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## Regulation of Plant Secondary Metabolism and Associated Specialized Cell Development by MYBs and bHLHs

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### Abstract

Plants are unrivaled in the natural world in both the number and complexity of secondary metabolites they produce, and the ubiquitous phenylpropanoids and the lineage-specific glucosinolates represent two such large and chemically diverse groups. Advances in genome-enabled biochemistry and metabolomic technologies have greatly increased the understanding of their metabolic networks in diverse plant species. There also has been some progress in elucidating the gene regulatory networks that are key to their synthesis, accumulation and function. This review highlights what is currently known about the gene regulatory networks and the stable sub-networks of transcription factors at their cores that regulate the production of these plant secondary metabolites and the differentiation of specialized cell types that are equally important to their defensive function. Remarkably, some of these core components are evolutionarily conserved between secondary metabolism and specialized cell development and across distantly related plant species. These findings suggest that the more ancient gene regulatory networks for the differentiation of fundamental cell types may have been recruited and remodeled for the generation of the vast majority of plant secondary metabolites and their specialized tissues.

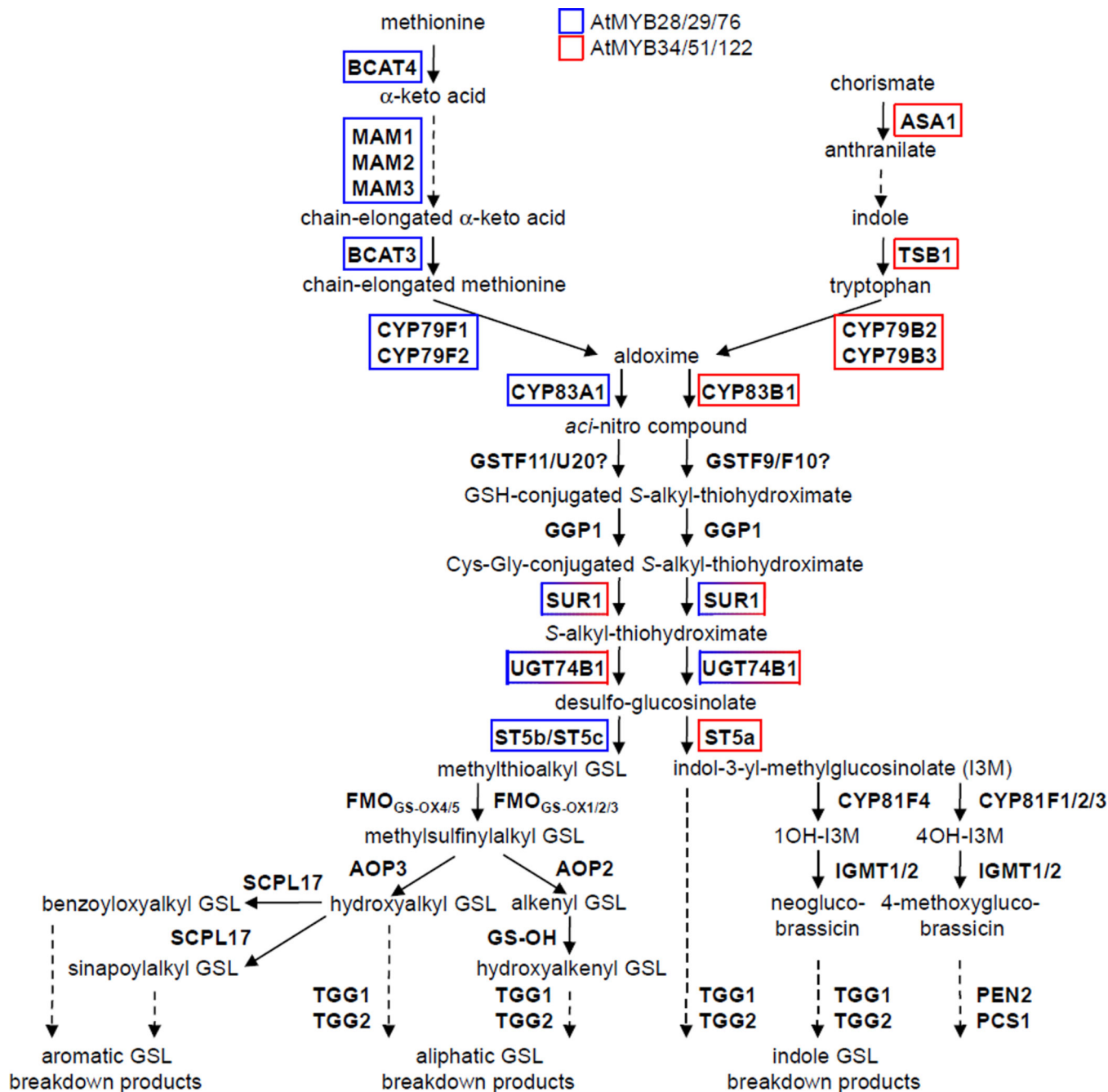
### Graphical Abstract

This review highlights the recent developments in the investigation of gene regulatory networks controlling the production of plant secondary metabolites and the differentiation of specialized cell types that are important to their defensive function. Also discussed are their possible origins in the more ancient gene regulatory networks controlling the differentiation of fundamental cell types.

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## 1. Introduction

Plant secondary metabolites (also called phytochemicals or natural products) constitute a large reservoir of natural chemical diversity that is constantly generated through genetic adaptations to the prevailing and ever-fluctuating abiotic and biotic environment. As a consequence, plant secondary metabolites have become essential to the survival and reproductive fitness of a plant species within its natural environment. Often derived from intermediates or end products of primary metabolic pathways, these secondary metabolites

possess a variety of bioactivities, including cytotoxicity, and are biochemically expensive to synthesize. For these reasons, the formation of many plant secondary metabolites is regulated by external stresses (Patra 2013). The constant evolutionary pressure towards chemical innovation has also caused the majority of secondary metabolites to be restricted to narrow phylogenetic lineages, such as a family, genus or single species. Interestingly, despite this strong ecological and phylogenetic character of plant secondary metabolism, not all lineage-specific metabolites are distributed consistently across ancestral and sister taxa. While structural diversity is the rule for secondary metabolites, even in plants from related taxa, the metabolic responses of flowering plants to certain environmental stresses are for the most part functionally conserved. For example, the biosynthesis and accumulation of antimicrobial compounds is one of the evolutionary conserved plant responses to pathogenic microbes (Hammerschmidt 1999; Mansfield 2000; Dixon 2001). Thus, the regulatory principles behind plant secondary metabolism appear to be conserved and may have evolved earlier than many of the specific biosynthetic pathways (Wink 2003).

Plant secondary metabolites are often produced at high levels in specific tissues or cell types of individual species or across related species. Economically important examples of tissue-specific metabolites include alkaloids (e.g., morphine and codeine) and latex in laticifer cells in poppy and rubber trees, terpenes and saponins in epidermal cells of many plant families, and resins in trees (DellaPenna and Last, 2008). In addition, plant secondary metabolites have been shown to control physiological processes, such as the circadian clock and phototropism (Hasegawa et al., 2000; Yamada et al., 2003; Kerwin et al., 2011). Plant secondary metabolites, both constitutively and inducibly formed, may also be interlinked with plant immunity, controlling evolutionarily conserved immune responses, such as callose deposition and programmed cell death, in higher plants (Quidde et al., 1998; Bouarab et al., 2002; Clay et al., 2009; Ahmad et al., 2011). For example, lineage-specific defense-related alkaloids in the California poppy (*Eschscholzia californica*) and rose periwinkle (*Catharanthus roseus*) regulate their own production in a species-specific manner by using a negative feedback mechanism that blocks the generation of immune signals that initiate the induction of biosynthetic enzymes (Heinze et al., 2015); poppy-specific alkaloids do not regulate the production of periwinkle-specific alkaloids, and vice versa.

Recently, WD40 domain proteins and activator- and repressor-type transcription factors of the R2R3-type *MYELOBLASTOSIS* (MYB) (Figure 1), R3-type MYB, and basic helix-loop-helix (bHLH) families have been identified to regulate plant secondary metabolism and associated specialized cell development across diverse plant species. These stable regulatory components also control differentiation of fundamental plant tissues, such as single-celled trichomes, non-hair root epidermal cells, and stomata, which suggests that they form an ancient transcriptional component of gene regulatory networks in land plants (Oppenheimer 1991; Galway 1994; Lee 1999; Ramsay and Glover, 2005; Serna and Martin, 2006; Kliebenstein, 2013). Highlighted here are the biosynthetic pathways of phenylpropanoids and glucosinolates, their associated specialized cell types, and the transcriptional mechanisms that regulate the production of both.

## 2. Secondary Metabolic Pathways

### 2.1. General phenylpropanoid pathway

Phenylpropanoids are a class of phenolic secondary metabolites that are mainly derived from the aromatic amino acid L-phenylalanine (**1**), and are ubiquitous in terrestrial plants and present in other eukaryotes. Lignins and the related lignans, flavonoids (e.g., flavonols, anthocyanins and proanthocyanidins), stilbenoids, coumarins, and hydroxycinnamic acid conjugates constitute the bulk of the known plant phenylpropanoids, and are synthesized by both general and specific pathways in phenylpropanoid metabolism. Phenylpropanoid metabolism often begins with three reactions catalyzed by phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H) and *p*-coumarate:coenzyme A ligase (4CL) enzymes, leading to the synthesis of the activated hydroxycinnamic acid *p*-coumaroyl-CoA (**2**) (Figures 2 and 3). This reaction sequence is known as the general phenylpropanoid pathway, and the remaining reactions of phenylpropanoid metabolism comprise the lignin, flavonoid, and many species- and clade-specific pathways (Vogt 2010).

### 2.2. Lignin pathways

Lignin is an important structural and defensive component of plant secondary cell walls and the second most abundant biopolymer on earth. Lignin is composed primarily of three hydroxycinnamyl alcohol monomers, referred to as monolignols. These monolignols are oxidatively polymerized to form a variety of substructures that give the lignin polymer its complexity (See Davin et al., 2008 for a review on the structure and assembly of lignin). The lignin biosynthetic pathway is indispensable to plants for not only its role in the production of the monolignols, but also its role in the production of a host of other small molecules such as the UV-B-protecting sinapate esters and the iron-chelating coumarins (Ruegger et al., 1999; Kai et al., 2008; Fourcroy et al., 2013; Schmid et al., 2014). The lignin pathway is also involved in the production of the polyphenolic domains of suberin, which is a lipid and phenolic cell wall heteropolymer found in the roots and other organs of all vascular plants. Suberin's polyphenolic domain is similar to lignin, but differs by containing a large proportion of hydroxycinnamic acids (especially ferulate) in addition to the monolignols normally found in lignin (Bernards et al., 1995; Yan and Stark, 2000).

In *Arabidopsis*, there are three iterations of the lignin pathway that result in the production of the three monolignols (*p*-coumaryl (**3**), coniferyl (**6**) and sinapyl (**8**) alcohols) from their respective precursors (*p*-coumaroyl-CoA (**4**), feruloyl-CoA (**11**) and sinapaldehyde (**16**)) by an enzyme cascade of cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), or CAD alone (Figure 2; Vanholme et al., 2010). The three monolignols are then oxidatively polymerized to form their respective lignin polymers, H, G and S lignins (Figure 2). The monolignol precursor feruloyl-CoA (**4**) is synthesized from *p*-coumaryl-CoA (**2**) by an enzyme cascade of hydroxycinnamoyl-CoA:shikimate/quininate hydroxycinnamoyltransferase (HCT), caffeoyl shikimate esterase (CSE), 4CL and caffeoyl-CoA *O*-methyltransferase (CCoAOMT) (Figure 2; Vanholme et al., 2013). The monolignol precursor sinapaldehyde (**7**) is synthesized from coniferaldehyde (**5**) by an enzyme cascade of ferulate 5-hydroxylase (F5H) and caffeic acid *O*-methyltransferase (COMT) (Figure 2). Although there are genomics-based descriptions of lignin pathway genes in monocots

(Barrière et al., 2007; Lawrence and Walbot, 2007; Andersen et al., 2008), the corresponding enzymatic functions by many of the gene candidates have yet to be verified biochemically or genetically. In addition, it is not yet known whether monocot CSE homologues are involved in lignification.

### 2.3. Flavonoid pathways

Flavonoids are important for plant pigmentation and protection against biotic and abiotic stressors (e.g., herbivores, microbial pathogens, UV-B, free radicals). In *Arabidopsis*, there is only one flavonoid biosynthetic pathway that begins with the chalcone synthase (CHS) enzyme, a type-III polyketide synthase that catalyzes the formation of naringenin chalcone (**10**) from malonyl-CoA and *p*-coumaroyl-CoA (**2**). Downstream steps produce the commonly occurring flavonols kaempferol (**12**) and quercetin (**13**), the 3-hydroxyflavonoids pelargonidin (**15**) and cyanidin (**16**) as well as their derived plant pigments, the anthocyanins pelargonin 3-glucoside (**19**) and cyanidin 3-glycoside (**20**) (Figure 3A; Shirley et al., 1995; Bharti and Khurana, 1997). Pelargonidin (**15**) and its reduced precursor leucopelargonidin (**14**) are also precursors to the flavan-3-ol polymers, the proanthocyanidins (also called condensed tannins), which accumulate in the seed coat as antimicrobials and feeding deterrents (Figure 3A; Winkel-Shirley 1998). In maize, there are at least two flavonoid biosynthetic pathways that branch from the flavanone intermediate naringenin (**11**). One pathway results in the commonly occurring 3-hydroxyflavonoids pelargonidin (**15**) and cyanidin (**16**) and their derived pigments, the anthocyanins (Figure 3B; Dooner et al., 1991; van der Meer 1993). The other pathway produces the floral organ-specific 3-deoxyflavonoids, which include the flavan-4-ols apiforol (**21**) and luteoforol (**22**), their derived pigments the red phlobaphenes, the flavones apigenin (**23**) and luteolin (**24**), and their derived insecticidal and antifungal products, the *C*-glycosyl flavones apimaysin (**25**) and maysin (**26**) (Figure 3B; Styles and Ceska, 1975; Waiss et al., 1979; Elliger et al., 1980; Snyder and Nicholson, 1990; Reid et al., 1992; Byrne et al., 1996a, 1996b). Phlobaphenes and *C*-glycosyl flavones are lineage-restricted flavonoids found in a number of unrelated plant species (e.g., lotus, maize and redwoods). There are also lineage-specific flavonoids, such as the isoflavonoids, which are synthesized primarily in legumes from chalcone and flavanone precursors.

Closely related to CHS, the stilbene synthase (STS) enzymes, another of several specialized type-III polyketide synthases found in plants (reviewed in Yu et al., 2012), utilize the same precursors as CHS to instead form resveratrol, the simplest stilbene. Downstream steps produce the stilbenoids, which are hydroxylated derivatives of stilbenes. Unlike lignins and flavonoids, stilbenoids have been detected in a limited number of unrelated plant species and rapidly accumulate only in response to a wide range of biotic and abiotic stresses. Not present in *Arabidopsis* or maize, the stilbenoid biosynthetic pathway is best characterized in grapevine (*Vitis vinifera*), peanut (*Arachis hypogea*) and pine (*Pinus* spp.) (Jeandet et al., 2010).

### 2.4. Glucosinolate pathways

Glucosinolates are  $\beta$ -thioglucoside *N*-hydroxysulfates that are found principally in plants of the Brassicales order. Together with their hydrolytic enzymes, the myrosinases, they

constitute the ‘mustard oil bomb’ (Matile, 1980; Luthy and Matile, 1984; Ratzka et al., 2002) that is involved in both constitutive and inducible plant defenses. Glucosinolates are characterized by a core sulfonated oxime group (also called a sulfated isothiocyanate group), which is conjugated to thioglucose and a side (R-) group (Figure 4). The chemical diversity of the more than 200 reported structures of GSLs lie in the elongations and modifications to the side-chain, and the esterification of the thioglucose moiety by benzoates and hydroxycinnamates (Clarke, 2010).

Glucosinolates (GSLs) are among the best studied secondary metabolites of *Arabidopsis*, which contains at least 37 different GSLs (Hogge et al., 1988; Kliebenstein et al., 2001a; Reichelt et al., 2002; Kliebenstein et al., 2007). The two major GSL groups in *Arabidopsis* are the methionine (**27**) (Met)-derived aliphatic GSLs and the tryptophan (**28**) (Trp)-derived indole GSLs. Extensive functional genomics, metabolomics and systems biology approaches have led to the identification and functional characterization of nearly all of the biosynthetic enzymes of aliphatic and indole GSL metabolism (Wittstock and Halkier, 2002; Halkier and Gershenzon, 2006; Sønderby et al., 2010). GSL biosynthesis proceeds through three independent stages: chain elongation (for aliphatic GSLs), core structure formation and side-chain modification (Figure 4). Short-chain Met-derived aliphatic GSLs undergo one to four cycles of chain elongation, whereas long-chain aliphatic GSLs undergo five to six cycles of chain elongation. Minor GSL groups are the Phe- and Met-derived benzoyloxyalkyl GSLs and sinapoylalkyl GSLs, which are synthesized from the short-chain Met-derived methylthioalkyl GSL (**29**) by the enzyme cascade of flavin-monoxygenase glucosinolate *S*-oxygenases FMO<sub>GS-OX4/5</sub>, 2-oxoglutarate-dependent dioxygenase AOP3 and serine carboxypeptidase-like acyltransferase SCPL17 (Figure 4; Kliebenstein et al., 2001b; Lee et al., 2012).

### 3. Regulatory Components in Plant Secondary Metabolism

#### 3.1. bHLH-bHLH, MYB-bHLH and MYB-bHLH-WD40 regulatory complexes

Gene regulation of plant secondary metabolism typically involves the formation of multiprotein complexes through combinatorial interactions between MYB and bHLH family of proteins, or between two different subgroups of bHLH proteins. Furthermore, similar transcription factor complexes have been shown to regulate cell differentiation and cell fate specification in eukaryotes. For example, the interactions between MYB and bHLH family proteins have been shown to activate or repress different gene modules in glucosinolate biosynthesis in *Arabidopsis* (Figures 4 and 5A; Schweizer et al., 2013; Frerigmann et al., 2014a), and in skeletal muscle differentiation in animals (Kaspar et al., 2005). In addition, depending on the plant lineage, the interactions between MYB, bHLH and WD40 domain proteins have been shown to activate all or a set of biosynthetic genes in anthocyanin and proanthocyanidin biosynthesis (Figure 3, 5A; Goff et al., 1992; Grotewold et al., 2000; Aharoni et al., 2001; Zimmermann et al., 2004; Quattrocchio et al., 2006), as well as the cell differentiation of single-celled trichomes, non-hair root epidermal cells (atrachoblasts) in the embryonic stem and mucilage secretory cells in the outer seed coat (Figure 5B; Payne et al., 2000; Bernhardt et al., 2003; Esch et al., 2003; Zhang et al., 2003; Gonzalez et al., 2009; Li et al., 2009). Of the plant MYB-bHLH-WD40 ternary complexes, there is considerable



redundancy for the bHLH cofactors, while the MYB protein typically provides regulatory specificity for a given target pathway in secondary metabolism and cell differentiation by binding to specific *cis*-regulatory elements in gene promoters (Figure 5; Broun 2005; Feller et al., 2011). In addition, the conserved WD40 domain proteins are shared by the plant MYB-bHLH-WD40 complexes between secondary metabolism and cell differentiation (Broun 2005; Feller et al., 2011).

Based on the phylogenetic tree of the MYB proteins, Serna and Martin (2006) suggested that the most ancient function of the MYB-bHLH-WD40 complex is in the regulation of anthocyanin and proanthocyanin production, and that the additional role of the MYB-bHLH-WD40 complex in single-celled trichome formation has been adopted after the Asterid-Rosid division (Serna and Martin, 2006). However, similar analyses have not been made for the other MYB-bHLH-WD40 complexes in epidermal cell differentiation, and thus it remains an open question whether the ancestral function of the MYB-bHLH-WD40 complex is in secondary metabolism or in differentiation of fundamental tissues.

Recently, interactions between bHLHs of different subfamilies in *Arabidopsis* have been shown to regulate the development of two different cell types that synthesize and accumulate the myrosinase enzymes that activate glucosinolate metabolites (Figure 5B; Shirakawa et al., 2014; Li and Sack, 2014). It is not yet known whether MYBs interact with one or more bHLH subgroups to regulate the development of specialized cell-types for secondary metabolism.

### 3.2. Activator-type R2R3-type MYBs

MYB transcription factors are exclusive to eukaryotes (Yang et al., 2003; Jiang et al., 2004), and the plant-specific double-repeat R2R3-type MYBs constitute one of the largest families of transcription factors in angiosperms, with 126 members in *Arabidopsis* (Stracke et al., 2001; Dubos et al., 2010) and at least 125 and 173 in rice and maize, respectively (Yilmaz et al., 2009). The R2R3 domain is responsible for DNA binding, for promoter specificity, and for interaction with other co-factors (e.g., bHLHs). The majority of the *Arabidopsis* R2R3-type MYBs are classified into 22 subgroups, based on the presence of distinctive motifs outside of the conserved MYB domains (Kranz et al., 1998; Stracke et al., 2001), while the remainder are grouped based on overall amino acid sequence alignments (e.g., the related MYBs AtMYB20, AtMYB42, AtMYB43 and AtMYB85 as well as general activators of lignin biosynthesis, AtMYB46 and AtMYB83) (Figures 1 and 6A).

While MYB function in animals is restricted to the control of cell division and differentiation (Weston 1998), the plant-specific R2R3-type MYBs are involved in many diverse such as epidermal cell differentiation (Oppenheimer et al., 1991; Nesi et al., 2001; Ramsay and Glover, 2005), seed development (Penfield et al., 2001), abiotic and biotic stress regulation (Abe et al., 1997; Lee et al., 2001; Vaillau et al., 2002; Mengiste et al., 2003; Zhu et al., 2005; Agarwal et al., 2006), light and sugar regulation (Ballesteros et al., 2001; Teng et al., 2005), stomatal movements (Cominelli et al., 2005; Liang et al., 2005), and secondary metabolism (Martin and Paz-Ares, 1997). Of these functions, nearly half of the *Arabidopsis* R2R3-type MYBs activate genes in epidermal cell differentiation or secondary metabolism (Figures 1 and 5). Among those involved in secondary metabolism, only one

MYB subgroup (subgroup 12) thus far is known to activate the lineage-specific glucosinolate pathway (Figures 1 and 5A), whereas at least 8 MYB subgroups have been shown to activate one or more branches of phenylpropanoid metabolism, including subgroups 2, 3, 5, 6, 7, 13, AtMYB20/42/43/85, and AtMYB46/83 (Figures 1 and 5; Grotewold et al., 1994; Sablowski et al., 1994; Tamagnone et al., 1998; Borevitz et al., 2000; Preston et al., 2004; Deluc et al., 2006; Patzlaff et al., 2003a, 2003b; Goicoechea et al., 2005; Demura and Fukuda, 2007; Zhong and Ye, 2007).

### 3.3. Repressor-type R2R3-MYBs and R3-MYBs

While the majority of R2R3-type MYBs in *Arabidopsis* activate transcription, some operate as transcriptional repressors. Subgroup 4 MYBs all contain a repressor motif known as the ethylene response factor (ERF)-associated amphiphilic repressor (EAR) motif (Kranz et al., 1998) involved in the repression of transcription (Figure 6B; Jin et al., 2000). These repressor-type MYBs transcriptionally repress different branches of phenylpropanoid metabolism, including the lignin and flavonoid pathways. Subgroup 4 is further divided into 4 clades (A–D) according to the presence of additional motifs (Figure 6B; Cavallini et al., 2015). A number of subgroup 4A/B MYBs have been shown to down-regulate the lignin pathway (and in the case of *Arabidopsis*, the derived sinapate ester pathway). They include the snapdragon (*Antirrhinum majus*) AmMYB308 and AmMYB330, *Arabidopsis* AtMYB4 and AtMYB32, *Eucalyptus gunnii* EgMYB1 and maize ZmMYB31 and ZmMYB42 proteins (Figure 6B; Tamagnone et al., 1998; Jin et al., 2000; Preston et al., 2004; Legay et al., 2007; Sonbol et al., 2009; Fornalé et al., 2010; Colquhoun et al., 2011). Subgroup 4A MYBs have also been shown to down-regulate one or more genes in the flavonoid pathway, such as *Arabidopsis* AtMYB4 and AtMYB7 and maize ZmMYB31 and Zm38 (Franken et al., 1994; Fornalé et al., 2000; Fornalé et al., 2014). Grapevine and a few other plant species additionally contain the subgroup 4D2 MYBs, which down-regulate the anthocyanin pathway (Figure 6B; Cavallini et al., 2015).

The EAR-repressor motif is also present in a small family of single-repeat R3-type MYBs. These repressor-type MYBs are thought to fine-tune metabolic and cell differentiation pathways to achieve different metabolic outputs and cell fates, respectively, in accordance with the tissue-type or environmental stress. Five of the 8 *Arabidopsis* R3-type MYBs (i.e., TRY, CPC, ETC1, ETC2, ETC3/CPL3) are functionally similar to one another and counteract the activity of the MYB-bHLH-WD40 ternary complexes in epidermal cell patterning in plants by sequestering their bHLH components (Esch et al., 2003; Zimmermann et al., 2004; Kirik et al., 2004; Simon et al., 2007; Tominaga et al., 2007). In addition, the R3-type MYB TCL1 may act to inhibit the expression of the MYB component of the MYB-bHLH-WD40 complex, through direct binding to its promoter (Wang et al., 2007). The R3-type MYB TCL2 functions redundantly with AtTCL1 in controlling trichome formation on inflorescences (Gan et al., 2011). Interestingly, the R3-type MYB MYBL2 not only inhibits trichome development but also flavonoid biosynthesis (Sawa 2002; Dubos et al., 2008; Matsui et al., 2008), presumably by sequestering the bHLH components in both active MYB-bHLH-WD40 complexes. Similarly, its petunia ortholog, the R3-type MYB PhMYBx, acts as an inhibitor of anthocyanin biosynthesis by sequestering the bHLH protein PhAN1 in active complexes (Koes et al., 2005).



Overexpression of subgroup 15 MYBs (AtGL1 and AtMYB0) promoted the formation of MYB homodimers or heterodimers at the MYB domains and counteracted the activity of the MYB-bHLH-WD40 complexes by preventing the formation of MYB-bHLH interactions (Larken et al., 1994; Liang et al., 2014).

### 3.4. Cis-regulatory elements of R2R3-type MYBs in phenylpropanoid metabolism

The tissue specificity and metabolic output of a particular biosynthetic pathway are dependent on not only the expression profiles and properties of the regulatory genes, but also the promoter regions of the target genes. *In vitro* promoter binding studies to date indicate that the distinct subgroups of R2R3-type MYBs from different subfamilies appear to bind to the same or very similar *cis*-regulatory elements, which are described generally as “AC-like” sequences. For example, systematic evolution of ligands by exponential enrichment (SELEX) experiments demonstrated that both the maize subgroup 4 phenylpropanoid-repressing MYB ZmMYB31 and the maize subgroup 7 flavonoid-activating MYB ZmP1 preferentially bind to ACC(T/A)ACC consensus sequence *in vitro* (Fornalé et al., 2010; Grotewold et al., 1994; Williams and Grotewold, 1997). In addition, electrophoretic mobility shift assay (EMSA) experiments have shown that this motif is similar in sequence to the ACCCGCC sequence that is bound by the *Arabidopsis* subgroup 4 phenylpropanoid-repressing MYB AtMYB4 (Zhao et al., 2007), and identical in sequence to two of the three AC elements that are bound by the *Arabidopsis* subgroup 3 lignin-activating MYBs AtMYB58 and AtMYB63 (Raes et al., 2003; Zhou et al., 2009). Similar to AtMYB58/63, the *Arabidopsis* subgroup 2 MYB AtMYB15 has been shown by EMSA to bind to all three AC elements (Romero et al., 1998), which are enriched in the promoters of lignin biosynthetic genes (Raes et al., 2003). Consistent with this finding, the loquat (*Eriobotrya japonica*) subgroup 2 MYB EjMYB1 has recently been shown to transactivate the promoters of both *Arabidopsis* and loquat lignin biosynthetic genes (Xu et al., 2014), presumably by binding to similar *cis*-regulatory elements.

AC-like sequences are also present in the promoters of target R2R3-type MYB genes to generate regulatory feedback and feedforward loops. For example, the *Arabidopsis* subgroup 4 MYB AtMYB4 directly represses its own transcription by binding to its promoter (Zhao et al., 2007). In addition, the related MYBs AtMYB46 and AtMYB83 proteins bind to AC-like sequences in the promoters of the lignin-activating AtMYB58/63 regulatory genes and the lignin biosynthetic genes, activating both and amplifying the lignin induction signal in the process (Zhong and Ye, 2012).

However, *in vitro*-defined consensus motifs for these R2R3-type MYBs may not be sufficient and/or present in the majority of their *in vivo* target promoters, as was shown for several animal transcription factors (Carr and Biggin, 1999; Yang et al., 2006; Rabinovich et al., 2008). For example, chromatin immunoprecipitation (ChIP)-PCR experiments demonstrated that the maize subgroup 4 MYB ZmMYB31 binds only a small subset of the maize lignin gene promoters containing the ACC(T/A)ACC consensus sequence (Fornalé et al., 2010). In addition, ChIP-Seq experiments indicated that the maize subgroup 7 MYB ZmP1 preferentially binds to sequences containing 6 to 8 repeats of CxxC (where X corresponds to any nucleotide) with no preference for A or T between the C base pairs *in*

*in vivo* (Morohashi et al., 2012). It is not known whether ZmP1 directly binds the (CxxC)<sub>6-8</sub> motif *in vivo* or whether this motif reflects a tethering of ZmP1 through an unknown regulatory cofactor.

### 3.5. MYB-interacting bHLHs

bHLHs are characterized by a conserved DNA-binding domain and diverse sequence motifs that participate in transcriptional regulation (Heim et al., 2003; Pires and Dolan, 2010). The majority of the approximately 160 *Arabidopsis* bHLHs contain His (H), Glu (E), and Arg (R) residues in the core bHLH region, and these residues have been shown to facilitate interaction with DNA at G-box (CACGTG) elements (Martínez-García et al., 2000; Toledo-Ortiz et al., 2003; Qian et al., 2007). The G-box cis-element, alone or in tandem, is found in the promoters of genes that control a variety of developmental and homeostatic processes (Menkens et al., 1995; Chakravarthy et al., 2003; Chandrasekharan et al., 2003). Recently, bHLHs have been shown to play key roles in several cell fate decision points in the differentiation of two different myrosin cell types (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Li and Sack, 2014; Shirakawa et al., 2014).

Activator-type MYBs controlling flavonoid biosynthesis and epidermal cell development have a signature motif ([DE]Lxx[RK]xxxLxxxxxxLxxxR) located in their R3 repeats (Zimmerman et al., 2004; Serna and Martin, 2006). This motif is responsible for their interaction with the subgroup 3F bHLHs (Zimmermann et al., 2004). This bHLH-interacting motif is also shared by repressor-type MYBs controlling phenylpropanoid metabolism and single-celled trichome development, including all characterized subgroup 4 R2R3-type MYBs in plants and six of the eight *Arabidopsis* R3-type MYBs (Zimmermann et al., 2004; Serna and Martin, 2006).

## 4. Metabolic Pathway Regulation

### 4.1. Regulation of lignin pathways in the context of secondary cell wall biosynthesis

At least four subgroups of *Arabidopsis* R2R3-MYBs have been demonstrated to transactivate the promoters of general phenylpropanoid and lignin-specific pathway genes, including subgroup 3 MYBs AtMYB58 and AtMYB63, subgroup 13 MYB AtMYB61, related MYBs AtMYB46 and AtMYB83, and related MYBs AtMYB20, AtMYB42, AtMYB43, and AtMYB85 (Newman et al., 2004; Zhong et al., 2007a; Zhong et al., 2008; McCarthy et al., 2009; Zhou et al., 2009). Of these, only AtMYB58 and AtMYB63 have been shown to directly activate nearly all of the structural genes for lignin biosynthesis (Zhong and Ye, 2009). In *Arabidopsis*, lignin biosynthesis is one of a number of metabolic and developmental pathways involved in secondary cell wall biosynthesis, which is regulated by a three-tiered hierarchical network of transcription factors. AtMYB46 and AtMYB83 as well as AtMYB58 and AtMYB63 occupy the bottom two tiers of this gene regulatory network, with AtMYB46 and AtMYB83 directly activating AtMYB58 and AtMYB63.

AtMYB46 and AtMYB83 together with their regulators, the secondary wall NAC transcription factors (SWNs), are functionally conserved in other angiosperm and

gymnosperm species, including the SWN orthologs in *Medicago*, poplar, *Eucalyptus*, rice, maize and *Brachypodium* (Zhong et al., 2010a; Zhao et al., 2010; Zhong et al., 2011a; Zhong et al., 2011b; Valdivia et al., 2013), as well as the AtMYB46/83 orthologs in poplar, *Eucalyptus* and pine (Patzlaff et al., 2003a; Goicoechea et al., 2005; McCarthy et al., 2010; Zhong et al., 2010b; Zhong et al., 2013). By contrast, the AtMYB58/63 orthologs are present only in species within the rosid eudicot clade (Plant Genome Duplication Database; [chibba.agtec.uga.edu/duplication/](http://chibba.agtec.uga.edu/duplication/)). In addition, even though the *Arabidopsis* AtMYB58 and AtMYB63 proteins are lignin-specific, their orthologs in other plant lineages may have different regulatory roles. For example, the AtMYB58/63 ortholog in maize, Zm1, has been shown to transactivate the maize DFR/FNS gene *A1*, which is involved in flavonoid biosynthesis and not lignin biosynthesis (Franken et al., 1994).

#### 4.2. Regulation of flavonoid pathways

In *Arabidopsis*, the regulation of the single flavonoid biosynthetic pathway is mediated by three regulatory complexes, each responsible for regulating either the early biosynthetic genes that are common to the different flavonoid subpathways or the late genes that are specific for the anthocyanin or proanthocyanidin subpathways. In *Arabidopsis*, the MYB-bHLH-WD40 complex that activates genes specific for proanthocyanidin biosynthesis consists of the subgroup 5 MYB AtTT2, subgroup 3F bHLH EGL3, GL3, or TT8, and the WD40 domain protein AtTTG1 (Figure 5A; Nesi et al., 2000, 2001; Baudry et al., 2004; Zimmermann et al., 2004). Similarly, the MYB-bHLH-WD40 complex that activates genes specific for anthocyanin biosynthesis consists of subgroup 6 MYB PAP1 or PAP2, subgroup 3F bHLH EGL3, GL3 or TT8, and the WD40 protein AtTTG1 (Figure 5A; Borevitz et al., 2000; Gonzalez et al., 2008; Teng et al., 2005; Zhang et al., 2003; Zimmermann et al., 2004). In both cases, the activator-type MYBs are responsible for pathway specificity. Finally, the subgroup 7 MYBs AtMYB11, AtMYB12, and AtMYB111 control flavonol biosynthesis, independently of any bHLH and WD40 cofactors (Figure 5A; Mehrtens et al., 2005; Stracke et al., 2007; Zimmermann et al., 2004).

The *Arabidopsis* subgroup 3F bHLHs EGL3, GL3, and TT8 also interact with another class of transcription factors, the JA-ZIM domain (JAZ) proteins, which can interact with MYBs and bHLHs to repress jasmonic acid (JA)-regulated signaling (Chini et al., 2007; Seo et al., 2011; Song et al., 2011). Here, the JAZ proteins inhibit the MYB-bHLH interaction between the subgroup 3F bHLHs and the subgroup 6 MYB PAP1 to repress JA-regulated anthocyanin accumulation, as well as inhibit the MYB-bHLH interaction between the subgroup 3F bHLHs and the R2R3-type MYB AtGL1 to repress JA-regulated trichome initiation (Qi et al., 2011).

Similar to *Arabidopsis*, the anthocyanin and 3-deoxyflavonoid pathways in maize are controlled by two different regulatory complexes. A MYB-bHLH-WD40 complex consisting of subgroup 5 MYB ZmC1 or ZmPL1, subgroup 3F bHLH ZmR or ZmB, and the WD40 protein ZmPAC1 activates early biosynthetic genes that are shared by all of the flavonoid pathways (e.g., CHS, chalcone isomerase [CHI], flavanone 3-hydroxylase [F3H] and dihydroflavonol 4-reductase [DFR] genes) as well as genes that are specific for the anthocyanin pathway (e.g., anthocyanidin synthase [ANS] and UDP-glucose flavonoid 3-*O*-

glucosyltransferase [UFGT] genes) (Figure 3; Goff et al., 1990; Roth et al., 1991; Tuerck and Fromm, 1994; Grotewold et al., 1998; Selinger and Chandler, 1999; Walker et al., 1999; Carey 2004). By contrast, the subgroup 7 MYB ZmP1 activates the production of the floral organ-specific 3-deoxyflavonoids, independently of any bHLH and WD40 cofactors (Grotewold et al., 1994). Additionally, ZmP1 also activates general phenylpropanoid pathway genes (Figures 2 and 3). Contrary to previous expectations that ZmP1 is a bottom-tiered transcription factor in a hierarchical gene regulatory network, recent CHIP-Seq results suggest that ZmP1 functions both as a transcriptional activator and a repressor with a wider regulatory role that includes not only the PAL, C4H and 4CL genes of the general phenylpropanoid pathway but also the *p*-coumaroyl ester 3'-hydroxylase (C3'H) gene of the lignin pathway (Morohashi et al., 2012).

In petunia petal cells, anthocyanin synthesis and vacuole acidification are regulated by a MYB-bHLH-WD40 complex, consisting of subgroup 6 MYB PhAN2 or PhAN4, subgroup 3F bHLH PhJAF13 or PhAN1, and the WD40 protein PhAN11 (Koes et al., 2005; Quattrocchio et al., 2006; Gerats and Strommer, 2009). In addition, subgroup 6 MYBs PhAN2 and PhAN4 regulate overlapping sets of target genes, providing gene specificity to the pathway (Quattrocchio et al., 2006). Finally, the R3-type MYB PhMYBx downregulates anthocyanin synthesis (Koes et al., 2005; Quattrocchio et al., 2006), presumably by sequestering the bHLH component of the MYB-bHLH-WD40 complex.

Similar to *Arabidopsis*, the proanthocyanidin and anthocyanin pathways in grapevine are activated by the subgroup 5 MYB VvMYBPA2 and the subgroup 6 MYBs VvMYBA1 and VvMYBA2, respectively. MYBs from subgroups 5 and 6 typically interact with subgroup 3F bHLHs and a conserved WD40 protein for their activity. However, as yet, no bHLH or WD40 cofactors have been identified for these grapevine flavonoid-activating MYBs, raising questions over the conservation of the MYB-bHLH-WD40 regulatory model in flavonoid biosynthesis for different plant species (Walker et al., 2007; Kobayashi et al., 2005).

In addition to activating complexes, flavonoid regulation is also controlled by multiple repressive transcription factors to fine-tune phenylpropanoid metabolism or serve as a regulatory loop. The subgroup 4 MYBs, which include AtMYB4, AtMYB6, AtMYB7, and AtMYB32 (Jin et al., 2000; Fornalé et al., 2014; Preston et al., 2004) have been thought to also form MYB-bHLH-WD40 complexes (Zimmermann et al., 2004). There appears to be distinctions between the gene targets of these repressors. AtMYB4 primarily targets the main phenylpropanoid pathway enzyme gene *C4H* and the flavonoid enzyme gene *CHS* to promote sinapate ester production (Jin et al., 2000). In contrast, the *atmyb7* mutant has increased transcription of early steps of phenylpropanoid biosynthesis as well as enzymes in flavonol and anthocyanin biosynthesis (Fornalé et al., 2014), and the *atmyb32* mutant has no change in core phenylpropanoid gene expression and has reduced transcription of anthocyanin biosynthetic genes (Preston et al., 2004). In spite of their transcriptional phenotypes, the *atmyb4* mutant produces greater levels of flavonols and reduced anthocyanin content, while the *atmyb7* mutant has the opposite metabolic phenotype, and the *atmyb4/myb7* double mutant has substantial increases in both flavonols and anthocyanins (Fornalé et al., 2014). These results suggest a more complex system of regulation of flavonoids.

These repressors are themselves activated by other MYBs, It was recently shown that *AtMYB4*, *AtMYB7*, and *AtMYB32* genes are direct targets of the secondary cell wall regulators *AtMYB46* and *AtMYB83* (Zhong and Ye, 2012), which suggested their involvement in fine-tuning of lignin biosynthesis. However, more recently MYB112, a subgroup 20 MYB, was found to directly activate *MYB4/6/7/32* expression in response to abiotic stresses such as salinity and UV light (Lotkowska et al., 2015) to accumulate anthocyanins. These contrasting results suggest that the particular role of these regulatory proteins and their complexes can vary depending upon tissue or cell type and can be stress-dependent.

### 4.3. Regulation of other phenylpropanoid pathways

In *Arabidopsis*, the phenylpropanoid biosynthetic pathway is indispensable to plants for not only its role in the production of the monolignols, but also its role in the production of the phenolic moieties of suberin and a host of other small molecules such as the UV-protecting sinapate esters and the iron-chelating coumarins (Ruegger et al., 1999; Kai et al., 2008; Fourcroy et al., 2013; Schmid et al., 2014).

A recent study identified *AtMYB41*, a subgroup 11 MYB, as a regulator of suberin and cutin production, including the activation of phenylpropanoid biosynthesis and fatty acid-ferulate transferases such as ASFT (Kosma et al., 2014). Analysis of *AtMYB41* expression by GUS staining found expression solely in the root following treatment with abscisic acid or salt, consistent with its role in suberin metabolism. Overexpression of MYB41 results in increased suberin production including ferulate-conjugated fatty acids in both *Arabidopsis* and *Nicotiana benthamiana*. In addition, overexpression of MYB41 increases total lignin content in both species, identifying MYB41 as a novel regulator of lignin metabolism.

The *Arabidopsis* subgroup 3-like MYB *AtMYB72* has recently been shown to modulate iron deficiency responses in roots by not only regulating the lignin biosynthetic genes involved in the production of feruloyl-CoA (4), the biosynthetic precursor of the coumarin scopoletin (9), but also activating genes involved in the secretion of the iron-chelating coumarins into the soil (Zamioudis et al., 2014), presumably by binding to AC-like elements in the target gene promoters.

The subgroup 2 MYBs have particularly interesting roles in phenylpropanoid metabolism. They are commonly known to be induced by both biotic and abiotic stresses including elicitor treatment, cold stress, and drought (Sugimoto et al., 2000; Maeda et al., 2005; Agarwal et al., 2006; Ding et al., 2009), and have been characterized as regulators of diverse phenylpropanoid metabolites. Recently, subgroup 2 MYBs have been found to upregulate lignin biosynthesis in loquat (e.g., *EjMYB1*), stilbene biosynthesis in grapevine (e.g., *VvMYB14/15*), and isoflavonoid biosynthesis in *Lotus japonicus* in response to abiotic and biotic stresses (Shelton et al., 2012; Höll et al., 2013; Xu et al., 2014). These results are in agreement with earlier studies that had identified subgroup 2 MYBs in tobacco and carrot (Sugimoto et al., 2000; Maeda et al., 2005) that activate early steps of phenylpropanoid biosynthesis. Phylogenetically, the subgroup 2 MYBs are closely related to the subgroup 3 MYBs; however, the subgroup 2 MYBs are more evolutionarily ancient, and have been identified in conifers (Bedon et al., 2007), although gymnosperm subgroup 2 MYBs have

not yet been characterized. These findings suggest that subgroup 2 MYBs in other plant lineages may regulate both lignin and clade-specific pathways under certain stress conditions, and may make attractive targets for engineering phenylpropanoid metabolism and exploring plant metabolic diversity.

#### 4.4. Regulation of glucosinolate pathways

The direct regulatory network controlling GSL synthesis in *Arabidopsis* involves activator-type MYBs from subgroup 12 (Figure 2A). The subgroup 12 MYBs AtMYB34, AtMYB51, and AtMYB122 regulate the tryptophan-derived indolic GSLs (Celenza et al., 2005; Gigolashvili et al., 2007a; Malitsky et al., 2008), of which AtMYB34/51 are the main regulators (Frerigmann et al., 2014b). By contrast, the subgroup 12 MYBs AtMYB28, AtMYB29, and AtMYB76 regulate the methionine-derived aliphatic GSLs (Gigolashvili et al., 2007b, 2008; Hirai et al., 2007; Sønderby et al., 2007; Beekwilder et al., 2008; Malitsky et al., 2008). In addition, all six subgroup 12 MYBs interact directly with the subgroup 3E bHLHs, including AtMYC2 (also known as AtbHLH06), AtMYC3 (also known as AtbHLH05), AtMYC4 (also known as AtbHLH04) and AtbHLH28 (Schweizer et al., 2013; Frerigmann et al., 2014). Moreover, certain MYB-bHLH protein pairs are preferred over others, with strong interactions found between the pairing of AtMYB28/51/76 with AtMYC4, AtMYB29 with AtMYC2, and AtMYB34/122 with AtMYC3/4 (Frerigmann et al., 2014).

Similar to the subgroup 3F bHLHs in anthocyanin biosynthesis and trichome development, the *Arabidopsis* subgroup 3E bHLHs AtMYC2/3/4 also interact with the JAZ proteins. The JAZ proteins inhibit the MYB-bHLH interaction to repress JA-regulated glucosinolate biosynthesis (Chini et al., 2009). Interestingly, the subgroup 3E bHLH AtbHLH28 does not interact with the JAZ proteins, which has led to the suggestion that it may regulate GSL biosynthesis independently of JA signaling (Fernández-Calvo et al., 2011; Niu et al., 2011). In addition, the interaction strength between bHLH-JAZ protein pairs may be negatively correlated with that of bHLH-MYB pairs. For example, a strong interaction between AtMYB29 and AtMYC2 may produce a correspondingly weak interaction between AtMYC2 and JAZ proteins, allowing for AtMYB29 to function in JA-mediated control of GSL biosynthesis (Hirai et al., 2007; Gigolashvili et al., 2008).

While glucosinolates are lineage-specific secondary metabolites, the subgroup E bHLHs are present in diverse plant lineages. In plant species that do not synthesize glucosinolates, the subgroup E bHLHs may regulate other secondary metabolic pathways. For example, the pea subgroup E bHLH PsGBP1 has been shown to bind to the promoter of the CHS gene (Qian et al., 2007), possibly regulating flavonoid biosynthesis.

## 5. Developmental Pathway Regulation

### 5.1. Compartmentalization of glucosinolates and myrosinases

The defining feature of glucosinolate-producing plants is the cellular compartmentalization of glucosinolates and their hydrolytic enzymes, the myrosinases (also called thioglucoside glucohydrolase [TGG]) (Kissen et al., 2009; Ahuja et al., 2010). Normally in *Arabidopsis*,



glucosinolates and myrosinases are synthesized and stored separately in adjacent cells termed S-cells and myrosin cells, respectively, at the leaf periphery and along veins (Andréasson et al., 2001; Husebye et al., 2002; Ueda et al., 2006; Shroff et al., 2008; Koroleva et al., 2010). Upon herbivore-induced release, the myrosinase, in the presence of water, cleaves the thioglucose bond in the glucosinolate, leading to the rapid generation of the unstable thiohydroximate-*O*-sulfate intermediate. Depending on the substrate, pH, availability of ferrous ions and presence of myrosinase-interacting proteins, a variety of biologically active breakdown products can be produced, including isothiocyanates, thiocyanates, nitriles and elemental sulfur, oxazolidine-2-thiones and/or epithioalkanes (Figure 4A). However, recent studies in *Arabidopsis* have shown that one particular glucosinolate species, 4-methoxyglucobrassicin (**30**) (Figure 4B), and the myrosinase PEN2 can be synthesized *de novo* in the same cells upon pathogen elicitation (Bednarek et al., 2009; Clay et al., 2009). The resulting breakdown products, as yet unknown, function as antifungals and as signaling molecules directing the deposition of callose polymers at the cell wall and thus restricting cellular entry by microbial pathogens (Bednarek et al., 2009; Clay et al., 2009). In addition, S-cells have been recently shown to synthesize both glucosinolates and myrosinases and then store them in separate subcellular compartments (Koroleva and Cramer, 2011).

## 5.2. bHLH heterodimers in myrosin cell development

There are two types of myrosinase-expressing myrosin cells: guard cells in stomata and idioblast myrosin cells. Idioblast myrosin cells are a recent developmental innovation that is restricted to the Brassicales order. By contrast, guard cells in stomata mediate gas exchange between the plant and the environment, and are present in nearly all extant land plants. In *Arabidopsis*, three “master regulator” subgroup 1A bHLHs, AtSPCH, AtMUTE and AtFAMA, regulate individual consecutive steps in stomatal development (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007). The final differentiation step in stomatal development is regulated by AtFAMA, which also confers guard cell fate (Ohashi-Ito and Bergmann 2006). Similarly, idioblast myrosin cell development and cell fate are also regulated by AtFAMA, which is transiently expressed in ground meristem cells before the cells differentiate into myrosinase-expressing idioblast myrosin cells (Li et al., 2014; Shirakawa et al., 2014). Differentiation of both myrosin cell types also requires two functionally redundant subgroup 3B bHLHs, AtICE1 (also called AtSCRM1) and AtSCRM2 (also called AtICE2), which are ubiquitously expressed in all cell types in the leaf and heterodimerize with AtFAMA (Kanaoka et al., 2008; Shirakawa et al., 2014). By contrast, guard cell differentiation additionally requires AtSPCH and AtMUTE, which also interact with AtICE1 and AtSCRM2 to regulate the first two differentiation steps in stomatal development (MacAlister et al., 2007; Pillitteri et al., 2007).

The subgroup 1A bHLHs are not functionally exchangeable in stomatal and idioblast myrosin cell differentiation (MacAlister et al., 2007; MacAlister and Bergmann, 2011; Shirakawa et al., 2014). In addition, the AtFAMA amino acid sequence is better conserved among land plants than those of AtSPCH or AtMUTE (Ran et al., 2013). In idioblast myrosin cell precursors, AtFAMA binds to its own promoter (Hachez et al., 2011) and positively regulates its own expression (Shirakawa et al., 2014), whereas in guard cell

precursors, AtFAMA does not exhibit positive feedback regulation of its own expression (Ohashi-Ito and Bergmann, 2006). Stomatal development is also regulated by two R2R3-MYBs, AtFLP (also AtMYB124) and AtMYB88, that are coexpressed with AtFAMA, AtICE1 and its paralog AtSCRM2 in leaves, but AtFAMA does not interact with these MYBs (Ohashi-Ito and Bergmann 2006).

The recent data on myrosin cell differentiation indicate that despite differences between the two different developmental pathways that generate the different myrosin cell types, the underlying AtFAMA-dependent regulatory mechanisms appear to be shared. Because *FAMA*-expressing cells outside of the Brassicales do not express myrosinases (Liu et al., 2009), at some point during the evolution of the Brassicales, these cells were specified to accumulate myrosinases. Ongoing efforts to identify the downstream targets of AtFAMA may shed some light on whether this “myrosin specifying factor” is independent of *AtFAMA* expression.

### 5.3. bHLH heterodimers in laticifer cell development

S-cells are elongated phloem-associated cells that are rich in glucosinolates and thus in sulfur (Koroleva et al., 2000). S-cells are similar in structure and function to the non-articulated laticifer cells in latex plants. Laticifers have evolved repeatedly in nearly every major plant group (Evert et al., 2006; Hagel et al., 2008), and may share a common evolutionary origin with S-cells (Koroleva et al., 2010). Unlike myrosin cells, the molecular mechanisms regulating S-cell development remain elusive, largely due to a paucity of gene markers for S-cells. Recently, transcripts of the rubber tree (*Hevea brasiliensis*) subgroup 3E-like bHLH genes, *HblMYC1* and *HblMYC2*, were found to increase or decrease, respectively, during mechanical wounding or JA treatment of laticifer cells, suggesting that these transcription factors may participate in JA-induced production of latex (Zhao et al., 2011). Parallels between laticifer and S-cell development suggest that S-cell development in *Arabidopsis* may also be regulated by the same subgroup 3E bHLHs that regulate glucosinolate biosynthesis. In addition to S-cells, both aliphatic and indole GSLs have been shown to accumulate in single-celled (non glandular) trichomes (Frerigmann et al., 2012), and indole GSLs in the cells of the outer leaf margin (Shroff et al., 2008; Koroleva et al., 2010).

## 6. Concluding Remarks

Plants are largely the basis for human nutrition and have widespread potential as a renewable source for fuel and chemical feedstocks. Technological advances in genomics and metabolomics combined with systems biology approaches have ushered plant metabolic research into a second golden age, at a time when there is increased awareness of diminishing food security and vanishing fossil fuels, the feedstocks of petrochemicals. A more fundamental understanding of plant secondary metabolism is needed to rationally redesign and predictably modify plant metabolic networks to address the many problems confronting humanity. This will entail not only the elucidation of the secondary metabolic networks, but also an increased understanding of the gene regulatory networks that are integrated with the metabolic networks and are responsible for generating chemical diversity

in response to genetic and environmental perturbations. Presently, very little is known about the relationships between these regulatory proteins and their target sequences, as well as potential regulatory complexes that may exist for each transcription factor. A fuller understanding of the regulatory mechanisms that underlie secondary metabolism will enable rational engineering of these metabolic pathways through coordinated activation or repression of biosynthetic pathways by transcriptional regulators. In addition, it should be possible to engineer the regulation of heterologous genes and pathways to be activated alongside their precursors by modifying promoter sequences. Once more is known about the relationships between these regulators and their target promoter sequences, one could imagine the engineering of the regulators themselves to customize their gene targets. These tools could then be applied to improve biomass yields, increase stress tolerance, and enable large-scale production of compounds with industrial and pharmaceutical importance in order to meet the challenges of a growing population, climate change and bioenergy.

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## Biographies



William R. Chezem received his B.S. in Biochemistry (2010) from Ball State University and is currently a Ph.D. candidate in the Department of Molecular, Cellular & Developmental Biology at Yale University. His research focuses on the transcriptional regulation of phenylpropanoid metabolism in plant immunity and the evolutionary conservation of pathway regulators. His research goal is to address needs in bioenergy, plant disease, and human health by engineering plant metabolism.

<http://www.ncbi.nlm.nih.gov/myncbi/browse/collection/41467483/>



Nicole K. Clay received her B.S. in Biology (1996) from Massachusetts Institute of Technology, and her Ph.D. in Biology (2005) from Yale University. She received her postdoctoral training in the field of plant-microbe interactions (2005–2010) at Massachusetts General Hospital, an affiliate of the Harvard Medical School. In 2011, she joined the faculty at Yale University as an Assistant Professor of Molecular, Cellular & Developmental Biology. Her research program exploits plant-pathogen interactions to discover new small molecules with antimicrobial and immune-signaling activities as well as their underlying biosynthetic “regulons” and regulatory networks. In addition, her program re-engineers plant immune receptors to discover novel protein modifications and quality-controls that regulate recombinant (and native) protein production and function.

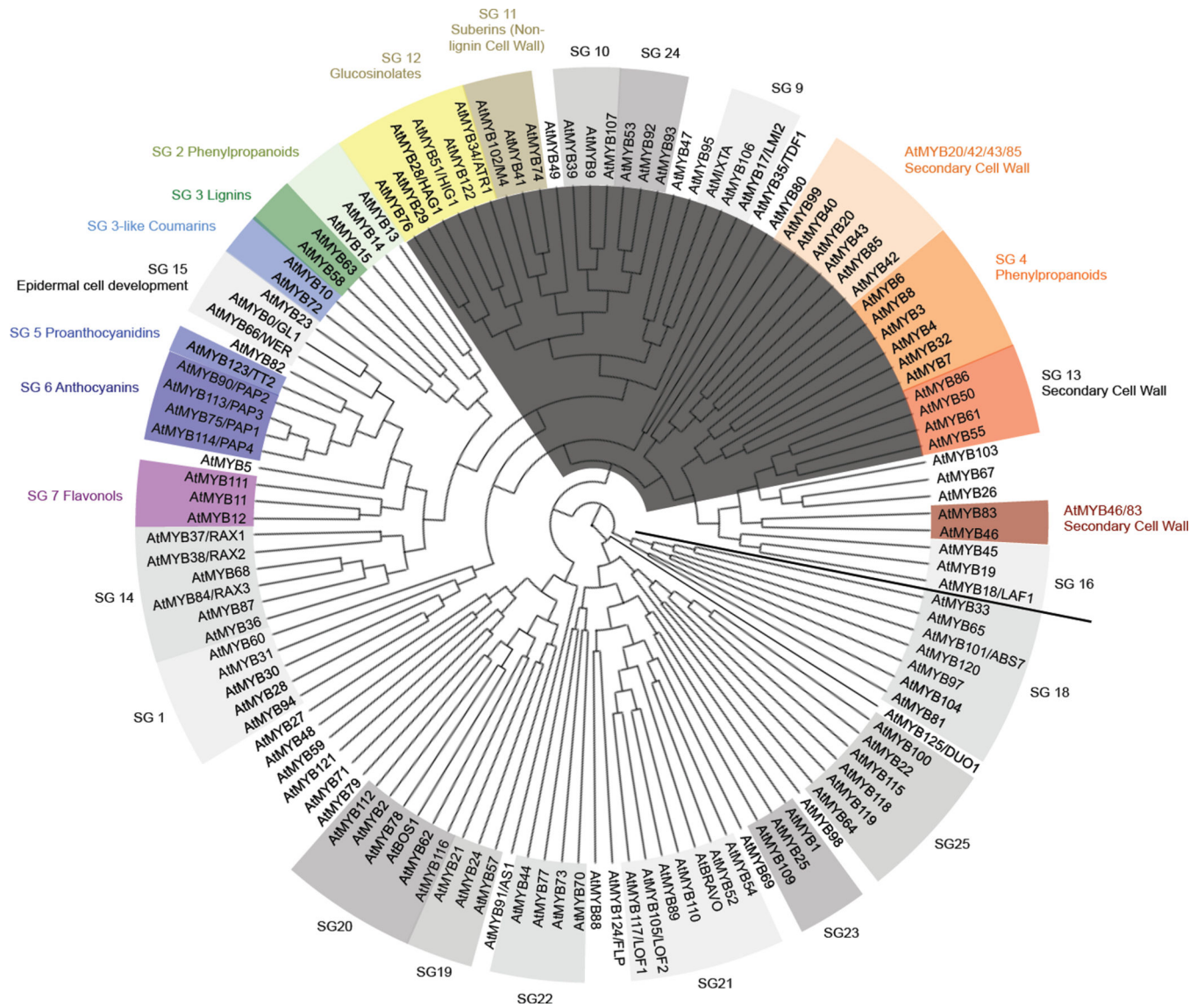
Prof. Clay has served on the scientific advisory panel at the United States Department of Agriculture. She is the most recent recipient of the Elsevier/Phytochemistry Young Investigator Award.

MYBs and bHLHs regulate secondary metabolism and epidermal cell differentiation

Multiple MYB subgroups regulate different pathways in phenylpropanoid metabolism

A single MYB subgroup regulates glucosinolate metabolism

bHLHs regulate specialized cell differentiation associated with secondary metabolism



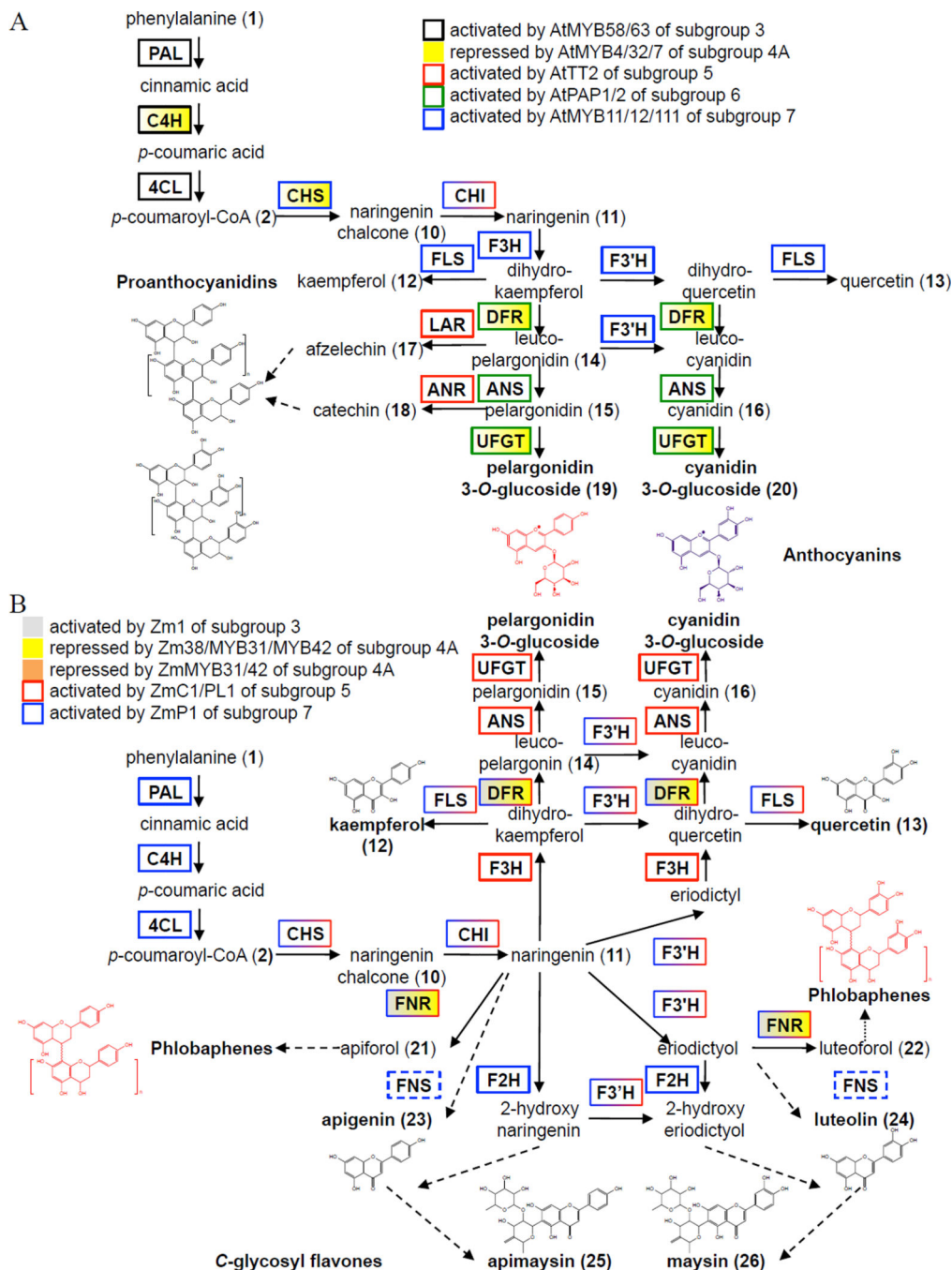
**Figure 1. Putative evolutionary relationships among *Arabidopsis* R2R3-type MYB proteins in secondary metabolism and associated cell development**  
 Phylogenetic maximum likelihood tree of the complete *Arabidopsis* family of R2R3-type MYB proteins was generated using MUSCLE multiple sequence alignment, PhyML phylogeny, and TreeDyn tree viewer programs (<http://phylogeny.lirmm.fr>; Dereeper et al., 2008). Highlighted in color are MYB subgroups that regulate secondary metabolism. Highlighted in dark gray are MYB subgroups that contain the GIDPxxH motif of unknown function after the end of the MYB DNA-binding domain.







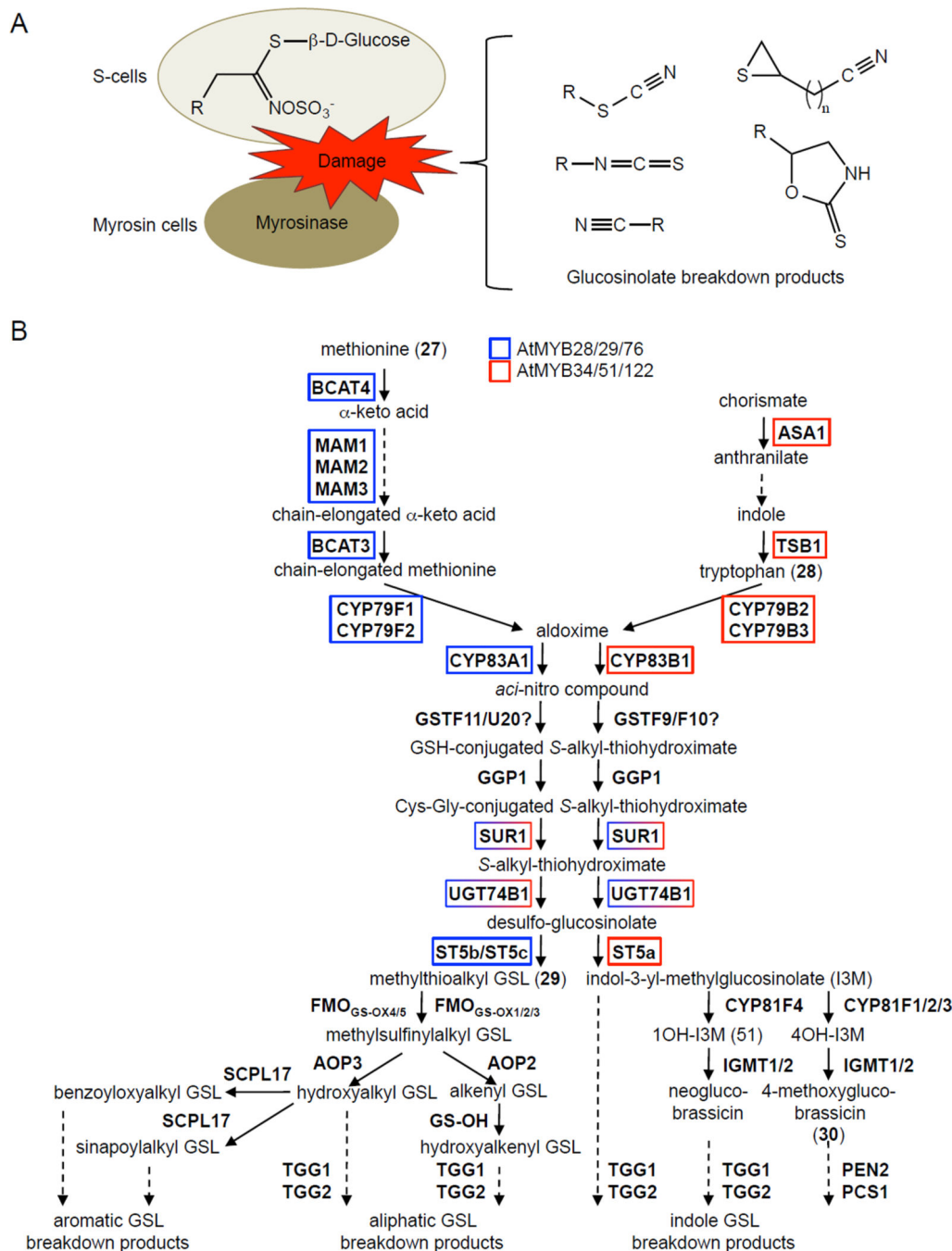
ADH, alcohol dehydrogenase; C3'H, *p*-coumaroyl ester 3'-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamoyl-alcohol dehydrogenase; 4CL, 4-coumarate:CoA ligase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; COMT, caffeic acid *O*-methyltransferase; CSE, caffeoyl shikimate esterase; F5H, ferulate 5-hydroxylase; F6'H, feruloyl-CoA 6'-hydroxylase; HCT, hydroxycinnamoyl-CoA:shikimate/quinic acid hydroxycinnamoyltransferase; LAC, laccase; PAL, phenylalanine ammonia-lyase; SCPL, serine carboxypeptidase-like; SGT, sinapate UDP-glucose sinapoyltransferase.



**Figure 3. Multiple R2R3-type MYB Subgroups Regulate the Flavonoid Biosynthetic Pathways in *Arabidopsis* and Maize**

**(A)** *Arabidopsis* flavonoid pathway. **(B)** Maize flavonoid pathways. Adapted from Morohashi et al. (2012). Naringenin (11) is at the intersection between the 3-hydroxyflavonoid and 3-deoxyflavonoid pathways. Unbroken arrows indicate single enzymatic conversions, and broken arrows indicate multiple enzymatic conversions. Flavan-3-ols afzelechin (17) and catechin (16) polymerize to form proanthocyanidins. 3-hydroxyflavonoids include flavonols kaempferol (12) and quercetin (13), and anthocyanins

pelargonin 3-glucoside (**19**) and cyanidin 3-glycoside (**20**). 3-deoxyflavonoids include the flavan-4-ols apiforol (**21**) and luteoforol (**22**), the flavones apigenin (**23**) and luteolin (**24**), and the *C*-glycosyl flavones apimaysin (**25**) and maysin (**26**). Flavan-4-ols polymerize to form the red phlobaphene pigments. Unbroken arrows indicate single enzymatic conversions, and broken arrows indicate multiple enzymatic conversions. ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C3'H, *p*-coumaroyl ester 3'-hydroxylase; C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; 4CL, 4-coumarate-CoA ligase; COMT, caffeic acid *O*-methyltransferase; DFR, dihydroflavonol 4-reductase; F2H, flavanone 2-hydroxylase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; FNR, flavanone 4-reductase; FNS, Type I flavone synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia-lyase; UFGT, UDP-glucose flavonoid 3-*O*-glucosyltransferase.



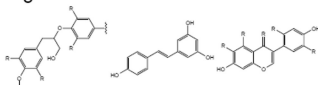
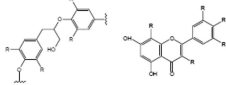
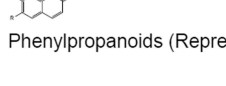
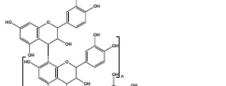
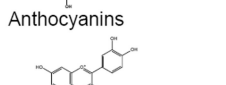
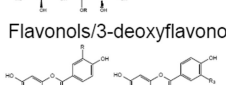

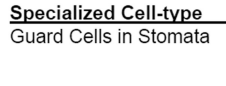
**Figure 4. One R2R3-type MYB Subgroup Regulates the Glucosinolate Biosynthetic Pathways in *Arabidopsis***

(A) Classical glucosinolate-myrosinase chemical defense system or “mustard oil bomb”. R is the side-chain of amino acids Trp, Tyr, Phe, Ile, Leu, Val, Ala or Met. Upon tissue damage, glucosinolates are hydrolyzed by myrosinase enzymes to glucose and unstable thiohydroximate-*O*-sulfate intermediates, which spontaneously eliminate the sulfate group and rearrange to form thiocyanates at pH >8; isothiocyanates at pH 5–8; nitriles and elemental sulfur if guided by epithiospecifier-like proteins, or at pH 2–5 and in the presence

of Fe<sup>2+</sup>; epithionitriles if a terminal double bond captures the elemental sulfur released during nitrile formation; and oxazolidine-2-thiones if a hydroxyl function is present on carbon 3 of the glucosinolate. Adapted from Grubb and Abel et al. (2006). **(B)** *Arabidopsis* glucosinolate pathways. Adapted from S nderby et al. (2010) and Maruyama-Nakashita et al. (2006). Unbroken arrows indicate single enzymatic conversions, and broken arrows indicate multiple enzymatic conversions. AOP2, alkenyl glucosinolate-producing 2-oxoglutarate-dependent dioxygenase; AOP3, hydroxyalkyl glucosinolate-producing 2-oxoglutarate-dependent dioxygenase; ASA1, anthranilate synthase alpha subunit; BCAT3, branched-chain aminotransferase; CYP79B2/3, tryptophan *N*-hydroxylases; CYP81F2, indol-3-ylmethylglucosinolate 4-hydroxylase; CYP83B1, indole-3-acetaldoxime *N*-hydroxylase; FMO<sub>GS-OX</sub>, flavin-monooxygenase GSL *S*-oxygenase; GST, glutathione transferase; GGP1, gamma-glutamyl peptidase 1; GS-OH, hydroxyalkenyl glucosinolate-producing 2-oxo acid-dependent dioxygenase; MAM, ; IGMT2 and IGMT3, indole glucosinolate *O*-methyltransferases; PEN2, 4-methoxyindol-3ylmethylglucosinolate thioglucosidase; PCS1, phytochelatin synthase 1; SCPL17, serine carboxypeptidase-like acyltransferase; ST5, desulfoglucosinolate sulfotransferase; SUR1, *S*-alkyl-thiohydroximate lyase; TGG, thioglucosidase; TSB1, tryptophan synthase  $\beta$ -subunit 1; UGT74B1, UDP-glucose:thiohydroximate *S*-glucosyltransferase.



**A**

<u>Plant Secondary Metabolites</u>	<u>Regulatory Complex</u>		
	<u>R2R3-MYBs</u>	<u>bHLHs</u>	<u>WD40</u>
Lignins/Stilbenoids/Isoflavonoids 	Subgroup 2	?	?
Lignins/Flavonols 	Subgroup 3	No	No
Coumarins 	Subgroup 3-like	?	?
Phenylpropanoids (Repressor) 	Subgroup 4	Subgroup 3F	Likely
Proanthocyanidins 	Subgroup 5	Subgroup 3F	Yes
Anthocyanins 	Subgroup 6	Subgroup 3F	Yes
Flavonols/3-deoxyflavonoids 	Subgroup 7	No	No
Glucosinolates 	Subgroup 12	Subgroup 3E	?

**B**

<u>Specialized Cell-type</u>	<u>Regulatory Complex</u>		
	<u>R2R3-MYBs</u>	<u>bHLHs</u>	<u>WD40</u>
Guard Cells in Stomata	?	Subgroups 1A/3B	?
Non-glandular Trichomes	Subgroup 15	Subgroup 3F	Yes
Trichoblast Cells	Subgroup 15	Subgroup 3F	Yes
Idioblast Myrosin Cells	?	Subgroups 1A/3B	?
S-cells	?	?	?
Laticifers	?	Subgroup 3E?	?

**Figure 5. Regulatory Components in Plant Secondary Metabolism and Associated Specialized Cell Development**  
 Summary of the R2R3-type MYB subgroups, bHLH subgroups and WD40 domain proteins that regulate (A) the production of plant secondary metabolites and (B) the differentiation of specialized cell types.

## A. Activator-type R2R3-MYBs

Subgroup	Consensus	Accession
<b>Subgroup 2 consensus</b>	<b>ΦDEsFW...mEfwQd</b>	
AtMYB15	215 IDEsFW...FDfWFD 270	NP_188966
VvMYB14	202 MDENFW...MEFWYN 258	EABW34392
VvMYB15	190 IDEsFW...MEFWYD 242	KCS14110
EjMYB1	191 VDDsFW...MEIwYD 250	KF767453*
<b>Subgroup 3 consensus</b>	<b>WxxxLExELGL</b>	
AtMYB58	231 WFKHLESELGL 241	NP_173098
AtMYB63	256 WFKHLESELGL 266	NP_178039
Zm1	272 WLENLEKELGL 282	P20024
<b>Subgroup 5 consensus</b>	<b>DEdWLRxxT</b>	
TT2/AtMYB123	249 DEDWLRDFT 257	NP_198405
ZmC1	260 DEDWLRQQT 268	P10290
ZmFL1	253 DEEWLRCHT 261	AAA19821
VvMYBPA2	273 EDEWIT 278	ACK56131
<b>Subgroup 6 consensus</b>	<b>KPxPrxF</b>	
PAP1/AtMYB75	140 KPRPRsF 146	NP_176057
PAP2/AtMYB90	141 KPRPRsF 147	NP_176813
VvMYBA1	131 KPHPHKF 137	BAD18977
VvMYBA2	131 KPHPHKF 137	ACK56131
PhAN2	139 KPRPRTF 145	AAF66727
PhAN4	142 RPQPRNL 148	ADQ00392
<b>Subgroup 7 consensus</b>	<b>GRTxxxxxK</b>	
AtMYB111	158 GRTSRSSMK 166	NP_199744
AtMYB11	141 GRTSRsAMK 149	NP_191820
ZmF1	148 GRTsGRPEPK 156	M73028
<b>Subgroup 12 consensus</b>	<b>LLNKVA</b>	
AtMYB34/ATR1	153 LLNRVA 158	NP_200897
AtMYB51	174 FLNKVA 179	NP_173292
AtMYB28	207 LLNKVA 212	NP_200950
AtMYB29	189 LLNKVA 194	NP_196386

## B. Repressor-type R2R3-MYBs

Subgroup	Consensus	Accession
<b>Subgroup 4A consensus</b>	<b>LNLeL...CxxCxxGxxxxxxCxC...FLGL...LDQRxLEMK</b>	
AtMYB4	198 LNLEL...CFKCSLGMINGMECRc...FLGL...LDFSTLEMK 282	AAC83582
AtMYB32	185 LNLEL...CSACRFgFGNGKEcSc...FLGL...LDFSTLEMK 274	NP_195225
AtMYB7	190 LNLEL...CTASRFYMEndMEcSS...FLGL...LDFRSLEMK 269	NP_179263
Zm38	168 LNLDL...CFGCSLGVQKGAPcSc...CLGL...LDFRGLKMK 255	1613412D
ZmMYB31	205 LNLDL...CFGCSLGLPRtADcKc...FLGL...LDFRSLEMK 331	CAJ42202
ZmMYB42	176 LNLDL...CLGCSLGGQKGAAGcSc...FLGL...LDFRGLKMK 260	CAJ42204
VvMYB4a	175 LNLEL...CFACSLGIPNSEEcSc...FLGL...LDYRGLKMK 251	ABL61515
VvMYB4b	170 LNLEL...CFYCSLGLKESKEcSc...FLGM...LDYRNLEMK 240	ACN94269
EgMYB1	183 LNLEL...CXEXNLGLKNSQNCRC...FLGL...LDYRS 255	CAE09058**
EjMYB2	178 LNLEL...CFSCSLGIQDAKNCcSc...FLGL...LDYRSLEMK 255	KF767454
<b>Subgroup 4B consensus</b>	<b>LNLeL...CxxCxxGxxxxxxCxC</b>	
AtMYB3	189 LNLEL...CLCCRIGLFRNESCRNC 249	NP_564176
AmMYB308	178 LNLEL...CFVCRlGIQNSKDCcSc 226	P81393
<b>Subgroup 4C consensus</b>	<b>LNLeL</b>	
AtMYB6	198 LNLEL 202	NP_192684
AtMYB8	185 LNLEL 189	NP_849749
<b>Subgroup 4D2 consensus</b>	<b>LNLeL...TLL</b>	
VvMYBC2-L1	182 LNLDV...TLLLF 257	ABW34393
VvMYBC2-L2	188 LNLDL...TLLLF 225	ACX50288
VvMYBC2-L3	184 LNLDL...TLILF 227	KM046932

## C. Activator-type bHLHs

	Subgroup 1A consensus	QΦΦQxLExxKxRk...TΦLysFxVKIxxEc...ΦEdΦxxxΦqq	Accession
AtFAMA/bHLH097	247	QLLQCLESQKRRR...TVLYSFNVKITSET...AEDIASSIQQ	NM_113319
AtSPCH/bHLH098	152	QVLQSLKAKKQRK...TMLNSFVVKIGLEC...LEDLLEILQ	NM_124700
AtMUTE/bHLH045	51	QLVQVLESKKRRK...TVLYFFVVKIGLEC...LEELTLEVQK	DQ864972
	Subgroup 3B consensus	VEVRxreGxaΦNIHMFCgRRPGLLLxTMkALDxLGLDVQQAVISC FN GFALDVFRAEqc	
AtICE1/bHLH116	411	VEVRLREGRAVNIHMFCGRRPGLLLATMKALDNLGLDVQQAVISC FN GFALDVFRAEQC	NM_001035697
AtSCRM2/bHLH033	367	VEVRLREGRAVNIHMFCGRRPGLLLATMKALDNLGLDVQQAVISC FN GFALDVFRAEQC	EABW34392
SmBHLH075	109	IEVKMqKqKDFNIHMFCGSRPGLLLSMMKALDNLGLDVQQAVISC FN GFALDVFRAEQC	XP_002984017
PpICE1-like	431	VEVRKREGQALNIHMFCARRPGLLLSTVRALDALGLDVQQAVISC FN GFALDLFRAEAK	XP_001758066
	Subgroup 3D consensus	LxxRLxxΦ...WxYAIFW...LSWGDG...RKxxxK...ExFQ...gVVELG	
AtJAM1/bHLH17	48	LNKKLSSLV...WNYAIFW...LSWGDG...RKRVQK...EIFF...GVVELG	Q9ZPY8
Hb1MYC1	29	LQQRLQFIL...WVYAIFW...LSWGDG...RKMTNK...EWFY...GVVELG	GU434304
Hb1MYC2	29	LQQRLQFIL...WVYAIFW...LSWGDG...RKVINK...EWFY...AVVELG	HM061097
	Subgroup 3E consensus	DdxasΦMEAF...LGWGDG...RKKVΦRELN...DTEWFF...GVVELg...dHSDLEa	
AtMYC2/bHLH006	16	DDNASMMEAF...LGWGDG...RKKVLELRLN...DTEWFF...GVVEVG...DHSDDLEA	Q39204
AtMYC3/bHLH005	14	DASAAAMEAF...LGWGDG...RKRVIRELN...DTEWFF...GVVELG...DHSDDLEA	Q9FIP9
AtMYC4/bHLH004	27	DDASVMEAF...LGWGDG...RKRVIRELN...DTEWFF...GVVELG...NHSDDLEA	049687
PsGBF	17	DDNSVMEAF...LGWGDG...RNKVLRELN...DTEWFF...GVVELA...DHSDDLEA	DQ399528
	Subgroup 3F consensus	WSYxΦFW...LxWxdGxYNGdΦKtRK...ΦxRSxqLreLYe...LSPEDLxDtEWYY	
AtGL3/bHLH001	27	WSYGIFW...LEWGDGYNGDIKTRK...LRSSEQLSLEYE...LSPEDLADTEWYY	NM_148087
AtEGL3/bHLH002	26	WSYGIFW...LEWGDGYNGDIKTRK...LRSSEQLSLEYE...LSPEDLADTEWYY	Q9CAD0
AtMYC1/bHLH012	36	WSYALFW...LEWGEGCYNGDMKRRK...LWKSKELRKLYL...LSPDLSDEWYY	Q8W2F1
AtTT8/bHLH042	33	WTVSVFW...LVWGNGYNGAIKTRK...LRSQQRLRELYE...LSPEDLDEWYY	Q9FT81
ZmR	37	WSYALFW...LTWTDGFYNGEVKTRK...MQRSDQLRELYE...LSPEDLGDTEWYY	AAA33504
ZmB	33	WSYALFW...LTWTDGFYNGEVKTRK...MQRSEQRLRELYE...LSPEDLGDTEWYY	CAA4054

**Figure 6. Motifs defining MYB and bHLH subgroups in pathway regulation of secondary metabolism and associated specialized cell development**

The numbers shown at the left and right of each sequence are the positions of the first and last amino acid relative to the translational start site. (A) Activator-type R2R3-MYBs.

Asterisk indicates that the reported cDNA sequence may contain an extra nucleotide that when removed produces the full conserved subgroup 2 motif. (B) Repressor-type R2R3-MYBs. Double asterisks indicate that the reported cDNA sequence is incomplete. (C)

Activator-type bHLHs in subgroups as defined by Heim et al. (2003) and Pires and Dolan (2010). At, *Arabidopsis thaliana*; Eg, *Eucalyptus gunnii*; Ej, *Eriobotrya japonica* (loquat); Ph, *Petunia x hybrida*; Pm, *Physcomitrella patens* (moss); Ps, *Pisum sativum* (pea); Sm, *Selaginella moellendorffii* (spikemoss); Vv, *Vitis vinifera* (grapevine); Zm, *Zea mays* (maize). F denotes aliphatic amino acid; W denotes aromatic amino acid; uppercase letters represent strictly conserved amino acids; lowercase letters represent amino acids that are conserved in the majority of the sequences.