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Specific and coordinated control of indolic and aliphatic glucosinolate biosynthesis by R2R3-MYB transcription factors in *Arabidopsis thaliana*

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Abstract Five members of subgroup 12 R2R3-MYB transcription factors, namely MYB51, MYB122, MYB28, MYB29 and MYB76, are novel regulators of glucosinolate biosynthesis in *Arabidopsis thaliana*. Overexpression of *MYB51* and *MYB122* led to an increased accumulation of tryptophan-derived indolic glucosinolates whereas *MYB28*, *MYB29* and *MYB76* overexpression lines showed an increase in methionine-derived aliphatic glucosinolates. Likewise, disruption of the corresponding genes caused a significant downregulation of indolic and aliphatic glucosinolates, respectively. Expression analysis of *promoter-GUS* fusions revealed promoter activities at the sites of glucosinolate synthesis and accumulation. Indolic glucosinolate regulators were mainly found in vegetative organs and roots, whereas aliphatic glucosinolate regulators were preferentially expressed in generative organs. Mechanical stimuli such as touch or wounding induced a transient expression of the regulators and overexpression of *MYB28* and *MYB51* reduced insect performance demonstrating the role of these transcription factors in plant biotic responses. The subgroup 12 R2R3-MYB transcription factors interdependently control the response to biotic challenges. For the regulation of methionine-derived glucosinolates, the coordinated activation of MYB28, MYB76 and MYB29 is required, whereas MYB51, MYB122 and the sixth member of subgroup 12 R2R3-MYB transcription factors, the previously described ATR1/MYB34, are involved in the regulation of tryptophan-derived glucosinolates. Because these two pathways are reciprocally inhibiting each other, a metabolic balance between both biosynthetic pathways can be accomplished in plants exposed to continuous biotic challenges.

Keywords Glucosinolate biosynthesis · Gene regulation · MYB factors · Biotic stress

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Introduction

Glucosinolates encompass the amino acid-derived class of sulfur and nitrogen containing secondary metabolites, characteristic of the order Brassicales, which include brassica vegetables, oilseed rape and the model plant *Arabidopsis thaliana* (Grubb and Abel 2006; Halkier and Gershenzon 2006). Glucosinolates are important defense compounds against generalist herbivores and pathogens or they serve as attractants for specialists (Halkier and Gershenzon 2006; Kim and Jander 2007). The plant-defensive properties of glucosinolates, their function as flavor compounds and recent findings suggesting cancer-protective properties of glucosinolates led to an increased interest in this class of compounds.

Changes in the concentration of glucosinolates derived from tryptophan, methionine and phenylalanine and their composition have been demonstrated to vary among different organs and tissues, during plant development and in response to environmental stimuli (Petersen et al. 2002; Brown et al. 2003). Furthermore, the regulation of aliphatic and indolic glucosinolates requires the co-regulatory feedback with other important primary metabolites and co-factors. For example, methionine is not only an important amino acid but also serves as a precursor of the common methyl donor S-adenosyl methionine (Kim and Leustek 2000) and as an important contributor of a plant sulphate pool. In addition, the biosynthesis of indolic glucosinolates is closely connected with that of the phytoalexin camalexin and indole-3-acetic acid (auxin, IAA), all sharing the common precursor indole-3-acetaldoxime (IAOx), and the biosynthesis of some other indolic glucosinolate derivatives, which also require a specific and coordinated control. Thus, an incorporation of methionine or tryptophan into the glucosinolate backbone should be carefully regulated not only spatially and temporally and in response to developmental and environmental stimuli, but also with respect to biosynthetic processes leading to other structurally related plant compounds. Consequently, a complex regulatory network controlling these processes can be expected. This review focuses on the characterization of novel regulators of indolic and aliphatic glucosinolate biosynthesis in *A. thaliana* with special attention to members of the R2R3-MYB transcription factor family, taking into account the recent progress achieved in this field.

Regulators of the glucosinolate biosynthetic pathways

Several components involved in the regulation of glucosinolate biosynthesis have been identified so far. IQD1 is a nuclear-localized calmodulin-binding protein, which controls the biosynthesis of aliphatic and indolic glucosinolates (Levy et al. 2005). Interestingly, IQD1 has been reported to upregulate genes coding for key enzymes of the indolic glucosinolate biosynthetic pathway, whereas respective structural genes of the aliphatic glucosinolate biosynthetic pathway were downregulated. Another regulatory component is SLIM1, which represses the biosynthesis of glucosinolates and activates enzymes catabolizing glucosinolates in response to sulphate deficiency (Maruyama-Nakashita et al. 2007). A third known protein, positively regulating glucosinolate biosynthesis, is AtDof1.1 (DNA-binding-with-one-finger), which has been shown to induce the transcription of at least *CYP83B1* and to moderately increase levels of aliphatic and indolic glucosinolates (Skirycz et al. 2006). Interestingly, AtDof1.1 caused also a significant increase in IAA levels in overexpression plants and its expression was activated in response to wounding and herbivore attack. Finally, ATR1/MYB34 has been shown to activate *CYP79B2*, *CYP79B3* and *CYP83B1* and has been proposed to regulate homeostasis between indolic glucosinolates and IAA biosynthesis (Celenza et al. 2005).

ATR1/MYB34 together with MYB51, MYB122, MYB28, MYB29 and MYB76 belong to the subgroup 12 of R2R3-type MYB transcription factors, one of the largest transcription factor families in plants (Stracke et al. 2001). Subgroup 12 can be further divided into two clades (Gigolashvili et al. 2007a): MYB51 (At1g18570), MYB122 (At1g74080) and MYB34 (At5g60890) as members of clade 1 have been shown to be regulators of indolic glucosinolates (Celenza et al. 2005; Gigolashvili et al. 2007a), whereas MYB28 (At5g61420), MYB29 (At5g07690) and MYB76 (At5g07700), members of clade 2, were shown to act as regulators of aliphatic glucosinolate biosynthesis with overlapping, but distinctive functions in *A. thaliana* (Hirai et al. 2007; Gigolashvili et al. 2007b, 2008; Sonderby et al. 2007).

The R2R3-MYB transcription factors ATR1/MYB34, MYB51 and MYB122 have divergent roles in the regulation of indolic glucosinolate biosynthesis and IAA homeostasis

As mentioned above, members of clade 1 of subgroup 12 R2R3-MYB transcription factors, i.e. ATR1/MYB34, MYB51 and MYB122, were shown to be important components of the regulatory network controlling indolic glucosinolate biosynthesis. The first member characterised was ALTERED TRYPTOPHAN REGULATION 1 (ATR1/MYB34), which was identified as a positive regulator of tryptophan biosynthesis (Bender and Fink 1998). A constitutive

overexpression mutant of *ATR1/MYB34* (*atr1D*) displayed a dominant activation of the tryptophan biosynthesis gene *ASA1* (*anthranilate synthase*), thereby rendering the plants resistant to the toxic tryptophan homologue 5-methyl tryptophan, which functions as a feedback inhibitor of ASA1 and thereby impairs tryptophan biosynthesis. It could be shown later that ATR1/MYB34 not only activates *ASA1* transcription and tryptophan biosynthesis, but also the pathway of indolic glucosinolates biosynthesis, which uses tryptophan as a precursor (Celenza et al. 2005). *ATR1/MYB34* overexpression lines were shown to contain significantly higher levels of total indolic glucosinolates than wild-type plants, due to the accumulation of the major indolic glucosinolate indol-3-ylmethyl glucosinolate (I3M; Celenza et al. 2005; Gigolashvili et al. 2007a). Furthermore, the *atr1-2* loss-of-function mutant contained decreased I3M levels in comparison to the wild-type. *ATR1/MYB34* overexpression lines also showed a modest elevation of IAA levels but were reported to do not display an obvious high IAA phenotype (Celenza et al. 2005). These observations indicate a positive regulatory role of ATR1/MYB34 in the biosynthesis of indolic glucosinolates. Subsequent studies demonstrated that not only ATR1/MYB34, but also its close homologues MYB51 and MYB122 are important components of a regulatory network controlling indolic glucosinolate biosynthesis. A dominant overexpression mutant of *HIGH INDOLIC GLUCOSINOLATE 1* (also referred to as MYB51) was identified in an activation tagging screen as a line with an increased accumulation of I3M (Gigolashvili et al. 2007a). A further characterisation of this mutant as well as *HIG1/MYB51* overexpression plants revealed that the content of other indolic glucosinolates e.g. 4-methoxy I3M (4MOI3M) and 1-methoxy I3M (1MOI3M), was also increased in comparison to the wild-type. This observation was not made in *ATR1/MYB34* overexpression lines, where I3M appeared as the sole indolic glucosinolate being upregulated (Gigolashvili et al. 2007a). The regulatory role of HIG1/MYB51 was further validated by the analyses of a *HIG1/MYB51* knock-out mutant (*hig1-1*) that displayed decreased levels of I3M, as it was previously shown for *atr1-2*. Since the two closest homologues ATR1/MYB34 and HIG1/MYB51 both appeared to be positive regulators of indolic glucosinolate biosynthesis, it was evident to functionally characterise also the third member of this subclade, MYB122 (also referred to as HIG2/MYB122). Indeed, ectopic overexpression of *HIG2/MYB122* led to a high indolic glucosinolate chemotype with an increased accumulation of I3M but not of other indolic glucosinolates as is the case for ATR1/MYB34 (Gigolashvili et al. 2007a). This high I3M content was interestingly only observed in the wild-type background. Attempts to complement the low I3M level of the *hig1-1* knock-out mutant by overexpression of *HIG2/MYB122* failed. This demonstrates that HIG2/MYB122 has the potential to upregulate indolic glucosinolate biosynthesis but only in the presence of a functional *HIG1/MYB51*.

The low I3M chemotype of *hig1-1* could be partially rescued by overexpression of *ATR1/MYB34*, however, all lines overexpressing *ATR1/MYB34* either in the *hig1-1* mutant or in the wild-type background showed a high IAA-phenotype with up to sevenfold higher IAA levels associated with highly retarded shoot and root growth, curly leaves, a bushy stature and the inability to produce seeds. Likewise, several lines overexpressing *MYB122* in the wild-type background displayed a high-IAA phenotype, even though not as pronounced as in the case of *ATR1/MYB34* overexpressing lines. By contrast, *HIG1/MYB51* overexpressing lines did not show an aberrant growth phenotype neither in wild-type nor in the *hig1-1* background concomitant with almost unaltered IAA levels.

It can be concluded that HIG1/MYB51 is the key player in the regulation of indolic glucosinolate biosynthesis, at least in leaves which are the main sites on indolic glucosinolate biosynthesis and accumulation (Brown et al. 2003). The observation that *ATR1/MYB34* is hardly expressed in vegetative parts of the plant argues against a major role of ATR1/MYB34 in the control of indolic glucosinolates biosynthesis in leaves (Gigolashvili et al. 2007a). Rather, ATR1/MYB34 appears to be preferentially linked to the regulation of IAA homeostasis with an impact on genes common to both biosynthetic pathways. In addition to the analyses of transgenic and mutant lines, evidence for important roles of MYB factors in the regulation of indolic glucosinolates is provided by QTL analyses. Kliebenstein et al. (2001) mapped a major QTL controlling total indolic glucosinolate accumulation in leaves as well as the accumulation of I3M and

4MOI3M to the position of *ATR1/MYB34* using L*er* x Cvi RILs; an additional QTL implicated in 4MOI3M leaf contents overlaps the physical position of *HIG1/MYB51*. Furthermore, mapping analyses with Bay x Sha RILs revealed multiple QTLs controlling indolic glucosinolate accumulation, three of which overlap with the physical position of *HIG1/MYB51* (At1g18570), *HIG2/MYB122* (At1g74080) and *ATR1/MYB34* (At5g60890; Wentzell et al. 2007).

Similar to I3M and IAA, the phytoalexin camalexin is also synthesized from tryptophan via IAOx. Regulators of indolic glucosinolate biosynthesis may therefore affect camalexin homeostasis. So far, little is known about regulatory components of camalexin biosynthesis. However, Nafisi et al. (2007) could show that both characterised genes of the camalexin pathway, *CYP71A13* and *PAD3/CYP71B15* (Schuhegger et al. 2006), are highly coregulated based on the global analysis of microarray data. Coexpression analysis of the pathogen set of AtGenExpress data using Expression Angler (Toufighi et al. 2005; http://bar.utoronto.ca/ntools/cgi-bin/ntools_expression_angler.cgi) reveals a coregulation of *PAD3/CYP71B15* and *CYP71A13* with tryptophan biosynthetic genes (*ASB1*, *TSA1*, *TSB2*) and *CYP79B2*. In addition to these structural genes, the transcripts of *HIG1/MYB51* and *HIG2/MYB122* showed a high degree of coregulation in this data set, an observation that makes both MYB factors good candidates as regulators of camalexin biosynthesis in *A. thaliana*. Remarkably, both genes are highly inducible by silver nitrate treatment (Genevestigator microarray database, https://www.genevestigator.ethz.ch/), a potent activator of camalexin biosynthesis. However, there are so far no experimental data available on the potential impact HIG1/MYB51 and HIG2/MYB122 might have on camalexin biosynthesis.

MYB28, MYB76 and MYB29 are regulators of aliphatic glucosinolate biosynthesis

MYB28, MYB76 and MYB29, also referred to as HAG1 (HIGH ALIPHATIC GLUCOSINOLATE1)/MYB28, HAG2/MYB76 and HAG3/MYB29 form a second clade within subgroup 12 of the R2R3-MYB transcription factor family and were assigned as regulators of aliphatic glucosinolate biosynthesis. Hirai et al. 2007 explored the role of MYB28 and MYB29 using an omics-based approach, whereas HAG1/MYB28, HAG2/MYB76 and HAG3/MYB29 were identified in a screen for the *trans*-activation potential of these transcription factors toward glucosinolate biosynthetic genes and further characterised *in planta* (Gigolashvili et al. 2007b, 2008). Finally, based on a QTL analysis and microarray-based coexpression data, Sonderby et al. (2007) suggested a role for MYB28, MYB76 and MYB29 in the control of aliphatic glucosinolate biosynthesis. It turned out that HAG1/MYB28 is the strongest regulator of aliphatic glucosinolate biosynthesis and therefore a key component of this pathway. Its closest homolog, HAG3/MYB29 was suggested to be only an accessory player of this pathway (Hirai et al. 2007). However, it could be demonstrated that HAG2/MYB76 and HAG3/MYB29 are additional independent aliphatic glucosinolate biosynthesis control elements (Sonderby et al. 2007; Gigolashvili et al. 2008).

As a result of HAG1/MYB28 overexpression in cultured *A. thaliana* cells, Hirai et al. (2007) observed an increased steady-state level of glucosinolate biosynthetic genes and increased accumulation of both aliphatic and indolic glucosinolates. By contrast, leaves of *HAG1/MYB28*, *HAG2/MYB76* and *HAG3/MYB29* overexpression lines showed increased levels of total aliphatic glucosinolates but not of indolic glucosinolates (Gigolashvili et al. 2007b, 2008; Sonderby et al. 2007). Overexpression of *HAG1/MYB28 and HAG3/MYB29* even led to a significant repression of the indolic glucosinolate pathway, indicating a reciprocal negative control of the two glucosinolate biosynthetic pathways (Gigolashvili et al. 2008; see below).

Interestingly, a strong overexpression of *HAG1/MYB28* and *HAG3/MYB29* resulted in a retardation of plant growth, impaired gravitropic response and fertility (Gigolashvili et al. 2007b, 2008). This growth phenotype was suggested to be a result of a severely increased flow of methionine into aliphatic glucosinolates biosynthesis leading to a deficiency in methionine as precursor for ethylene biosynthesis and impaired plant gravitropism.

Mutants defective in HAG1/MYB28 function showed decreased contents of both long- and short-chained aliphatic glucosinolates (Hirai et al. 2007; Gigolashvili et al. 2007b; Sonderby et al. 2007). In addition, *hag3/myb29* mutants contained significantly reduced levels of short-chained aliphatic

glucosinolates, indicating that HAG3/MYB29 is a second important player in the regulation of aliphatic glucosinolates. The decrease in the content of total aliphatic glucosinolates was, however, not as pronounced as in the case of the *hag1/myb28* mutant, indicating that a functional HAG1/MYB28 in the *hag3/myb29* mutant background is partially compensating for the loss of HAG3/MYB29. The double knock out mutant *myb28/myb29* contained almost no aliphatic glucosinolates, which indicates the primary importance of both MYB28 and MYB29 in the regulation of aliphatic glucosinolates, as well as on an inability of MYB76 to complement their functions (Sonderby et al. 2007). This observation was confirmed by the analysis of the *hag2/myb76* mutant which was hardly affected in glucosinolate accumulation (Gigolashvili et al. 2008).

Altogether, the analysis of loss-of-function mutants demonstrate that MYB28 and MYB29 are the major important players of methionine-derived glucosinolate biosynthesis and that HAG2/MYB76 has an accessory role in the regulation of this pathway. The disruption of *HAG1/MYB28* affects the production of both short- and long-chained aliphatic glucosinolates, whereas the disruption of *MYB29* and *MYB76* only that of short-chained glucosinolates. Based on the analysis of loss-of-function mutants it can be suggested that MYB28 is a potential regulator of all methylsulfinyl glucosinolates, whereas MYB29 and MYB76 are the regulators of short-chained glucosinolates.

Subgroup 12 R2R3-MYB transcription factors are activators of glucosinolate pathway genes

Overexpression of *ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122* did not only result in an altered indolic glucosinolate chemotype but also in an increased transcript accumulation of key pathway genes of indolic glucosinolate biosynthesis, namely *TSB1*, *CYP79B2*, *CYP79B3* and *CYP83B1* (Bender and Fink 1998; Celenza et al. 2005; Gigolashvili et al. 2007a; see Fig. 1). Interestingly, there seems to be a feedback regulation controlling *ATR1/MYB34* transcription, which is activated in *cyp79b2/cyp79b3* double mutant and *cyp83b1* single mutant plants (Smolen and Bender 2002; Celenza et al. 2005). In addition, both ATR1/MYB34 and HIG2/MYB122 have the potential to activate *ASA1* transcription, which is not the case for HIG1/MYB51. As mentioned above, the overexpression of *ATR1/MYB34* and *HIG2/MYB122* led to high-IAA phenotypes which is not the case for *HIG1/MYB51* overexpressing plants showing also an upregulation of genes further downstream of *CYP83B1*, i.e. *UGT74B1* and *AtST5a*, encoding enzymes for the last two steps in I3M biosynthesis (Gigolashvili et al. 2007a). Hence, *HIG1/MYB51* overexpression lines not only differ from *ATR1/MYB34* and *HIG2/MYB122* overexpressors with respect to their indolic glucosinolate profile but also regarding the transcript profile. Thus, the three MYB factors seem to have partially overlapping but distinct functions.

Concerning the aliphatic glucosinolate biosynthetic pathway, *HAG1/MYB28* showed the highest transactivation potential toward aliphatic glucosinolate biosynthetic genes supporting the view that HAG1/MYB28 represents the key regulator of this pathway (Hirai et al. 2007; Gigolashvili et al. 2007b, 2008). HAG3/MYB29 and HAG2/MYB76 have also the potential to activate aliphatic glucosinolate biosynthetic genes albeit to a lesser and variable extent. For example, *MAML*, which is mainly responsible for the production of long-chained aliphatic glucosinolates was strongly activated by *HAG1/MYB28* but less by *HAG3/MYB29* being in agreement with the observation that HAG1/MYB28 but not HAG3/MYB29 is a regulator of long-chained aliphatic glucosinolate biosynthesis (Sonderby et al. 2007; Gigolashvili et al. 2008). Furthermore, *trans*-activation of *CYP79F2* was much stronger for HAG1/MYB28 and HAG3/MYB29 compared with HAG2/MYB76 (Gigolashvili et al. 2008), and the expression of sulfotransferases was activated by HAG1/MYB28 and HAG3/MYB29, but repressed by HAG2/MYB76 (Sonderby et al. 2007). These observations support the previous conclusion that HAG2/*MYB76* has only an accessory role in aliphatic glucosinolate biosynthesis. Together, the transactivation potential of HAG1/MYB28, HAG3/MYB29 and HAG2/MYB76 differed toward promoters of some target genes suggesting that they are not operating via identical mechanisms (Gigolashvili et al. 2007b, 2008; Sonderby et al. 2007).

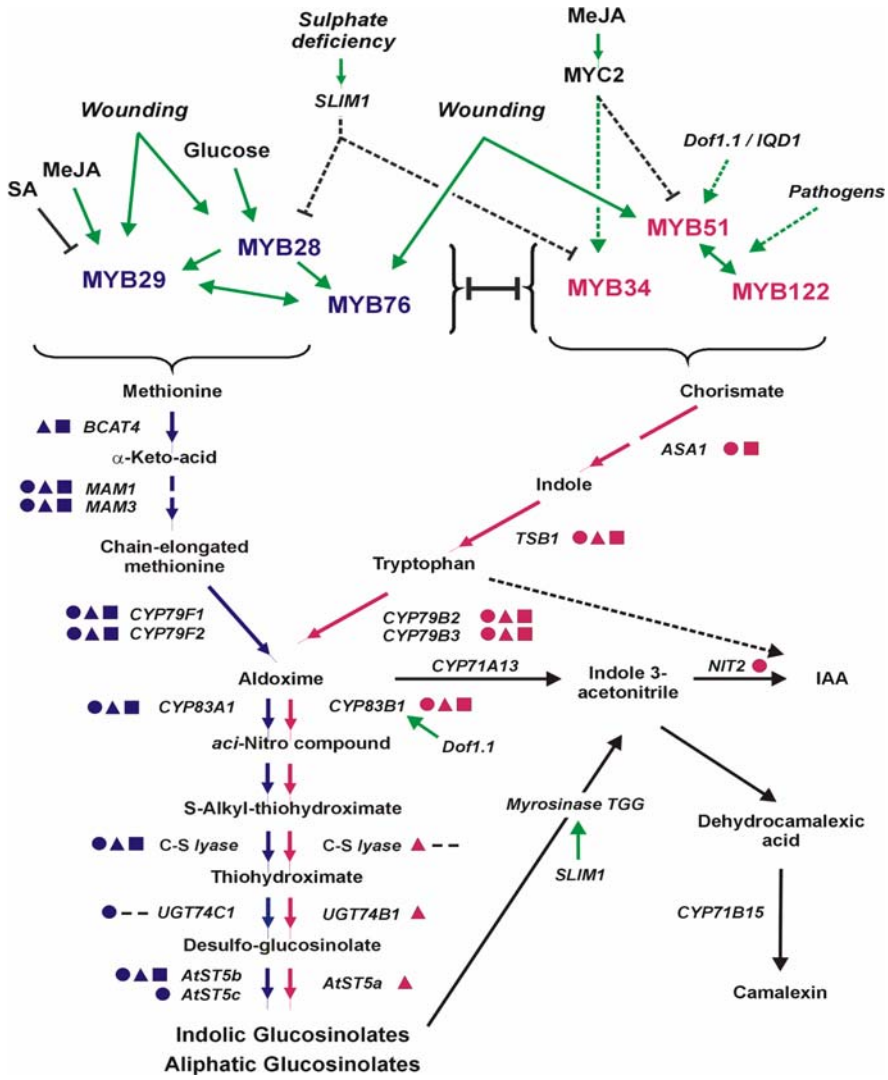

Fig. 1 Network of regulatory genes and their targets in glucosinolate biosynthetic pathways with special attention to group 12 R2R3-MYB transcription factors. The aliphatic and indolic biosynthetic pathways are shown in red and blue, respectively. Blue symbols (◯ , △ , □) indicate activation by HAG3/MYB29, HAG1/MYB28 and HAG2/MYB76, respectively and red symbols (◯ , △ , □) indicate activation by ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122, respectively. (-), not determined. In the upper part of the figure, the regulation of the R2R3-MYB factors by external stimuli and further upstream components are shown. Symbols used are →, activation; ↔ reciprocal activation; ⊥, inhibition; ⊢⊣, reciprocal negative control. SA, salicylic acid; MeJA, methyl jasmonate

Signaling network in the control of glucosinolate biosynthesis regulators

Glucosinolate metabolism has been shown to be controlled by different environmental or endogenous stimuli such as mechanical stresses, jasmonate, salicylic acid, or ethylene. In addition, the expression of *HAG1/MYB28* was shown to be activated by glucose (Gigolashvili et al. 2007b). This observation fits well with microarray data, demonstrating a rapid upregulation of *HAG1/MYB28* after glucose treatment (Li et al. 2006) and indicating a novel transcriptional regulatory mechanism integrating carbohydrate availability and biotic stress signals for glucosinolate biosynthesis (Gigolashvili et al. 2007b).

The biosynthesis of glucosinolates had been shown to be induced by methyl jasmonate (MeJA; Cipollini et al. 2004; Mewis et al. 2005; Sasaki-Sekimoto et al. 2005). It could indeed be demonstrated that the expression of *HAG3/MYB29* but not that of *HAG1/MYB28* and *HAG2/MYB76* increased several fold in response to exogenous MeJA . Thus, HAG3/MYB29 can be proposed as a second important regulator of aliphatic glucosinolate biosynthesis, quickly integrating MeJA signaling for the production of plant chemoprotectives.

HAG2/MYB76 appears to play only a minor role in the control of aliphatic glucosinolate biosynthesis under non-stressed conditions. However, upon mechanical stimuli like wounding *HAG2/MYB76*

expression is induced more than 50-fold indicating a particular role for HAG2/MYB76 in glucosinolate production upon mechanical stress. Furthermore, HAG2/MYB76 could accelerate aliphatic glucosinolate biosynthesis in concert with HAG1/MYB28 and HAG3/MYB29. This view is supported by the analysis of transcript levels of wounded *A. thaliana* seedlings and *HAG2/MYB76* overexpression plants, and by transactivation assays of HAG1/*MYB28, HAG3/MYB29* and *HAG2/MYB76* (Gigolashvili et al. 2008).

Smolen and Bender (2002) could show that *ATR1/MYB34* transcription is induced in *A. thaliana* seedlings upon MeJA treatment, which observation was confirmed by a recent study demonstrating that the expression of both *ATR1/MYB34* and *HIG1/MYB51* are induced by MeJA treatment (Dombrecht et al. 2007). However, a striking difference was the differential regulation by the known jasmonate signaling component MYC2/JIN1 (Fig. 2). Whereas the MeJA-mediated induction of *HIG1/MYB51* was enhanced in the *jin1-9* knock-out mutant compared to the wild-type, the induction of *ATR1/MYB34* transcripts was lower in the mutant compared with the wild-type. Hence, MYC2/JIN1 acts as a positive regulator of MeJA-dependent *ATR1/MYB34* expression, but as a negative regulator of the MeJA-dependent *HIG1/MYB51* induction. The latter situation was also true for several tryptophan and indolic glucosinolate biosynthetic pathway genes and the accumulation of I3M, which is probably a result of the upregulation of *HIG1/MYB51* in the mutant background. On the other hand, MYC2/JIN1-mediated regulation of *ATR1/MYB34* was contrary to that of tryptophan and tryptophan-derived secondary metabolism genes, which questions the role of ATR1/MYB34 in the jasmonate-dependent glucosinolate accumulation. The authors, however, speculate that the feedback regulation of *ATR1/MYB34* by indolic glucosinolates might be the reason for that.

Apart from MeJA-mediated stress signaling, HIG1/MYB51 was also shown to play an important role in the response to other biotic stresses. The overexpression of *HIG1/MYB51* led to an increased resistance against the generalist herbivore *Spodoptera exigua* (Gigolashvili et al. 2007a) and a QTL implicated in the resistance against the generalist *Trichoplusia ni* was mapped to a region including the physical position of the *HIG1/MYB51* locus (Kliebenstein et al. 2002). Additionally, *HIG1/MYB51* expression appears to be influenced by biotic stress treatment. Several microarray studies reported on an increased *HIG1/MYB51* transcription upon pathogen attack or treatment with elicitors (Chen et al. 2002; Cheong et al. 2002; Thilmony et al. 2006). Moreover, *HIG1/MYB51* is strongly upregulated in response to wounding in a rapid but transient manner (Gigolashvili et al. 2007a). *HIG1/MYB51* might therefore be part of an early stress response pathway mediating the accumulation of indolic glucosinolates upon mechanical stimuli or pathogen attack.

In comparison to the regulation of *HIG1/MYB51* and *ATR1/MYB34* by mechanical stimuli and/or jasmonate, little is known about the involvement of *HIG2/MYB122* in biotic stress responses and respective signaling pathways. However, co-expression studies using ExpressionAngler revealed a significant correlation of *HIG1/MYB51* and *HIG2/MYB122* expression in the AtGenExpress pathogen set (correlation coefficient $r = 0.8$; Toufighi et al. 2005). Furthermore, a direct interaction of HIG1/MYB51 and HIG2/MYB122 could be shown in a yeast-two-hybrid assay (unpublished results). Future analyses to investigate the specific role of HIG2/MYB122 in the biotic stress response should therefore concentrate on the interaction of HIG1/MYB51 and HIG2/MYB122.

Thus, the characterized MYB factors are obviously key players in the signal transduction chain leading from biotic stress perception to an increased biosynthesis of glucosinolates, thereby rendering the plant more resistant to herbivores. It could indeed be shown that generalist herbivores avoided *HIG1/MYB51* or gained less weight on *HAG1/MYB28* overexpression lines compared to wild-type (Gigolashvili et al. 2007a, b). These findings also confirm the regulatory function of these factors in the control of glucosinolate biosynthesis upon biotic challenges. The high transactivation potential of indolic and aliphatic glucosinolate regulators toward glucosinolate biosynthetic genes suggests that they act as direct regulators.

A direct activation of glucosinolate biosynthetic genes could not be demonstrated for other positive regulators of glucosinolate biosynthesis such as IQD1, Dof1.1 or SLIM1 suggesting that these factors are positioned further upstream of the MYBs. It will be a challenge to identify additional components connecting environmental stimuli with the function of IQD1, SLIM1, Dof1.1 and finally the MYB factors.

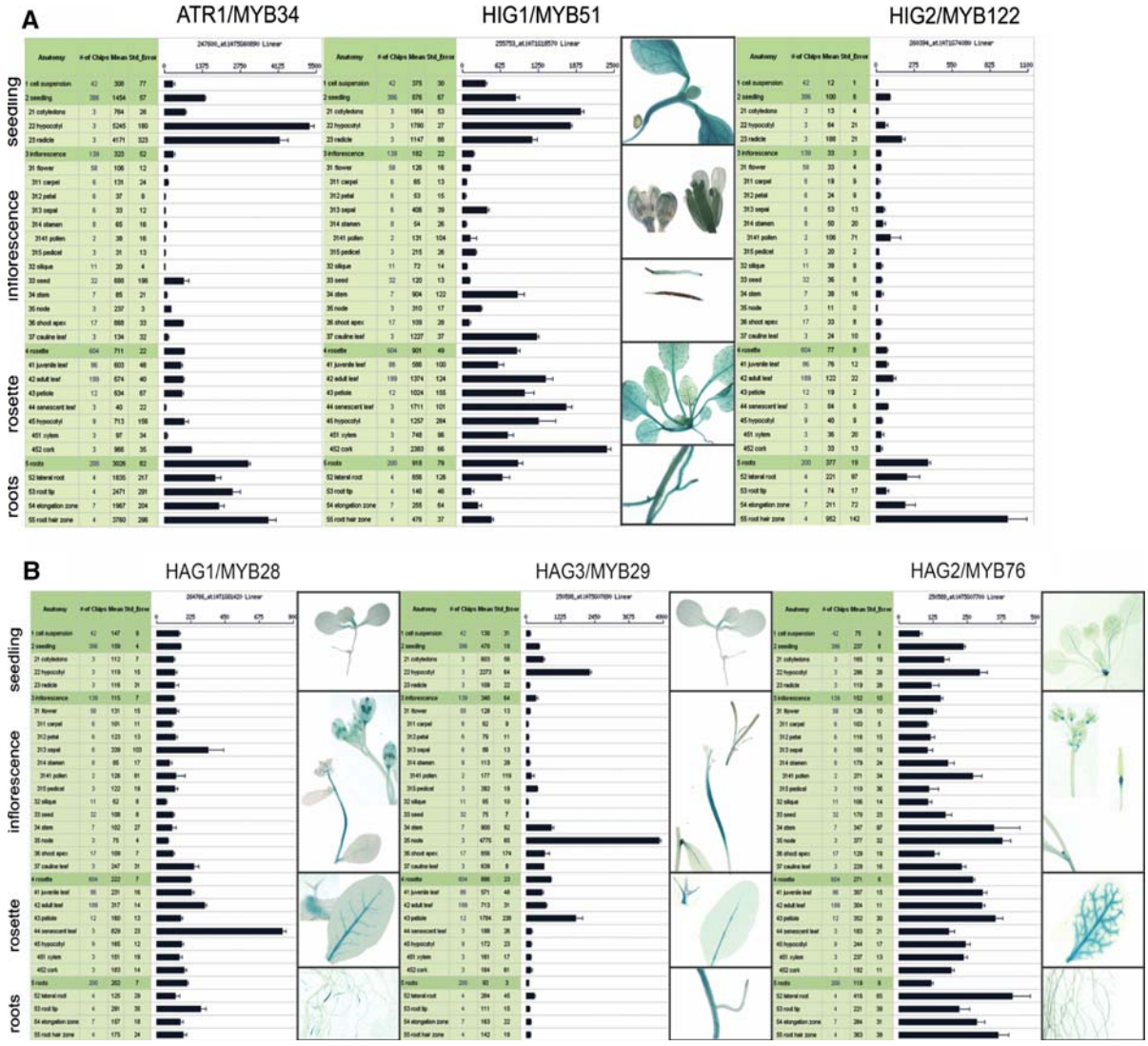

Fig. 2 Expression patterns of subgroup 12 R2R3 MYB transcription factors. **a** Expression patterns of *ATR1/MYB34*, *HIG1/MYB51* and *HIG2/MYB122* in seedlings, inflorescences, foliar parts and roots of wild-type plants (Col-0). For HIG1/MYB51, histochemical GUS staining of corresponding tissues of plants expressing a promoter-GUS construct is also shown. **b** Expression patterns of *HAG1/MYB28*, *HAG3/MYB29* and *HAG2/MYB76* in seedlings, inflorescences, foliar parts and roots of wild-type plants (Col-0). The histochemical GUS staining in tissues of plants expressing promoter-GUS constructs of *HAG1/MYB28, HAG2/MYB29* and *HAG3/MYB76* are also shown. All microarray data were analysed using genevestigator (Zimmermann et al. 2004; https://www.genevestigator.ethz.ch/at) and displayed as linear expression values

The tissue-specific expression of subgroup 12 R2R3-MYB transcription factors differs in various organs and during ontogenesis

Analyses of GUS reporter lines (Gigolashvili et al. 2007a) in combination with AtGenExpress data from the Genevestigator microarray database (Zimmermann et al. 2004; https://www.genevestigator.ethz.ch/; Fig. 2) revealed that all three MYB factors controlling indolic glucosinolate accumulation are primarily expressed in vegetative organs; only reduced expression can be observed in the inflorescence. Whereas *ATR1/MYB34* and *HIG2/MYB122* are mainly expressed in roots, the transcript level of *HIG1/MYB51* is highest in rosette leaves, suggesting a predominant role of *HIG1/MYB51* in the rosette. The

observed transcript patterns of all three genes overlaps at least partially with those of indolic glucosinolate biosynthesis genes (Mikkelsen et al. 2000; Grubb et al. 2004) and the major sites of indolic glucosinolate accumulation, the root system and the rosette (Brown et al. 2003). Interestingly, expression of *HIG1/MYB51* as well as of *HAG1/MYB28* and *HAG2/MYB29* is also found in trichomes which play a role in the immediate perception of biotic signals and acceleration of glucosinolate biosynthesis and thus in the defense against herbivore attack.

Aliphatic glucosinolates are found throughout the plant, with highest concentrations in the reproductive organs. In accordance with this, the regulators of aliphatic glucosinolates, *HAG1/MYB28*, *HAG3/MYB29* and *HAG2/MYB76* display a rather ubiquitous expression pattern, with expression in the vasculature of rosette leaves and stems and specific expression in floral organs (Fig. 2). Furthermore, the transcript of *HAG3/MYB29* is found at a remarkably high level in nodes, whereas *HAG2/MYB76* is notedly present in the transition zone from root to the foliar part. The observed expression patterns nicely overlap with the expression of aliphatic biosynthesis genes, like *MAM3*, *BCAT4* and *CYP79F1* (Reintanz et al. 2001; Chen et al. 2003; Schuster et al. 2006; Textor et al. 2007).

Subgroup 12 R2R3-MYB transcription factors encompass a regulatory network

As discussed above, transcription of subgroup 12 *R2R3-MYB* genes is induced by different stimuli such as glucose, MeJA or wounding. It can be assumed that cooperative gene activation could enhance rapid responses of plants to environmental stimuli. It could indeed be shown that *HAG1/MYB28, HAG3/MYB29* and *HAG2/MYB76* show a positive reciprocal activation (Gigolashvili et al. 2008). For example, *HAG1/MYB28* overexpression caused an increase in *HAG3/MYB29* and *HAG2/MYB76* transcripts, whereas an increase of *HAG3/MYB29* transcripts resulted in an accumulation of *HAG2/MYB76* mRNA. Interestingly, *HAG2/MYB76* as the weakest regulator of aliphatic glucosinolate biosynthesis is *trans*-activated by *HAG1/MYB28* and *HAG3/MYB29*, or can activate itself in trans-activation assays (Gigolashvili et al. 2008). On the other hand, the strongest regulator of the aliphatic glucosinolate biosynthetic pathway, *HAG1/MYB28*, does not appear to be upregulated by *HAG2/MYB76* and *HAG3/MYB29*.

HIG1/MYB51 and *HIG2/MYB122* controlling indolic glucosinolate biosynthesis also seem to be interdependent from each other and to be co-regulated in response to pathogen treatment (Toufighi et al. 2005; Gigolashvili et al. 2007a). Positive reciprocal gene activation may not only serve to rapidly respond to environmental stimuli but also to provide a dynamic range of different glucosinolate profiles under different environmental conditions.

In addition to this positive reciprocal activation there is evidence for a reciprocal negative control of methionine- and tryptophan-derived glucosinolate pathways. For example, mutants of *CYP79F1* and *CYP83A1* genes with impaired aliphatic glucosinolate biosynthesis accumulated higher levels of indolic glucosinolates. Also, the overexpression of *IQD1*, a positive regulator of indolic glucosinolate genes, caused a repression of *CYP79F1* and *CYP79F2* genes involved in aliphatic glucosinolate biosynthesis. On the other hand, positive regulators of aliphatic glucosinolate accumulation were shown to downregulate the expression of regulators of indolic glucosinolate accumulation i.e. ATR1/MYB34, HIG1/MYB51 and HIG2/MYB122 (Gigolashvili et al. 2008). These observations are consistent with decreased levels of aliphatic glucosinolates in *HIG1/MYB51* overexpression lines and decreased levels of indolic glucosinolates in *HAG1/MYB28* and *HAG3/MYB29* overexpression lines .

This data suggests that the observed reciprocal negative feedback regulation of indolic and aliphatic glucosinolate biosyntheses might be linked to the control of glucosinolate and sulphur homeostasis in plants overproducing positive regulators of glucosinolate biosynthesis. The repression of regulators of the indolic glucosinolate pathway in plants overexpressing aliphatic glucosinolates, or the repression of regulators of the aliphatic glucosinolate pathway in plants overexpressing indolic glucosinolates, may lead to a metabolic balance between both glucosinolate biosynthetic pathways and may thereby contribute to the sulphur balance in the plant cell.

Concluding remarks

Biosynthetic pathways leading to the formation of aliphatic and indolic glucosinolates have been elucidated in the past years and most of the genes involved could be identified in the model plant *A. thaliana*. Only recently the first regulators of glucosinolate biosynthesis have emerged including the subgroup 12 R2R3-MYB transcription factors and components acting further upstream such as IQD1, SLIM1 or AtDof1.1. The next steps will include identifying new interacting partners of MYB proteins and unravelling the signal transduction cascade from incoming signals to early response genes and further downstream regulators. These studies will contribute to our understanding of the complex genetic network controlling glucosinolate biosynthesis and accumulation.

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