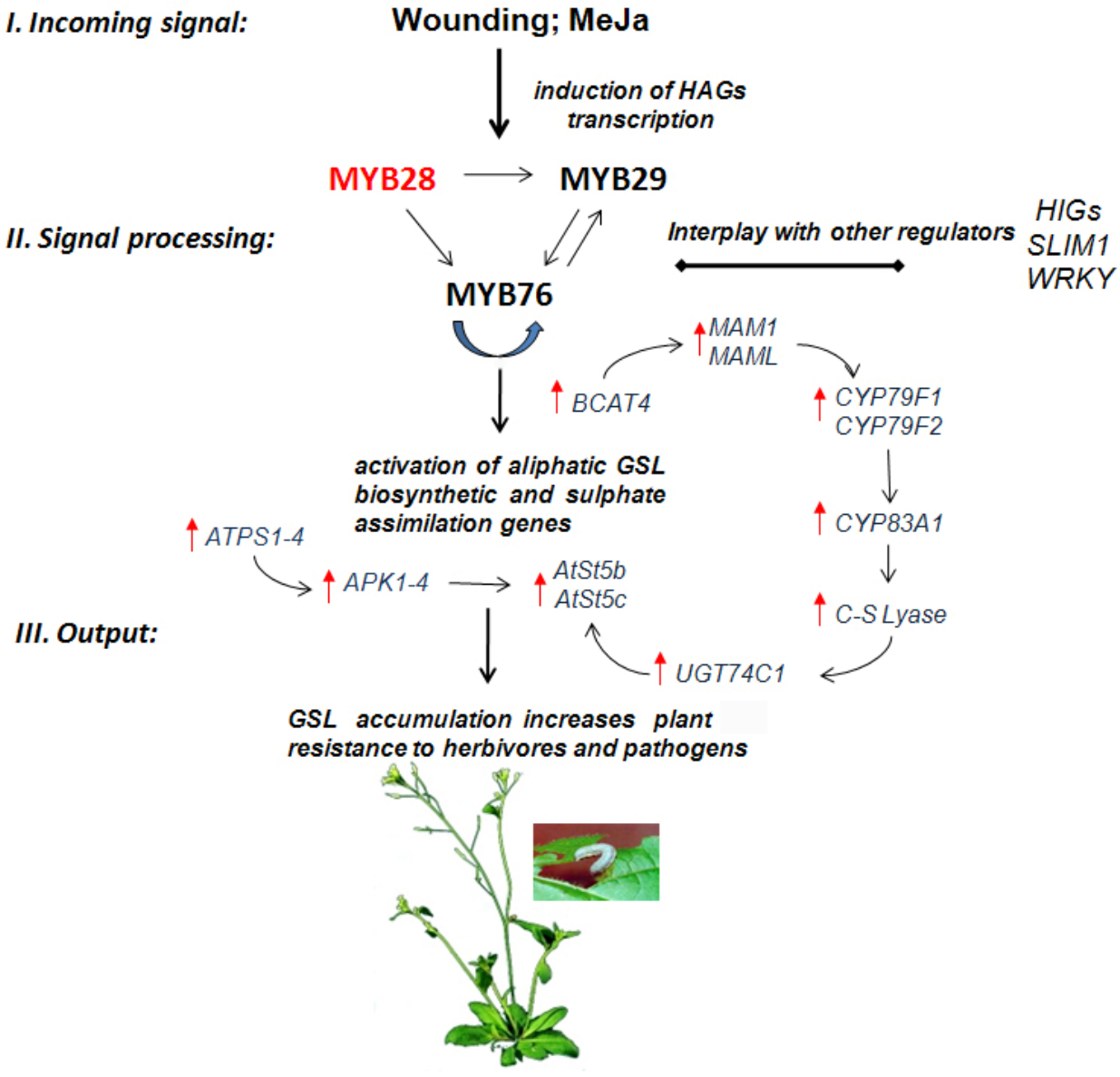

Figure 23. The role of *MYB28/HAG1, MYB29/HAG3 and MYB76/HAG2* transcription factors in plant defence against herbivores.

5. References

Andreasson, E., Jorgensen LB, Hoglund AS, Rask L, Meijer J. (2001). Different myrosinaseand idioblast distribution in Arabidopsis and Brassica napus. Plant Physiol. **127,** 1750–1763.

Bak, S., and Feyereisen, R. (2001). The involvement of two P450 enzymes, CYP83B1 and CYP83A1, in auxin homeostasis and glucosinolate biosynthesis. Plant Physiology **127,** 108-118.

Bak, S.T., F. E.Feldmann, K. A.Galbraith, D. W.Feyereisen, R. (2001). CYP83B1, a cytochrome P450 at the metabolic branch paint in auxin and indole glucosinolate biosynthesis in Arabidopsis. Plant Cell **13,** 101-111.

Bednarek, P., Schneider, B., Svatos, A., Oldham, N.J., and Hahlbrock, K. (2005). Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in Arabidopsis roots. Plant Physiology **138,** 1058-1070.

Berger, B. (2007). The role of HIG1/MYB51 in the regulation of indolic glucosinolate biosynthesis. In Botaniches Institute der Mathematisch-Naturwissenschaftlichen Fakultät (Köln: Universität zu Köln), pp. 119.

Berger, B., Stracke, R., Yatusevich, R., Weisshaar, B., Flugge, U.I., and Gigolashvili, T. (2007). A simplified method for the analysis of transcription factor-promoter interactions that allows high-throughput data generation. Plant Journal **50,** 911-916.

Bonnesen, C., Eggleston, M., and Hayes, D. (2001). Dietary Indoles and Isothiocyanates That Are Generated from Cruciferous Vegetables Can Both Stimulate Apoptosis and Confer Protection against DNA Damage in Human Colon Cell Lines. Cancer Research **61,** 6120-6130.

Bowles, D., Isayenkova, J., Lim, E.K., and Poppenberger, B. (2005). Glycosyltransferases: managers of small molecules. Current Opinion in Plant Biology **8,** 254-263.

Brader, G., Tas, E., and Palva, E.T. (2001). Jasmonate-dependent induction of indole glucosinolates in Arabidopsis by culture filtrates of the nonspecific pathogen Erwinia carotovora. Plant Physiology **126,** 849-860.

Brader, G., Mikkelsen, M.D., Halkier, B.A., and Palva, E.T. (2006a). Altering glucosinolate profiles modulates disease resistance in plants. Plant Journal **46,** 758-767.

Brader, G., Mikkelsen, M.D., Halkier, B.A., and Tapio Palva, E. (2006b). Altering glucosinolate profiles modulates disease resistance in plants. Plant J **46,** 758-767.

Brown, P.D., Tokuhisa, J.G., Reichelt, M., and Gershenzon, J. (2003a). Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. Phytochemistry **62,** 471-481.

Brown, P.D., Tokuhisa, J.G., Reichelt, M., and Gershenzon, J. (2003b). Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. Phytochemistry **62,** 471-481.

Bruce, T.J., Matthes, M.C., Chamberlain, K., Woodcock, C.M., Mohib, A., Webster, B., Smart, L.E., Birkett, M.A., Pickett, J.A., and Napier, J.A. (2008). cis-Jasmone induces Arabidopsis genes that affect the chemical ecology of multitrophic interactions with aphids and their parasitoids. Proc Natl Acad Sci U S A **105,** 4553-4558.

Celenza, J.L. (2001). Metabolism of tyrosine and tryptophan - new genes for old pathways. Current Opinion in Plant Biology **4,** 234-240.

Celenza, J.L., Quiel, J.A., Smolen, G.A., Merrikh, H., Silvestro, A.R., Normanly, J., and Bender, J. (2005). The Arabidopsis ATR1 Myb transcription factor controls indolic glucosinolate homeostasis. Plant Physiology **137,** 253-262.

Chassot, C. Buchala, A.Schoonbeek, J., H.M., and O., a.L. (2008). Wounding of Arabidopsis leaves causes a powerful but transient protection against Botrytis infection. The Plant Journal **55,** 555 - 567.

Chen, S.X., Glawischnig, E., Jorgensen, K., Naur, P., Jorgensen, B., Olsen, C.E., Hansen, C.H., Rasmussen, H., Pickett, J.A., and Halkier, B.A. (2003). CYP79F1 and CYP79F2 have distinct functions in the biosynthesis of aliphatic glucosinolates in Arabidopsis. Plant Journal **33,** 923-937.

Chung, W.C., Huang, H.C., Chiang, B.T., Huang, H.C., and Huang, J.W. (2005). Inhibition of soil-borne plant pathogens by the treatment of sinigrin and myrosinases released from reconstructed Escherichia coli and Pichia pastoris. Biocontrol Science and Technology **15,** 455-465.

Cipollini, D., Enright, S., Traw, M.B., and Bergelson, J. (2004). Salicylic acid inhibits jasmonic acid-induced resistance of Arabidopsis thaliana to Spodoptera exigua. Mol Ecol **13,** 1643-1653.

Clauss, M.J., Dietel, S., Schubert, G., and Mitchell-Olds, T. (2006). Glucosinolate and trichome defenses in a natural Arabidopsis lyrata population. J Chem Ecol **32,** 2351-2373.

Dawson, G.W., Hick, A.J., Bennett, R.N., Donald, A., Pickett, J.A., and Wallsgrove, R.M. (1993). Synthesis of Glucosinolate Precursors and Investigations into the Biosynthesis of Phenylalkylglucosinocates and Methylthioalkylglucosinolates. Journal of Biological Chemistry **268,** 27154-27159.

Dinkova-Kostova, A.T., Jenkins, S.N., Fahey, J.W., Ye, L., Wehage, S.L., Liby, K.T., Stephenson, K.K., Wade, K.L., and Talalay, P. (2006). Protection against UV-light-induced skin carcinogenesis in SKH-1 high-risk mice by sulforaphane-containing broccoli sprout extracts. Cancer Letters **240,** 243-252.

Dombrecht, B., Xue, G.P., Sprague, S.J., Kirkegaard, J.A., Ross, J.J., Reid, J.B., Fitt, G.P., Sewelam, N., Schenk, P.M., Manners, J.M., and Kazan, K. (2007). MYC2 differentially modulates diverse jasmonate-dependent functions in Arabidopsis. Plant Cell **19,** 2225-2245.

Fahey, J.W., Zalcmann, A.T., and Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56,** 5-51.

Falk, K.L., Vogel, C., Textor, S., Bartram, S., Hick, A., Pickett, J.A., and Gershenzon, J. (2004). Glucosinolate biosynthesis: demonstration and characterization of the condensing enzyme of the chain elongation cycle in Eruca sativa. Phytochemistry **65,** 1073-1084.

Field, B., Cardon, G., Traka, M., Botterman, J., Vancanneyt, G., and Mithen, R. (2004). Glucosinolate and amino acid biosynthesis in Arabidopsis. Plant Physiology **135,** 828-839.

Flügge, U.I., and Gigolashvili, T. (2006). Verfahren zur Herstellung einer transgenen Pflanze der Familie der Brassicaceae (Kreuzblьtler) mit einer erhцhten Glucosinolat-Biosynthese. (Germany).

Gigolashvili, T., Yatusevich, R., Berger, B., Muller, C., and Flugge, U.I. (2007a). The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in Arabidopsis thaliana. Plant J **51,** 247-261.

Gigolashvili, T., Berger, B., Mock, H.P., Muller, C., Weisshaar, B., and Flugge, U.I. (2007b). The transcription factor HIG1/MYB51 regulates indolic glucosinolate biosynthesis in Arabidopsis thaliana. Plant J **50,** 886-901.

Gigolashvili, T., Engqvist, M., Yatusevich, R., Muller, C., and Flugge, U.I. (2008). HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in Arabidopsis thaliana. New Phytol **177,** 627-642.

Glawischnig, E., Hansen, B.G., Olsen, C.E., and Halkier, B.A. (2004). Camalexin is synthesized from indole-3-acetaidoxime, a key branching point between primary and secondary metabolism in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America **101,** 8245-8250.

Glendening, T.M., and Poulton, J.E. (1988). Sulfation of Desulfobenzylglucosinolate by Cell-Free Extracts of Cress (Lepidium sativum L.) Seedlings Plant Physiology **86,** 319-321.

Griffiths, D.W., Birch, A.N.E., and Hillman, J.R. (1998). Antinutritional compounds in the Brassicaceae: Analysis, biosynthesis, chemistry and dietary effects. Journal of Horticultural Science & Biotechnology **73,** 1-18.

Grubb, C.D., and Abel, S. (2006). Glucosinolate metabolism and its control. Trends in Plant Science **11,** 89-100.

Grubb, C.D., Zipp, B.J., Ludwig-Muller, J., Masuno, M.N., Molinski, T.F., and Abel, S. (2004). Arabidopsis glucosyltransferase UGT74B1 functions in glucosinolate biosynthesis and auxin homeostasis. Plant Journal **40,** 893-908.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annual Review of Plant Biology **57,** 303-333.

Hansen, B., and Halkier, B. (2005). New insight into the biosynthesis and regulation of indole compounds in Arabidopsis thaliana. Planta **221,** 603-606.

Hansen, B.G., Kliebenstein, D.J., and Halkier, B.A. (2007). Identification of a flavin-monooxygenase as the S-oxygenating enzyme in aliphatic glucosinolate biosynthesis in Arabidopsis. Plant Journal **50,** 902-910.

Hayes, D., Kelleher, O., and Eggelston, M. (2008). The cancer chemoprotective actions of phytochemicals derived from glucosinolates Eur. J. Nutr**,** 73-88.

Hemm, M.R., Ruegger, M.O., and Chapple, C. (2003). The Arabidopsis ref2 mutant is defective in the gene encoding CYP83A1 and shows both phenylpropanoid and glucosinolate phenotypes. Plant Cell **15,** 179-194.

Herbers, K., Meuwly, P., Metraux, J.P., and Sonnewald, U. (1996). Salicylic acid-independent induction of pathogenesis-related protein transcripts by sugars is dependent on leaf developmental stage. Febs Letters **397,** 239-244.

Hirai, M.Y., Fujiwara, T., Awazuhara, M., Kimura, T., Noji, M., and Saito, K. (2003). Global expression profiling of sulfur-starved Arabidopsis by DNA macroarray reveals the role of O-acetyl-L-serine as a general regulator of gene expression in response to sulfur nutrition. Plant J. **33,** 651–663.

Hirai, M.Y., Sugiyama, K., Sawada, Y., Tohge, T., Obayashi, T., Suzuki, A., Araki, R., Sakurai, N., Suzuki, H., Aoki, K., Goda, H., Nishizawa, O.I., Shibata, D., and Saito, K. (2007). Omics-based identification of Arabidopsis Myb transcription factors regulating aliphatic glucosinolate biosynthesis. Proceedings of the National Academy of Sciences of the United States of America **104,** 6478-6483.

Hirai, M.Y., Klein, M., Fujikawa, Y., Yano, M., Goodenowe, D.B., Yamazaki, Y., Kanaya, S., Nakamura, Y., Kitayama, M., Suzuki, H., Sakurai, N., Shibata, D., Tokuhisa, J., Reichelt, M., Gershenzon, J., Papenbrock, J., and Saito, K. (2005). Elucidation of gene-to-gene and metabolite-to-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. Journal of Biological Chemistry **280,** 25590-25595.

Hirai, M.Y.e.a. (2005). Elucidation of gene-to-gene and metaboliteto-gene networks in Arabidopsis by integration of metabolomics and transcriptomics. J. Biol. Chem **280,** 25590–25595.

Hull, A.K., Vij, R., and Celenza, J.L. (2000). Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. Proceedings of the National Academy of Sciences of the United States of America **97,** 2379-2384.

Johnson, R., and Ryan, C.A. (1990). Wound-Inducible Potato Inhibitor-Ii Genes - Enhancement of Expression by Sucrose. Plant Molecular Biology **14,** 527-536.

Kirkegaard, J.A., and Sarwar, M. (1998). Biofumigation potential of brassicas - I. Variation in glucosinolate profiles of diverse field-grown brassicas. Plant and Soil **201,** 71-89.

Kirkegaard, J.A., Sarwar, M., and Matthiessen, J.N. (1998). Assessing the biofumigation potential of Crucifers. Brassica 97**,** 105-111.

Klein, M., Reichelt, M., Gershenzon, J., and Papenbrock, J. (2006). The three desulfoglucosinolate sulfotransferase proteins in Arabidopsis have different substrate specificities and are differentially expressed. Febs Journal **273,** 122-136.

Kliebenstein, D., Pedersen, D., Barker, B., and Mitchell-Olds, T. (2002a). Comparative analysis of quantitative trait loci controlling glucosinolates, myrosinase and insect resistance in Arabidapsis thaliana. Genetics **161,** 325-332.

Kliebenstein, D.J., Gershenzon, J., and Mitchell-Olds, T. (2001a). Comparative quantitative trait loci mapping of aliphatic, indolic and benzylic glucosinolate production in Arabidopsis thaliana leaves and seeds. Genetics **159,** 359-370.

Kliebenstein, D.J., Figuth, A., and Mitchell-Olds, T. (2002b). Genetic architecture of plastic methyl jasmonate responses in Arabidopsis thaliana. Genetics **161,** 1685-1696.

Kliebenstein, D.J., Kroymann, J., and Mitchell-Olds, T. (2005a). The glucosinolate-myrosinase system in an ecological and evolutionary context. Current Opinion in Plant Biology **8,** 264-271.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005b). Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity. Plant Journal **44,** 25-36.

Kliebenstein, D.J., Lambrix, V.M., Reichelt, M., Gershenzon, J., and Mitchell-Olds, T. (2001b). Gene duplication in the diversification of secondary metabolism: Tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in arabidopsis. Plant Cell **13,** 681-693.

Knill, T., Schuster, J., Reichelt, M., Gershenzon, J., and Binder, S. (2008). Arabidopsis branched-chain aminotransferase 3 functions in both amino acid and glucosinolate biosynthesis. Plant Physiology **146,** 1028-1039.

Koenraad, F., Bart P., B., M., Schmidt, J., Kistner, K., Porzel, A., Mauch-Mani, B., Cammue, B., and W., B. (2001). Study of the Role of Antimicrobial Glucosinolate-Derived Isothiocyanates in Resistance of Arabidopsis to Microbial Pathogens. Plant Physiol. **125(4)** 1688–1699.

Koroleva, O., Davies A, Deeken R, Thorpe MR, Tomos AD, Hedrich R. (2000). Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. Plant Physiol. **124,** 599–608.

Koroleva, O.A., Tomlinson, M.L., Leader, D., Shaw, P., and Doonan, J.H. (2005). High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant Journal **41,** 162-174.

Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J., and Gershenzon, J. (2001). The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences Trichoplusia ni herbivory. Plant Cell **13,** 2793-2807.

Levy, M., Wang, Q.M., Kaspi, R., Parrella, M.P., and Abel, S. (2005). Arabidopsis IQD1, a novel calmodulin-binding nuclear protein, stimulates glucosinolate accumulation and plant defense. Plant Journal **43,** 79-96.

Li, Y.H., Lee, K.K., Walsh, S., Smith, C., Hadingham, S., Sorefan, K., Cawley, G., and Bevan, M.W. (2006). Establishing glucose- and ABA-regulated transcription networks in Arabidopsis by microarray analysis and promoter classification using a Relevance Vector Machine. Genome Research **16,** 414-427.

London, S.J., Yuan, J.M., Chung, F.L., Gao, Y.T., Gao, Y.T., Coetzee, G., Ross, R., and Yu, M. (2000). Isothiocyanates, glutathione S-transferase M1 and T1 polymorphisms, and lung-cancer risk: a prospective study of men in Shanghai, China. Lancet **356(9231),** 724-729.

Luthy, B., and Matile, P. (1984). The Mustard Oil Bomb - Rectified Analysis of the Subcellular Organization of the Myrosinase System. Biochemie Und Physiologie Der Pflanzen **179,** 5-12.

Maruyama-Nakashita, A., Inoue, E., Watanabe-Takahashi, A., Yarnaya, T., and Takahashi, H. (2003). Transcriptome profiling of sulfur-responsive genes in Arabidopsis reveals global effects of sulfur nutrition on multiple metabolic pathways. Plant Physiology **132,** 597-605.

Maruyama-Nakashita, A., Nakamura, Y., Tohge, T., Saito, K., and Takahashi, H. (2006). Arabidopsis SLIM1 is a central transcriptional regulator of plant sulfur response and metabolism. Plant Cell **18,** 3235-3251.

Mewis, I., Appel, H.M., Hom, A., Raina, R., and Schultz, J.C. (2005). Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiology **138,** 1149-1162.

Mikkelsen, M.D., and Halkier, B.A. (2003). Metabolic engineering of valine- and isoleucine-derived glucosinolates in Arabidopsis expressing CYP79D2 from cassava. Plant Physiology **131,** 773-779.

Mikkelsen, M.D., Naur, P., and Halkier, B.A. (2004). Arabidopsis mutants in the C-S lyase of glucosinolate biosynthesis establish a critical role for indole-3-acetaldoxime in auxin homeostasis. Plant Journal **37,** 770-777.

Mikkelsen, M.D., Hansen, C.H., Wittstock, U., and Halkier, B.A. (2000). Cytochrome P450CYP79B2 from Arabidopsis catalyzes the conversion of tryptophan to indole-3-acetaldoxime, a precursor of indole glucosinolates and indole-3-acetic acid. Journal of Biological Chemistry **275,** 33712-33717.

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A. (2003). Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. Plant Physiology **131,** 298-308.

Mithen, R.F. (2001). Glucosinolates and their degradation products. In Advances in Botanical Research, Vol 35, pp. 213-262.

Mueller, C., Agerbirk, N., Olsen, C., Boeve, J., Schaffner, U., Brakefield, P. (2001). Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly Athalia rosae. J. Chem. Ecol. **27,** 2505–2516.

Müller, C., Agerbirk, N., Olsen, C.E., Boeve, J.L., Schaffner, U., and Brakefield, P.M. (2001). Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. Journal of Chemical Ecology **27,** 2505-2516.

Nair, R., and Rost, B. (2005). Mimicking cellular sorting improves prediction of subcellular localization. Journal of Molecular Biology **348,** 85-100.

Naur, P., Petersen, B.L., Mikkelsen, M.D., Bak, S., Rasmussen, H., Olsen, C.E., and Halkier, B.A. (2003). CYP83A1 and CYP83B1, two nonredundant cytochrome P450 enzymes metabolizing oximes in the biosynthesis of glucosinolates in Arabidopsis. Plant Physiology **133,** 63-72.

Nikiforova, V., Freitag, J., Kempa, S., Adamik, M., Hesse, H.,, and Hoefgen, R. (2003). Transcriptome analysis of sulfur depletion in Arabidopsis thaliana: Interlacing of biosynthetic pathways provides response specificity. Plant J. **33,** 633–650.

Petersen, B.L., Chen, S.X., Hansen, C.H., Olsen, C.E., and Halkier, B.A. (2002). Composition and content of glucosinolates in developing Arabidopsis thaliana. Planta **214,** 562-571.

Piotrowski, M., Schemenewitz, A., Lopukhina, A., Muller, A., Janowitz, T., Weiler, E.W., and Oecking, C. (2004). Desulfoglucosinolate sulfotransferases from Arabidopsis thaliana catalyze the final step in the biosynthesis of the glucosinolate core structure. Journal of Biological Chemistry **279,** 50717-50725.

Ratzka, A., Vogel, H., Kliebenstein, D.J., Mitchell-Olds, T., and Kroymann, J. (2002). Disarming the mustard oil bomb. Proceedings of the National Academy of Sciences of the United States of America **99,** 11223-11228.

Rausch, T., and Wachter, A. (2005). Sulfur metabolism: a versatile platform for launching defence operations. Trends in Plant Science **10,** 503-509.

Raybould, A., and Moyesà, C. (2001). The ecological genetics of aliphatic glucosinolates. Heredity **87,** 383-391.

Reintanz, B., Lehnen, M., Reichelt, M., Gershenzon, J., Kowalczyk, M., Sandberg, G., Godde, M., Uhl, R., and Palme, K. (2001). Bus, a bushy arabidopsis CYP79F1 knockout mutant with abolished synthesis of short-chain aliphatic glucosinolates. Plant Cell **13,** 351-367.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell **14,** S185-S205.

Sadka, A., Dewald, D.B., May, G.D., Park, W.D., and Mullet, J.E. (1994). Phosphate Modulates Transcription of Soybean Vspb and Other Sugar-Inducible Genes. Plant Cell **6,** 737-749.

Sakac, B., Filipovic, S., et al. (2006). The Influence of Extrusion on Total Glucosinolates and Total Phenols in Rapeseed Acta Agriculturae Serbica **XI,** 19-27.

Schuster, J., Knill, T., Reichelt, M., Gershenzon, J., and Binder, S. (2006). BRANCHED-CHAIN AMINOTRANSFERASE4 is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. Plant Cell **18,** 2664-2679.

Shen, G., Khor, T., Hu, R., Yu, S., Nair, S., Chi-Tang Ho, Bandaru S. Reddy, Mou-Tuan Huang, Harold L. Newmark, and Kong, a.A.-N.T. (2007). Chemoprevention of Familial Adenomatous Polyposis by Natural Dietary Compounds Sulforaphane and Dibenzoylmethane Alone and in Combination in ApcMin/+ Mouse Cancer Res **67,** 9937-9944.

Shroff, R., Vergara, F., Muck, A., Aleš Svatoš, and Gershenzon, J. (2008). Nonuniform distribution of glucosinolates in Arabidopsis thaliana leaves has important consequences for plant defense PNAS **105,** 6196-6201.

Shuttuck, V.I. (1993). Glucosinolates and glucosinolate degradation in seeds from turnip mosaic virus-infected rapid cycle Brassica campestris L. plants. Journal of experimental botany **44** (262), 963-970.

Skirycz, A., Reichelt, M., Burow, M., Birkemeyer, C., Rolcik, J., Kopka, J., Zanor, M.I., Gershenzon, J., Strnad, M., Szopa, J., Mueller-Roeber, B., and Witt, I. (2006). DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in Arabidopsis. Plant Journal **47,** 10-24.

Smith, B., Kirkegaard, J., and Howe, G. (2004). Impacts of Brassica break-crops on soil biology and yield of following wheat crops. Australian Journal of Agricultural Research **55,** 1 - 11

Smolen, G., and Bender, J. (2002). Arabidopsis cytochrome p450 cyp83B1 mutations activate the tryptophan biosynthetic pathway. Genetics **160,** 323-332.

Stracke, R., Werber, M., Weisshaar, B. (2001). The R2R3-MYB gene family in Arabidopsis thaliana. Curr Opin Plant Biol. **4,** 447-456.

Talalay, P., and Fahey, J.W. (2001). Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. J. Nutr **131,** 3027S–3033S.

Tantikanjana, T., Mikkelsen, M.D., Hussain, M., Halkier, B.A., and Sundaresan, V. (2004). Functional analysis of the tandem-duplicated P450 genes SPS/BUS/CYP79F1 and CYP79F2 in glucosinolate biosynthesis and plant development by Ds transposition-generated double mutants. Plant Physiology **135,** 840-848.

Textor, S., de Kraker, J.W., Hause, B., Gershenzon, J., and Tokuhisa, J.G. (2007). MAM3 catalyzes the formation of all aliphatic glucosinolate chain lengths in arabidopsis. Plant Physiology **144,** 60-71.

Tierens, K., Thomma, B., Brouwer, M., Schmidt, J., Kistner, K., et al. (2001). Study of the role of antimicrobial glucosinolate-derived isothiocyanates in resistance of Arabidopsis to microbial pathogens. Plant Physiol.**,** 1688–1699.

Toroser, D., Wood, C., Griffiths, H., and Thomas, D.R. (1995). Glucosinolate Biosynthesis in Oilseed Rape (Brassica-Napus L) - Studies with (So42-)-S-35 and Glucosinolate Precursors Using Oilseed Rape Pods and Seeds. Journal of Experimental Botany **46,** 787-794.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005). The Botany Array Resource: e-Northerns, Expression Angling, and Promoter analyses. Plant Journal **43,** 153-163.

Traw, M.B., Kim, J., Enright, S., Cipollini, D.F., and Bergelson, J. (2003). Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of Arabidopsis thaliana. Mol Ecol **12,** 1125-1135.

van Poppel, G., Verhoeven, D., Verhagen, H., and Goldbohm, R. (1999). Brassica vegetables and cancer prevention. Epidemiology and mechanisms. Adv Exp Med Biol.**,** 159-168.

Vaughn, S.F., Isbell, T.A., Weisleder, D., and Berhow, M.A. (2005). Biofumigant compounds released by field pennycress (Thlaspi arvense) seedmeal. Journal of Chemical Ecology **31,** 167-177.

Wentzell, A.M., Rowe, H.C., Hansen, B.G., Ticconi, C., Halkier, B.A., and Kliebenstein, D.J. (2007). Linking metabolic QTLs with network and cis-eQTLs controlling biosynthetic pathways. Plos Genetics **3,** 1687-1701.

Wetter, L., Chisholm, M. (1968). Sources of sulfur in thioglucosides of various higher plants. Can. J. Biochem **46,** 931–935.

Wittstock, U., and Gershenzon, J. (2002). Constitutive plant toxins and their role in defense against herbivores and pathogens. Current Opinion in Plant Biology **5,** 300-307.

Wittstock, U., Agerbirk, N., Stauber, E.J., Olsen, C.E., Hippler, M., Mitchell-Olds, T., Gershenson, J., and Vogel, H. (2004). Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proceedings of the National Academy of Sciences of the United States of America **101,** 4859-4864.

Xiao, W.Y., Sheen, J., and Jang, J.C. (2000). The role of hexokinase in plant sugar signal transduction and growth and development. Plant Molecular Biology **44,** 451-461.

Xue, J.P., Jorgensen, M., Pihlgren, U., and Rask, L. (1995). The Myrosinase Gene Family in Arabidopsis-Thaliana - Gene Organization, Expression and Evolution. Plant Molecular Biology **27,** 911-922.

Yan, X.F., and Chen, S.X. (2007). Regulation of plant glucosinolate metabolism. Planta **226,** 1343-1352.

Zabala, M.D., Grant, M., Bones, A.M., Bennett, R., Lim, Y.S., Kissen, R., and Rossiter, J.T. (2005). Characterisation of recombinant epithiospecifier protein and its over-expression in Arabidopsis thaliana. Phytochemistry **66,** 859-867.

Zang, Y.X., Kim, J.H., Park, Y.D., Kim, D.H., and Hong, S.B. (2008). Metabolic engineering of aliphatic glucosinolates in Chinese cabbage plants expressing Arabidopsis MAM1, CYP79F1, and CYP83A1. Bmb Reports **41,** 472-478.

Zasada, I.A., and Ferris, H. (2004). Nematode suppression with brassicaceous amendments: application based upon glucosinolate profiles. Soil Biology & Biochemistry **36,** 1017-1024.

Zheng, Z., Qamar, S., Chen, Z., and Mengiste, T. (2006). Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. **48(4),** 592-605.

Zheng, Z., Mosher, S., Fan, B., and Chen, Z. (2007). Functional analysis of Arabidopsis WRKY25 transcription factor in plant defense against Pseudomonas syringae. BMC Plant Biology **7:2,** doi:10.1186/1471-2229-1187-1182.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiology **136,** 2621-2632.

Internet recourses and databases:

http://www.atted.bio.titech.ac.jp/ - ATTEDII: *Arabidopsis thaliana* trans-factor and cis-element prediction database.

https://www.genevestigator.ethz.ch - GENEVESTIGATOR: expression database and meta-analysis system.

http://bar.utoronto.ca/ntools/ - EXPRESSION ANGLER: microarray co-expression database.

http://www.expasy.org/uniprot/ - SWISS PROT: protein knowledgebase.

http://www.cubic.bioc.columbia.edu/services/loctree/ - LOCtree: a service for predicting the subcellular localization of proteins

6. Appendix

Primers for Gateway cloning

Generation of CDS entry clones

Primer name	AGI number	Gene name	Primer sequence 5'-3'
attB1_GW	-	-	gggacaagtttgtacaaaaaagcaggcttc
attB2_GW	-	-	gggaccactttgtacaagaaagctgggtc
GW-At5g614020 Fw	At5g61420	MYB28	ATGTCAAGAAAGCCATGTTGCGTCGG
GW-At5g614020 RV	At5g61420	MYB28	TATGAAATGCTTTTCAAGCGAGTCTGAG
MYB28_RNAi_1_Fw_attB1	At5g61420	MYB28	TTAATGGCTTCACTGAGCAGATTC
MYB28_RNAi_1_Rv_att B2	At5g61420	MYB28	TGATGAGACTTCTTGGGAAACATC
MYB28_RNAi_2a_Fw_attB1	At5g61420	MYB28	AATTACCGAAGTGACTCGTCTAAG
MYB28_RNAi_2a_Rv_attB2	At5g61420	MYB28	ACTACTCTGAAGAACCCTTTGTAG
MYB29_ attB1_gene_Fw	At5g07690	MYB29	ATGTCAAGAAAGCCATGTTGTGTG
MYB29_ attB2_gene_Rv	At5g07690	MYB29	TATGAAGTTCTTGTCGTCGTAATC
pENTR-At5g07700 FW	At5g07700	MYB76	CACCATGTCAAAGAGACCATATTGTATC
pENTR-At5g07700 RV	At5g07700	MYB76	TAAGAAGTTCTTCTCGTCGGAATCTT

Generation of promoter's entry clones

Primer name	AGI number	Gene name	Primer sequence 5'-3'
pMAM1_Fw_attB1	At5g23010	MAM1	TTAGTATCCAATCCCACAGCACTG
pMAM1_RV_attB2	At5g23010	MAM1	CGTGTCGAATACACGCACATAGTT
pMAML_Fw_attB1	At5g23020	MAML	ATATTTTTTTTGTATGAATGTAAACC
pMAML_RV_attB2	At5g23020	MAML	CTGCCTGGCAATCTCTAACTTCTGC
pCYP79F1_FW_attB1	At1g16410	CYP79F1	GAATTAGACTACATGACACCAACAAGA
pCYP79F1_RV_attB2	At1g16410	CYP79F1	GGAGAGGATAAAGACTAGTAGGATGTGA
pCYP79F2_FW_attB1	At1g16400	CYP79F2	TGATCTATTCAACAATGACTCATG
pCYP79F2_RV_attB2	At1g16400	CYP79F2	ACCAGTGCTAAATGAGACAAAAC
pCYP83A1_FW_attB1	At4g13770	CYP83A1	CAGACGCAAAGCAACCACACAACTTAC
pCYP83A1_RV_attB2	At4g13770	CYP83A1	TTGGTAGAGGAAGAAAAGGAGAACC
pC-S_FW_attB1	At2g20610	C-S lyase	TTAGCTGCTCACTGACTT
pC-S_RV_attB2	At2g20610	C-S lyase	CGTTATGGGCTCTTTCTC
promMYB28_FW	At5g61420	MYB28	ATGGGACCGTTTAAGTAGGTTGACAT
promMYB28_RV	At5g61420	MYB28	GTTGCCACGAGAAGCATGAA
promMYB29_attB1_F	At5g07690	MYB29	GTGAATGAAGGAGATTCTACGTACGC
promMYB29_attB2_R	At5g07690	MYB29	AGAGATGAGTTTCTTGTCTTCTTCGG
promMYB76_attB1_F	At5g07700	MYB76	ACAGTCTGAGCCATGTTACCAAAA
promMYB76_attB2_R	At5g07700	MYB76	CACACCGTTTCAGCCCTAAACT

Sequencing primers

Primer name	AGI number	Gene name	Primer sequence 5'-3'
Intron_pJ17_start_RV	-	-	AGAGAGGCTTACGTTAGCAGAGGA
Intron_pJ17_end_FW	-	-	GAAGTCTGAACAATTCTTGGGATTG
pC-S_mid1_fw	At2g20610	C-S lyase	ACTTCCACCTTTCTCAATGTCAAAC
pC-S_mid1_rv	At2g20610	C-S lyase	GTTGGGAATATGTGTCAAAAACCTG
pC-S_mid2_fw	At2g20610	C-S lyase	TGATTCAAGAAGCTGGTGTAGTTCA
Prom_MYB28mid1_FW	At5g61420	MYB28	CATAGAAAGCTTCAACACGAAAACC
Prom_MYB28mid2_FW	At5g61420	MYB28	TTAGGTTGAGAGAGTCAAAGAGGGAA
Prom_MYB28mid2_RV	At5g61420	MYB28	CATGCTCCTTTCTTCAAGCCTTCT

Primers for real-time RT-PCR

Primer name	*AGI number*	*Gene name*	*Primer sequence 5'-3'*
RL_sh_MAM1_Fw	At5g23010	MAM1	GAGAAATTGAACGCTGTCTTCTCAC
RL_sh_MAM1_Rv	At5g23010	MAM1	AGCCGTTAGACTTTAAACCGTTAGC
RL_sh_MAML_Fw	At5g23020	MAML	TTCGTTACTTCTCACATCGTCGAG
RL_sh_MAML_Rv	At5g23020	MAML	GGAGCAACATGAGACGAACAGAGT
RL_sh_CYP79F1_Fw	At1g16410	CYP79F1	CCATACCCTTTTCACATCCTACTAGTCT
RL_sh_CYP79F1_Rv	At1g16410	CYP79F	GTAGATTGCCGAGGATGGGC
RL_sh_CYP79F2_Fw	At1g16400	CYP79F2	CATGCTTTCAAATCTTACTAGGATTTATCG
RL_sh_CYP79F2_Rv	At1g16400	CYP79F2	GTAGATTGCCGAGGATGGGC
RL_sh_CYP83A1_Fw	At4g13770	CYP83A1	TTCAAGAGGTTGTCAATGAGACGC
RL_sh_CYP83A1_Rv	At4g13770	CYP83A1	CTACAATATCCAAGATGACGGCTTT
RL_sh_AtSt5b_Fw	At1g18590	AtSt5b	GGAATCCAAAACCATAAACGACG
RL_sh_AtSt5b_Rv	At1g18590	AtSt5b	CGGATCTTTTGGTCTCCAGCC
RL_sh_AtSt5c_Fw	At1g74090	AtSt5c	CCCTACCGAGTCACGACGAGA
RL_sh_AtSt5c_RV	At1g74090	AtSt5c	GGTAGCCACCAGTAACCACCATACT
RL_MYB28sh_Fw	At5g61420	MYB28	TCCCTGACAAATACTCTTGCTGAAT
RL_MYB28sh_Rv	At5g61420	MYB28	CATTGTGGTTATCTCCTCCGAATT
RNAi_M28_contrFw1	At5g61420	MYB28	CACTTCCATCAAAGATATATTGTCGGC
RNAi_M28_contRv1	At5g61420	MYB28	GGTCAAGAAGATAATTTGACCATCCCT
RL_RNAi_M28_contF2	At5g61420	MYB28	TTATGTCCGATGTTTCCCAAGAAGT
RL_RNAi_M28_contR2	At5g61420	MYB28	AATGCTTTTCAAGCGAGTCTGAGT
RL_MYB29sh_Fw	At5g07690	MYB29	GAAATATGATGCTTCCTTGAGCTCC
RL_MYB29sh_RV	At5g07690	MYB29	ACGGTGTAGAGCTGATCAAGGTTC
RL_MYB76sh_Fw	At5g07700	MYB76	ACGTTTTGGACGATCGAGCTCTAC
RL_MYB76sh_RV	At5g07700	MYB76	TGATTGAGAGAACGAGTCTGGGAGT
SLIM1_FW_sh	At1g73730	EIL3	GAACAACCTGAAGCTCAACAAAGA
SLIM1_RV_sh	At1g73730	EIL3	AGGGATTGTAGAAGTTGTACCCTGA
Actin2_FW	At3g18780		ATGGAAGCTGCTGGAATCCAC
Actin2_RV	At3g18780		TTGCTCATACGGTCAGCGATG

7. Abbreviations

2, 4-D	dichlorophenoxyacetic acid
4MTOB	4-methylthio-2-oxobutanoic acid
3MSOP	3-methylsulfinylpropyl glucosinolate
4MSOB	4-methylsulfinylbutyl glucosinolate
4MTB	4-methylthiobutyl-GS
5MSOP	5- methylsulfinylpropyl glucosinolate
8MSOO	8-methysulfinyloctyl glucosinolate
35S	35S promoter of the cauliflower mosaic virus
aa	amino acids
ACC	1-aminocyclopropane-1-carboxylic acid, ethylene precursor
AGI	*Arabidopsis* Genome Initiative number
AhR	arylhydrocarbon receptor
Amp^R	ampicillin
ami RNA	artificial micro RNA
AOP	a-ketoglutarate-dependent dioxygenases
APS	adenosine phosphosulfate
A. thaliana	*Arabidopsis thaliana*
ATPS	ATP sulfurilases
ATR	altered tryptophan regulation
AtST	*A. thaliana* sulfotransferase
A. tumefaciens	*Agrobacterium tumefaciens*
BCAT	brain-chain aminotranferase
bHLH	basic helix-loop-helix
bp	base pairs
°C	centigrade
CaMV	cauliflower mosaic virus
$Carb^R$	carbenicillin
cDNA	complementary DNA
CDS	coding sequence
$Chlor^R$	chloramphenicol resistance
cm	centimetre
cPCR	colony PCR
Col-0	*Arabidopsis* ecotype Columbia 0
CYP	cytochrome P450 monooxygenase
DNA	desoxyribonucleic acid
dd	double distilled
DEPC	diethylpyrocarbonate
DMF	dimethylformamide
DMSO	dimethyl-sulfoxide
DNA	ribonucleic acid
dNTPs	deoxynucleotides
DTT	di-thiotreitol

E. coli	*Escherichia coli*
EDTA	ethylendiaminetetraacetic acid
EPS	myrosinase epithiospecifier proteins
ET	ethylene
$Gent^R$	gentamycin
GFP	green fluorescent protein
GSL	glucosinolate
GST	glutathione-S-transferase
GT	glucuronosyl transferases
GUS	β-glucuronidase
gDNA	genome DNA
gPCR	genome PCR
HEPES	N-2-Hydroxyethylpiperazin-N.-2-ethansulfonic acid
HAG	high aliphatic glucosinolate regulator
HIG	high indolic glucosinolate regulator
HPLC	High-performance liquid chromatography
Hyg^R	hygromycin
IAA	indole-3-acetic acid
IAOx	indole-3-acetaldoxime
ITC	isothiocyanates
I3M	indole-3-ylmethyl glucosinolate
Kan^R	Kanamycin
kDa	kilo Daltons
kg	Kilograms
L	Liter
LB	Luria-Bertani medium
L*er*	*Arabidopsis* ecotype *Landsberg erecta*
M	molar
MAM	methiolthiomalate synthase
MeJA	methyl jasmonate
MES	4-morpholinoethan-sulphonic acid
Met	methionine
mg	milligram
µF	micro Faraday
µg	microgram
µl	microlitre
min	minute
mL	millilitre
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog medium
MUG	4-methylumbelliferyl-β-D-glucuronide
NrF2	NF-E2 related factor 2
N. benthamiana	*Nicotiana benthamiana*
ng	nanogram
Ω	Ohm
ORF	open reading frame

PAPS	phosphoadenylyl sulphate
PCR	polymerase chain reaction
PEG	polyethylenglycol
QTL	quantitative trait loci
QR	quinone reductase
Rif^R	rifampicin
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcribed PCR
SA	salicylic acid
S. exigua	*Spodoptera exigua*
SD	standard deviation
sec	seconds
$Spect^R$	spectinomycin
ST	sulfotransferase
SUR1	superroot1
TAE	Tris-Acetate/EDTA
T-DNA	Transfer DNA
TE	Tris/EDTA
TGG	β-thioclucoside glycohydrolase (myrosinase)
Tris	tris-(hydroxymethyl)-aminomethan
U	units (enzymatic)
UPLC	Ultra-performance liquid chromatography
UGT	S-glucosyl transferase
v/v	volume/volume
w/v	weight/volume
WT	wild-type
X-Gluc	5-bromo-4-chloro-3-indoly-β-D-glucoronid acid

Abstract

Glucosinolates (GSL) are nitrogen- and sulphur-rich natural plant products that serve as chemoprotective compounds in plant biotic defence reactions against herbivores and pathogens. GS also function as flavour compounds and exhibit strong anticancerogenic properties beneficial to human health. Although considerable progress has been made concerning the biosynthesis of glucosinolates, little is known how plants regulate the synthesis of these metabolites. The MYB28, MYB29 and MYB76 (referred to as HIGH ALIPHATIC GLUCOSINOLATE 1, 3 and 2) transcription factors were identified as novel regulators of glucosinolate biosynthesis. Molecular and biochemical characterization of *Arabidopsis* gain- and loss-of-function mutants revealed a significant correlation between the *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* transcript levels and the accumulation of aliphatic Met-derived glucosinolates. *MYB28/HAG1, MYB29/HAG3* and *MYB76/HAG2* over-expression caused a considerable increase in the level of aliphatic glucosinolates due to the specific activation of genes involved in aliphatic glucosinolate biosynthesis. Disruption of *MYB28/HAG1* and *MYB29/HAG3* gene functions caused a dramatic decrease in the content of aliphatic glucosinolates, whereas *myb76/hag2* loss-of-function mutants showed no changes in glucosinolate profiles except for the slight decrease in the level of 4MSOB glucosinolate. Analysis of the Pro_{HAG}*:GUS* activity revealed similar expression patterns in generative organs and rosette leaves of *Arabidopsis* plants, covering the main sites of aliphatic glucosinolate accumulation and overlapping with the expression of glucosinolate biosynthetic genes. Mechanical stimuli transiently induced *MYB/HAG* expression demonstrating their role in early plant responses to biotic stresses. Expression of *MYB28/HAG1* was clearly induced by glucose, indicating a novel signaling mechanism for the integration of carbohydrate availability in glucosinolates production, whereas *MYB29/HAG3* was shown to be involved in MeJa-induced glucosinolate biosynthesis. Notably, *MYB76/HAG2* expression was independent from plant elicitors and seems to play an accessory role in glucosinolate biosynthesis. Besides, *MYB28/HAG1* over-expression reduced performance of the generalist lepidopteran herbivore *Spodoptera exigua* in weight-gain experiments. Finally, MYB28/HAG1, MYB76/HAG2 and MYB29/HAG3 reciprocally *trans*-activate each other and comprise a complex regulatory network in

concert with other regulators (MYB51, MYB34, MYB122, WRKY25 and SLIM1*)* to control glucosinolate biosynthesis in response to different environmental stimuli.

Kurzzusammenfassung

Glucosinolate (GS) sind natürliche stickstoff- und schwefelreiche Pflanzenstoffe, die als chemoprotektive Verbindungen an pflanzlichen Abwehrreaktionen gegen Herbivoren und Krankheitserreger beteiligt sind. Weiterhin sind Glucosinolate geschmacksgebende Verbindungen und weisen hochgradig antikanzerogene Eigenschaften auf, die positive Auswirkungen auf die Gesundheit des Menschen haben. Obwohl in den letzten Jahren deutliche Fortschritte in der Glucosinolat-Forschung erzielt wurden, sind die regulatorischen Mechanismen der Biosynthese bisher weitgehend unbekannt. Die Transkriptionsfaktoren MYB28, MYB76 und MYB29 (oder HIGH ALIPHATIC GLUCOSINOLATE) wurden als neue Regulatoren der aliphatischen Glucosinolatbiosynthese identifiziert. Die molekulare und biochemische Charakterisierung von *Arabidopsis* gain- und loss-of-function-Mutanten zeigten einen signifikanten Zusammenhang zwischen den *MYB28/HAG1-, MYB29/HAG3*- und *MYB76/HAG2*- Transkriptionsniveaus und einer Akkumulation an aliphatischen, über Methionin synthetisierten Glucosinolaten. Die Überexpression von *MYB28/HAG1, MYB76/HAG2* oder *MYB29/HAG3* verursachte einen deutlichen Anstieg des Gehaltes aliphatischer Glucosinolate infolge der spezifischen Aktivierung der an der Biosynthese beteiligten Gene. Ein Ausschalten der *MYB28/HAG1* und *MYB29/HAG3* Genfunktion resultierte in einem verringerten Gehalt aliphatischer Glucosinolate, während Pflanzen mit fehlender *MYB76/HAG2*-Aktivität außer einem etwas niedrigeren 4MSOB - Gehalt keine Änderungen im Glucosinolatprofil zeigten. Die Analyse der Pro_{HAG}*:GUS*-Aktivität in *Arabidopsis* zeigt vergleichbare Expressionsmuster in den generativen Organen und in Rosettenblättern, welche die wichtigsten Speicherorte aliphatischer Glucosinolate sind. Mechanische Stimuli wie Verletzung induzierten eine kurzfristige *HAG*-Expression und Weizen damit auf eine Funktion der MYB/HAG Faktoren in der frühen Antwort auf biotischen Stress hin. Eine Induktion der *MYB28/HAG1*-Expression durch Glucose deutet auf einen neuen regulatorischen Mechanismus zur Einbindung der Kohlenhydratverfügbarkeit in der Glucosinolatbiosynthese hin, während *MYB29/HAG3* an der MeJa-induzierten Glucosinolat-biosynthese beteiligt ist. Die Expression von *HAG2* war von pflanzlichen Elizitoren unabhängig und scheint eine Helferrolle in der Glucosinolatbiosynthese zu übernehmen. Überdies konnte gezeigt werden, dass eine *MYB28/HAG1*-Überexpression das Wachstum des universellen Herbivoren *Spodoptera exigua*

verringert. MYB28/HAG1, MYB76/HAG2 und MYB29/HAG3 wirken als gegenseitige Transaktivatoren und bilden im Zusammenspiel mit anderen Regulatoren (MYB51, MYB34, MYB122, WRKY25 und SLIM1) ein komplexes Netzwerk, welches die Glucosinolatbiosynthese in Reaktion auf verschiedene Umweltstimuli reguliert.

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Teilpublikation:

1. **Gigolashvili T., Yatusevich R., Berger B., Müller C., Flügge U-I** (2007) The R2R3-MYB transcription factor HAG1/MYB28 is a regulator of methionine-derived glucosinolate biosynthesis in *Arabidopsis thaliana*. Plant J. 51, 247-261.

2. **Berger B, Stracke R, Yatusevich R, Weisshaar B, Flügge U-I, Gigolashvili T** (2007) A simplified method for the analysis of transcription factor-promoter interactions that allows high-throughput data generation. Plant J. 50, 911-916.

3. **Gigolashvili T., Engqvist M., Yatusevich R., Müller C., Flügge U-I (2008)** HAG2/MYB76 and HAG3/MYB29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana.* New Phytologist (2008) **177**: 627–642.

Posters und Oral Präsentation:

Yatusevich R, (2007) Doktorandenkonferenz MPIZ, Oral Präsentation

Yatusevich R et al., (2008) 21. Tagung Molekularbiologie der Pflanzen, Poster Präsentation;

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