

Regulation of Specialized Metabolism by WRKY Transcription Factors

Craig Schluttenhofer and Ling Yuan*

Department of Plant and Soil Sciences and Kentucky Tobacco Research and Development Center, University of Kentucky, Lexington, Kentucky 40546

WRKY transcription factors (TFs) are well known for regulating plant abiotic and biotic stress tolerance. However, much less is known about how WRKY TFs affect plant-specialized metabolism. Analysis of WRKY TFs regulating the production of specialized metabolites emphasizes the values of the family outside of traditionally accepted roles in stress tolerance. WRKYs with conserved roles across plant species seem to be essential in regulating specialized metabolism. Overall, the WRKY family plays an essential role in regulating the biosynthesis of important pharmaceutical, aromatherapy, biofuel, and industrial components, warranting considerable attention in the forthcoming years.

Over the last 20 years, the WRKY transcription factor (TF) family has been well recognized for its role in regulating abiotic and biotic stress tolerance in plants. WRKY domain-containing genes comprise one of the largest TF families in plants and are characterized by a highly conserved WRKYGQK motif and a C₂H₂ or C₂HC zinc finger (Eulgem et al., 2000). Ishiguro and Nakamura (1994) identified the first WRKY TF, SWEET POTATO FACTOR1 (SPF1), as a negative regulator of sporamin and β -amylase expression in sweet potato (*Ipomoea batatas*). Based on the lack of homologous genes, they speculated that SPF1 may be a new class of DNA-binding proteins. The following year, two additional WRKY TFs, ABF1 and ABF2, from wild oat (*Avena fatua*), helped solidify these genes as encoding a novel class of plant DNA-binding proteins (Rushton et al., 1995). In 1996, the function of the family was extended to plant defense with the discovery of three parsley (*Petroselinum crispum*) WRKYs that regulate the *PATHOGENESIS RELATED1* promoter (Rushton et al., 1996). That study also clearly identified the core cis-element, (T)TGAC(C), also called the W-box, that is recognized by most WRKY TFs.

A seminal study was published in 2000 characterizing the Arabidopsis (*Arabidopsis thaliana*) WRKY family (Eulgem et al., 2000). The 72 members of the Arabidopsis WRKY family are phylogenetically classified into three major groups (groups I–III), with group II further divided into five subgroups (groups IIa–IIe). The phylogenetic information led to investigations about the origin of WRKY TFs. Early studies found that WRKYs exist in a limited number of protists, including *Giardia lamblia* and *Dictyostelium discoideum*, as well as the green alga *Chlamydomonas reinhardtii* (Zhang and Wang, 2005). Based on these species, early convention held that the ancestral WRKY gene belonged to the

group I WRKY family (Wu et al., 2005; Zhang and Wang, 2005). A more recent study, however, determined that the ancestral WRKY gene most likely encodes a group IIc-like WRKY TF (Brand et al., 2013).

The first structure of the WRKY domain was published in 2005. Since then, the functions of key domain residues have been elucidated (Yamasaki et al., 2005; Duan et al., 2007). The globular WRKY domain is composed of five antiparallel β -strands, a Glu-Trp-Lys triad that stabilizes the structure, and the conserved Cys and His residues binding the Zn²⁺ ion (Duan et al., 2007). The WRKY domain forms a unique wedge shape that inserts perpendicularly into the major groove of the DNA (Yamasaki et al., 2012) and primarily binds the W-box element through the RKYGQ motif on the second β -strand (Duan et al., 2007; Brand et al., 2013). After elucidation of the WRKY domain structure, the GLIAL CELL MISSING1, FLYWCH, and Mutator-like element transposases from metazoans were identified as having similar protein structures to WRKY TFs (Yamasaki et al., 2005; Babu et al., 2006). These findings suggest that the ancestral WRKY gene encoded a BED finger-type DNA-binding protein that evolved from a C₂H₂ zinc finger gene. Structural data also led to the identification of another large group of plant TFs, the NO APICAL MERISTEM, ATAF1, and CUP-SHAPED COTYLEDON2 (NAC) family, that are structurally related to WRKYs (Yamasaki et al., 2008).

Since 1994, WRKY TFs have been found to play roles in plant tolerance to a variety of abiotic stresses, including wounding, drought, salt, heat, cold, and osmotic pressure, topics that have been extensively reviewed recently (Chen et al., 2012; Bakshi and Oelmüller, 2014; Tripathi et al., 2014). Arabidopsis WRKY45 mediates the expression of a transporter needed for phosphate acquisition, providing insight into how WRKYs manage plant tolerance to soil abiotic stress (Wang et al., 2014). Additionally, WRKYs function in biotic stress tolerance to numerous pathogens (Zheng et al., 2006; Pandey and

* Address correspondence to lyuan3@uky.edu.
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Somssich, 2009; Ishihama and Yoshioka, 2012; Chen et al., 2013). WRKYs also have been found to be degraded by the 26S proteasome, divulging the role of proteolysis in regulating WRKY TF functions (Matsushita et al., 2012; Yu et al., 2013). The role of WRKYs in plant development, such as trichome and root hair formation, seed coat color, seed size, plant senescence, and male gametogenesis, is also well documented (Johnson et al., 2002; Robatzek and Somssich, 2002; Luo et al., 2005; Guan et al., 2014).

The involvement of WRKY TFs in numerous phytohormone signaling cascades involving abscisic acid, auxin, brassinosteroids, cytokinin, ethylene, jasmonate, and salicylic acid was recently reviewed (Rushton et al., 2012; Bakshi and Oelmüller, 2014). As salicylic acid and abscisic acid are intricately connected with plant defense and abiotic stress tolerance, respectively, much research has focused on these signaling cascades as regulators of WRKY factors (Yu et al., 2001; Rushton et al., 2012); yet, more recent efforts have focused on other phytohormones (Guo et al., 2014; Li et al., 2014; Schlüttenhofer et al., 2014). An Arabidopsis mitogen-activated protein kinase (MAPK) signaling cascade, through MITOGEN-ACTIVATED PROTEIN KINASE3 (MPK3)/MPK6 has been shown to regulate WRKY33 (Pecher et al., 2014), which activates the expression of two ethylene biosynthesis genes (Li et al., 2012). Arabidopsis WRKY57 is an important regulator balancing jasmonate and auxin signaling in leaf senescence by interacting with jasmonate zim-domain and auxin/indole-3-acetic acid proteins (Jiang et al., 2014).

THE PRESENT: WRKY TFs AS KEY REGULATORS OF SPECIALIZED METABOLISM

Plants are a rich source of specialized metabolites with antimicrobial and pharmaceutical properties (Fig. 1). However, several key plant-derived drugs are susceptible to shortages, drastically reducing the secure supply for patient treatment (Chabner, 2011). Therefore, developing methods to secure raw material supply and increase yield is essential. One approach is to use TFs to reengineer plants for increased metabolite production. The WRKY TF family is well known for the regulation of stress tolerance based on its association with numerous stress-related phenotypes and our understanding of the underlying mechanisms for stresses (Chen et al., 2012; Bakshi and Oelmüller, 2014; Tripathi et al., 2014). The relative ease of identifying mutant phenotypes in stress-response screens has been a key contributor to establishing the association of WRKYs with stress. In contrast, WRKY TFs have not commonly been considered key regulators of specialized metabolism because of (1) their known association with stress tolerance, (2) the emphasis on other characterized master regulators of specialized metabolism, such as basic helix-loop-helix (bHLH) and ethylene response

factors, (3) the difficulty of identifying metabolic mutant phenotypes, (4) the potential redundancy of multiple WRKY TFs regulating the same biosynthetic genes, and (5) a general lack of mechanistic understanding of the transcriptional regulation of specialized metabolism. Nevertheless, accumulating evidence suggests that certain WRKYs regulate the production of valuable natural products by regulating metabolite biosynthetic genes (Kato et al., 2007; Ma et al., 2009; Suttipanta et al., 2011). WRKYs regulating products from phenylpropanoids, alkaloids, and terpenes, the three major classes of plant metabolites, have been identified (Table I).

The phytohormone jasmonate is a key elicitor of specialized metabolism (Aerts et al., 1994; Singh et al., 1998; Qi et al., 2011; Lenka et al., 2012), primarily through the induction of key TFs, such as MYC2 and ethylene response factors, that regulate the biosynthetic genes (van der Fits and Memelink, 2000; Shoji et al., 2010; Shoji and Hashimoto, 2011; Zhang et al., 2011; Patra et al., 2013; Schweizer et al., 2013; Sears et al., 2014). Study of the grape (*Vitis vinifera*) WRKY family found that 80% of WRKY genes are jasmonate responsive (Guo et al., 2014). Recently, bioinformatics and comparative genomics approaches to identify jasmonate-inducible WRKY transcriptions in Arabidopsis and *Catharanthus roseus* uncovered the association of WRKY TFs with jasmonate in the production of specialized metabolites (Schlüttenhofer et al., 2014).

WRKYs Regulating Phenylpropanoid Pathways

WRKY TFs have been found to regulate the production of a variety of phenolic compounds, including lignin (Naoumkina et al., 2008; Guillaumie et al., 2010; Wang et al., 2010). As lignin is derived from the same phenylpropanoid pathway as other specialized metabolites, it is likely that WRKYs regulating lignin production or deposition may also directly or indirectly affect flux through the phenylpropanoid pathway, resulting in altered biosynthesis of other phenolic-based compounds (e.g. flavonoids, lignans, etc.; Besseau et al., 2007). This is supported by the observation that orthologs of WRKYs regulating lignin production also control the biosynthesis of other specialized metabolites (Table I; Supplemental Table S1). Four *Medicago truncatula* WRKY TFs, each individually overexpressed in tobacco (*Nicotiana tabacum*), demonstrate that multiple WRKY TFs can affect the accumulation of the same metabolites (Naoumkina et al., 2008). *M. truncatula* SECONDARY WALL THICKENING IN PITH (STP) and its Arabidopsis ortholog, WRKY12, illustrate that WRKY TFs involved in plant metabolism can have conserved roles across species (Wang et al., 2010). Studies on OsWRKY89 and VvWRKY2 indicate how modulating specialized metabolism regulation may also alter plant physiology and crop performance (Wang et al., 2007; Guillaumie et al., 2010).

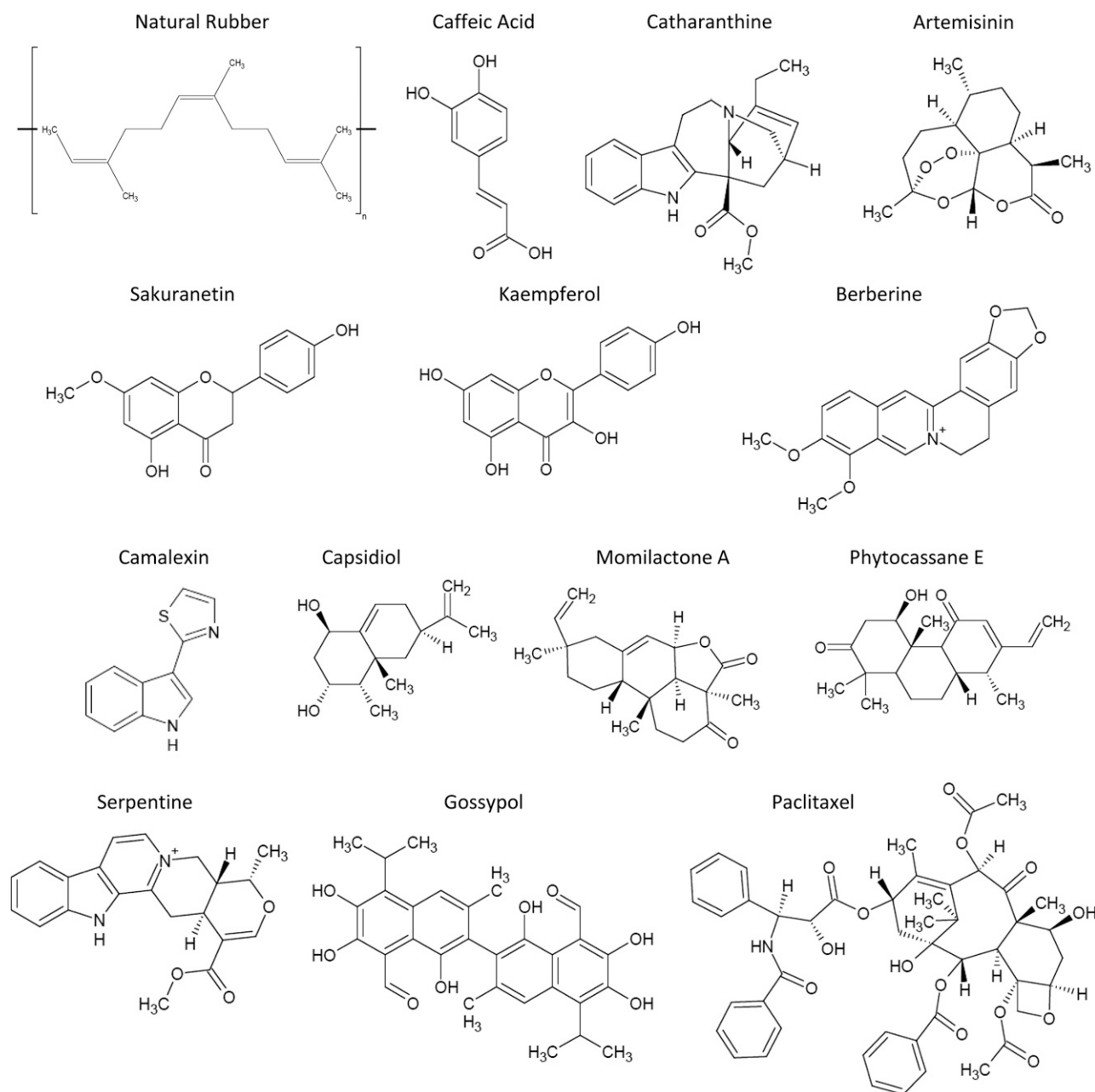


Figure 1. Phytochemicals synthesized by metabolic pathways regulated by WRKY TFs.

In addition to lignin, WRKYs control the production of flavonol and tannin compounds, also derived from the phenylpropanoid pathway. Flavonols, particularly kaempferol and quercetin, can function as polar auxin transport inhibitors (Brown et al., 2001; Peer et al., 2004), thus connecting specialized metabolism to phytohormone signaling. Arabidopsis WRKY23 regulates the production of flavonols in an auxin-inducible manner and illustrates how metabolites can have a negative feedback on phytohormone signaling (Grunewald et al., 2012). The first WRKY characterized in regulating specialized

metabolism was AtWRKY44, also called TRANSPARENT TESTA GLABRA2. AtWRKY44 regulates the production of proanthocyanins, a subset of tannin compounds, and pectinaceous seed mucilage (Johnson et al., 2002). The expression of AtWRKY44 is governed by a MYB-bHLH-WD40 TF complex that is a key regulator of anthocyanin production (Ishida et al., 2007), indicating the interconnections of WRKY TFs with other networks regulating specialized metabolism. Together, these findings indicate the incorporation of WRKY TFs into multiple TF and hormone signaling networks.

Table 1. A list of reported WRKY TFs regulating specialized metabolite production

–, Information not available.

Species	WRKY	GenBank No.	Metabolic Pathways Affected	Metabolite Class	Reference
<i>A. thaliana</i>	WRKY12	AF404857	Lignin	Phenylpropanoid	Wang et al. (2010)
<i>A. thaliana</i>	WRKY23	AY052647	Flavonol	Phenylpropanoid	Grunewald et al. (2012)
<i>A. thaliana</i>	WRKY33	AK226301	Camalexin	Indole alkaloid	Mao et al. (2011)
<i>A. thaliana</i>	WRKY44	NM_129282	Tannin, mucilage	Phenylpropanoids	Johnson et al. (2002)
<i>A. annua</i>	WRKY1	FJ390842, KC118517	Artemisinin	Sesquiterpene	Ma et al. (2009); Han et al. (2014)
<i>C. roseus</i>	WRKY1	HQ646368	Catharanthine, serpentine	TiAs	Suttipanta et al. (2011)
<i>C. japonica</i>	WRKY1	AB267401	Berberine	BIA	Kato et al. (2007)
<i>E. californica</i>	AtWRKY1	AF442389	Sanguinarine, chelirubine	BIAs	Apuya et al. (2008)
<i>G. arboreum</i>	WRKY1	AY507929	Gossypol	Sesquiterpene	Xu et al. (2004)
<i>H. brasiliensis</i>	WRKY41	GU372969	Rubber	Polyterpene	Zhang et al. (2012)
<i>H. brasiliensis</i>	WRKY1	JF742559	Rubber	Polyterpene	Wang et al. (2013b)
<i>H. brasiliensis</i>	WRKY33	AJJZ010966566	Rubber	Polyterpene	Li et al. (2014)
<i>H. brasiliensis</i>	WRKY14	AJJZ010236935	Rubber	Polyterpene	Li et al. (2014)
<i>H. brasiliensis</i>	WRKY55	AJJZ010198483	Rubber	Polyterpene	Li et al. (2014)
<i>M. truncatula</i>	WRKY100577	EU526033	Rutin, kaempferol, caffeic acid, lignin	Phenylpropanoids	Naoumkina et al. (2008)
<i>M. truncatula</i>	WRKY100630	EU526034	Rutin, kaempferol, caffeic acid, lignin	Phenylpropanoids	Naoumkina et al. (2008)
<i>M. truncatula</i>	WRKY108715	EU526035	Rutin, kaempferol, caffeic acid, lignin	Phenylpropanoids	Naoumkina et al. (2008)
<i>M. truncatula</i>	WRKY109669	EU526036	Rutin, kaempferol, caffeic acid, lignin	Phenylpropanoids	Naoumkina et al. (2008)
<i>M. truncatula</i>	STP	HM622066	Lignin	Phenylpropanoid	Wang et al. (2010)
<i>N. benthamiana</i>	WRKY8	AB445392	Capsidiol	Sesquiterpene	Ishihama et al. (2011)
<i>O. sativa</i>	WRKY13	EF143611	Momilactone A	Diterpene	Qiu et al. (2008a)
<i>O. sativa</i>	WRKY45	AK066255	Momilactone A, oryzalexin, phytocassane	Diterpenes	Akagi et al. (2014)
<i>O. sativa</i>	WRKY53	AB190436	Momilactone A	Diterpene	Chujo et al. (2014)
<i>O. sativa</i>	WRKY76	AK068337	Momilactone A, phytocassane, sakuranetin	Diterpenes and phenylpropanoid	Yokotani et al. (2013)
<i>O. sativa</i>	WRKY89	AY781112	Lignin	Phenylpropanoid	Wang et al. (2007)
<i>P. quinquefolius</i>	WRKY1	JF508376	–	Triterpene	Sun et al. (2013)
<i>P. somniferum</i>	WRKY1	JQ775582	–	BIA	Mishra et al. (2013)
<i>S. lycopersicum</i>	WRKY73	NM_001247873	–	Monoterpenes	Spyropoulou et al. (2014)
<i>T. chinensis</i>	WRKY1	JQ250831	Paclitaxel	Diterpene	Li et al. (2013)
<i>V. vinifera</i>	WRKY2	AY596466	Lignin	Phenylpropanoid	Guillaumie et al. (2010)

WRKYs Regulating Alkaloid Pathways

WRKYs have emerged as key regulators of alkaloid metabolism. Analysis of *Catharanthus* spp. terpene indole alkaloid (TIA) biosynthetic gene promoters revealed that W-boxes are a commonly present cis-element, suggesting that WRKY TFs are important in regulating alkaloid production (Suttipanta et al., 2011). At least 25% of WRKY TFs in *Catharanthus* spp. are jasmonate response factors and could potentially regulate TIA biosynthesis (Schlutenhofer et al., 2014). CrWRKY1 from *C. roseus* directly regulates the expression of TRYPTOPHAN DECARBOXYLASE, the enzyme synthesizing the indolic tryptamine precursor (Suttipanta et al., 2011). Additionally, metabolites from two branches of the TIA pathway, catharanthine and serpentine, accumulate differently in CrWRKY1 RNA interference lines of hairy root cultures, indicating that CrWRKY1 regulates genes governing metabolite flux within the pathway. Analysis of the CrWRKY1 promoter reveals cis-elements for bHLH, DNA binding

with one finger, MYB, and TGACG sequence-specific binding protein TFs, suggesting that additional trans-factors with greater pathway hierarchy may regulate this gene (Yang et al., 2013).

Similar to TIA, benzylisoquinoline alkaloids (BIAs) also are regulated by WRKY TFs. *Coptis japonica* WRKY1 dictates the expression of nine genes involved in berberine biosynthesis but does not appear to have an effect on primary metabolism (Kato et al., 2007). Importantly, this finding illustrates that WRKY TFs are capable of up-regulating entire specialized metabolic pathways. More recently, a WRKY from opium poppy (*Papaver somniferum*) was found to bind to the TYROSINE DECARBOXYLASE promoter and possibly to regulate BIA production (Mishra et al., 2013). That study also found that W-boxes are present in the seven available BIA gene promoters. Heterologous expression of Arabidopsis AtWRKY1 in California poppy (*Eschscholzia californica*) affects the accumulation of sanguinarine and chelirubine (Apuya et al., 2008).

Therefore, one way to engineer a plant, which synthesizes valuable compounds but is hindered by the limited availability of pathway information, is to express WRKY TF genes with known roles in governing specialized metabolism.

Camalexin is a primary defense metabolite in Arabidopsis and is governed by a set of MAPK cascades through MPK3/MPK6 and MPK4. MPK3/MPK6 directly phosphorylates WRKY33, which proceeds to activate *PHYTOALEXIN DEFICIENT3*, a key gene in camalexin biosynthesis (Ren et al., 2008; Mao et al., 2011). In the MPK4-WRKY33 cascade, MPK4 interacts with VQ MOTIF-CONTAINING PROTEIN21, and phosphorylation of the VQ protein destabilizes the complex, releasing WRKY33 (Qiu et al., 2008b; Petersen et al., 2010). These results emphasize the role of phosphorylation and protein complexes in regulating WRKY TF activity. VQ proteins, which interact with the WRKY domain, thus inhibiting TF-DNA binding, appear to be key regulators of group I and group IIc WRKYs activities (Petersen et al., 2010; Cheng et al., 2012; Chi et al., 2013) and also are likely to be important regulators of specialized metabolism. In Arabidopsis, MPK3 and MPK6 are important in regulating VQ-WRKY protein interactions (Lai et al., 2011; Pecher et al., 2014). In *Catharanthus* spp., phosphorylation regulates TIA biosynthesis, part of which occurs through the MAPK CrMPK3, an ortholog of Arabidopsis MPK3 (Raina et al., 2012). The work on CrMPK3 suggests that additional WRKY TFs, and probably VQ proteins, are involved in regulation of the TIA pathway.

WRKYs Regulating Terpene Pathways

While considerably less is known about the transcriptional regulatory networks governing terpene synthesis (Patra et al., 2013), the majority of regulators identified are WRKY TFs. Other than in Arabidopsis, early work established the role of WRKY TFs in the regulation of specialized metabolism in cotton (*Gossypium arboreum*; Xu et al., 2004). GaWRKY1 regulates a sesquiterpene cyclase at a pathway branch point leading to the production of gossypol, an antifeedant phytoalexin. Recently, the *Gossypium raimondii* WRKY family was characterized, which identified seven WRKY TFs primarily expressed in fiber trichomes (Cai et al., 2014). In plants, trichomes are a major site of specialized metabolite production. In tomato (*Solanum lycopersicum*), SlWRKY73 was found to activate the expression of three monoterpene synthase genes, suggesting that a single WRKY can regulate multiple distinct biosynthetic pathways (Spyropoulou et al., 2014). In *Artemisia annua*, biosynthesis of the antimalarial drug artemisinin, also produced in trichomes, is regulated by AaWRKY1 (Ma et al., 2009). Recently, the yield of artemisinin in a high-yielding cultivar of *A. annua* was doubled by overexpressing AaWRKY1 (Han et al., 2014). That work provides compelling evidence that WRKYs can be used successfully to engineer metabolic pathways. Interestingly, in spite of

increased artemisinin yield, only one pathway gene was significantly up-regulated in the AaWRKY1 overexpression lines, demonstrating the dramatic outcomes of overcoming a bottleneck in the pathway. Similarly, TcWRKY1 from *Taxus chinensis* fine-tunes the expression of a rate-limiting gene in the pathway for production of the anticancer drug paclitaxel, commonly called taxol (Li et al., 2013). Together, the studies on AaWRKY1 and TcWRKY1 suggest that WRKY TFs can control critical rate-limiting steps in metabolic pathways.

Rice (*Oryza sativa*) produces terpenes for defense to pathogens and herbivores. OsWRKY45 is a positive regulator of momilactone, phytocassane, and oryzalexin accumulation by priming biosynthetic gene expression (Akagi et al., 2014). Similarly, rice OsWRKY53 enhances the production of momilactones (Chujo et al., 2014). Overexpression of OsWRKY13 up-regulates genes in the phenylpropanoid pathway while down-regulating those for terpene biosynthesis, illustrating the potential of WRKY TFs to differentially modulate discrete classes of metabolites (Qiu et al., 2008a). Both OsWRKY53 and *Nicotiana benthamiana* WRKY8 (Ishihama et al., 2011) are phosphorylated by the MPK3/MPK6 cascade. Rice OsWRKY76 activates cold stress tolerance but represses terpene synthesis (Yokotani et al., 2013). OsWRKY76 also suppresses production of the phenylpropanoid sakuranetin. As these four WRKYs are responsive to the pathogen *Magnaporthe oryzae*, these findings suggest the existence of a complex, interconnected signaling network governing within an individual specialized metabolite pathway.

A major engineering problem with plants synthesizing valuable natural products is the long life cycle and resilience to stable transformations. In such species, the correlation of gene expression with metabolite accumulation provides an opportunity to identify important components of the metabolic pathway. The expression of two WRKY TFs has been associated with increased biosynthesis of natural rubber, a polyisoprenoid derived from wounding the bark of the tropical tree *Hevea brasiliensis* (Zhang et al., 2012; Wang et al., 2013b). Recent characterization of the *H. brasiliensis* WRKY family identified three WRKYs regulating the expression of the SMALL RUBBER PARTICLE PROTEIN, which binds rubber for storage (Li et al., 2014). An American ginseng (*Panax quinquefolius*) WRKY TF, PqWRKY1, is associated with increased production of ginsenosides, a group of triterpene compounds (Sun et al., 2013). Heterologous expression of WRKY genes in fast-growing model systems can aid functional characterization. Ectopic expression of PqWRKY1 in Arabidopsis up-regulates triterpene biosynthetic genes, suggesting that WRKYs are capable of regulating metabolic pathways in other species. Collectively, these studies suggest that expression-metabolite association and heterologous gene expression techniques are useful methods to identify WRKY TFs regulating specialized metabolism in long-lived plant species.

Orthologs of WRKYs in Specialized Metabolism

Predicted orthologs of several WRKY TFs have been demonstrated to regulate specialized metabolism in multiple plant species (Supplemental Tables S1 and S2). Three WRKYs regulating phenylpropanoids are orthologs of HbWRKY41, HbWRKY33 and TcWRKY1, regulating terpene biosynthesis, have an ortholog that regulates lignin biosynthesis. While AtWRKY1 regulates alkaloid accumulation in California poppy, its ortholog, HbWRKY1, affects rubber accumulation. CrWRKY1 and OsWRKY45 are orthologous proteins regulating TIA and diterpenes in *C. roseus* and rice, respectively. GaWRKY1 and OsWRKY76 are predicted orthologs but display an antagonistic function in regulating terpene biosynthesis. AaWRKY1 and WRKY109669 are predicted orthologs affecting artemisinin (Ma et al., 2009) and lignin biosynthesis (Naoumkina et al., 2008), respectively. Importantly, six WRKY TFs predicted as orthologs (AtWRKY33, NbWRKY8, OsWRKY53, PsWRKY1, VvWRKY2, and WRKY108715) have independently been found to regulate specialized metabolism (Table I; Supplemental Table S1). Duplicate WRKYs of this group function to regulate the biosynthesis of alkaloids, terpenes, and phenylpropanoids (Naoumkina et al., 2008; Guillaumie et al., 2010; Ishihama et al., 2011; Mao et al., 2011; Mishra et al., 2013; Chujo et al., 2014). Therefore, orthologs of the MPK3/MPK6-AtWRKY33 signaling cascade may be key factors in the regulation of specialized metabolism. Overall, these results reinforce the idea of conserved functions among at least some WRKY TFs. However, other WRKYs involved in specialized metabolism possibly possess species-specific roles (Table I). Collectively, these findings suggest that a core set of WRKYs may be highly conserved in the regulation of specialized metabolism, whereas others may play more restricted roles.

The identification of WRKY TFs regulating specialized metabolism in more species is essential to determine WRKYs with conserved or unique functions. Genomes of several species known for producing valuable compounds have recently been sequenced (Shulaev et al., 2011; Krishnan et al., 2012; Wang et al., 2012; Nystedt et al., 2013; Denoëud et al., 2014; Myburg et al., 2014). The tropical tree neem (*Azadirachta indica*) produces an array of metabolites with antibiotic, anticancer, and antifeedant properties (Atawodi and Atawodi, 2009) that have applications in pest control (Gahukar, 2014). Flax (*Linum usitatissimum*) produces podophyllotoxins, precursors used to synthesize the valuable anticancer compounds etoposide and teniposide (Schmidt et al., 2012). Coffee (*Coffea canephora* and *Coffea arabica*) synthesizes phenylpropanoids and alkaloids that impact product quality (Franca et al., 2005; Denoëud et al., 2014). Several coffee WRKY TFs have already been identified (Ramiro et al., 2010). Strawberry (*Fragaria vesca*) is a rich source of anthocyanins and ellagitannins. Eucalyptus (*Eucalyptus grandis*) and Norway spruce (*Picea abies*) are desirable

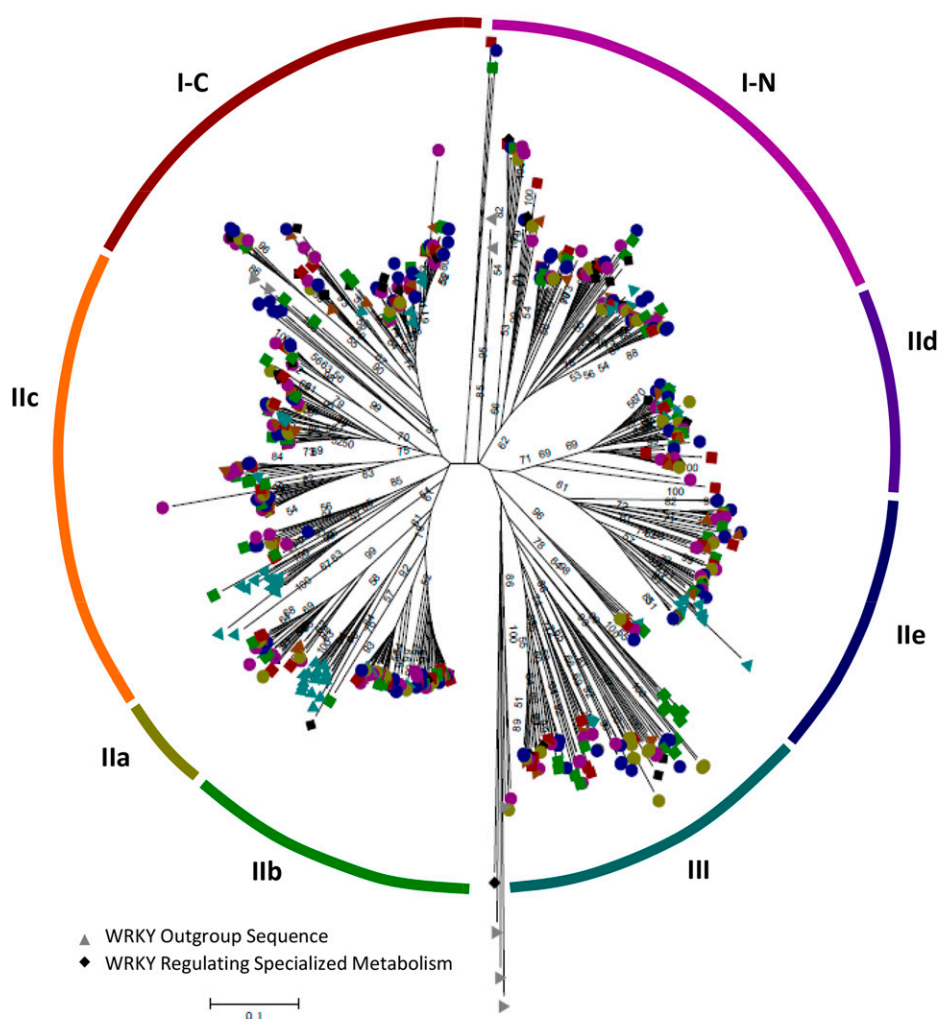
timber trees that produce terpene-rich oils with economic importance to the pharmaceutical and aromatherapy industries. We identified a number of WRKY orthologs in these plant species in order to elucidate their roles in regulating specialized metabolism (Supplemental Tables S1 and S2). There are 69, 49, 76, 54, 101, and 60 WRKY TFs in neem, coffee, eucalyptus, strawberry, flax, and spruce, respectively (Fig. 2). Characterization of these orthologs and those from other reported families (Supplemental Table S3) will strengthen our ability to improve natural products from these crops while enhancing our knowledge of WRKY TF function.

Biological Relevance of WRKY-Regulated Specialized Metabolites

WRKY TFs regulate a diverse array of plant-specialized metabolites that pertain to a broad assortment of biological functions. Biosynthesis of the metabolites listed in Table I is associated with WRKY TFs. In Arabidopsis, camalexin has antifungal properties and aids defense against pathogens including *Botrytis cinerea*, *Alternaria brassicicola*, and *Sclerotinia sclerotiorum* (Thomma et al., 1999; Ferrari et al., 2007; Stotz et al., 2011). Berberine, catharanthine, gossypol, momilactones, phytocassanes, and sanguinarine also have antimicrobial properties and confer tolerance to plant pathogens (Stermitz et al., 2000; Puckhaber et al., 2002; Umemura et al., 2003; Fukuta et al., 2007; Roepke et al., 2010; Obiang-Obounou et al., 2011). In addition to the involvement in auxin signaling (Brown et al., 2001; Buer and Muday, 2004), flavonols are protectants against UV light stress (Li et al., 1993; Bieza and Lois, 2001; Emiliani et al., 2013) and herbivores (Wuyts et al., 2006a; Onkokesung et al., 2014). AtWRKY23 functions in regulating flavonol production (Grunewald et al., 2012) and nematode resistance (Grunewald et al., 2008). Gossypol, another antifeedant compound, deters herbivores, including cotton bollworm (*Heliothis armigera*) and tobacco budworm (*Heliothis virescens*; Stipanovic et al., 2008; Kong et al., 2010). OsWRKY13, OsWRKY45, OsWRKY53, and OsWRKY76 regulate the synthesis of the allelopathic diterpene momilactone (Fukuta et al., 2007; Kato-Noguchi et al., 2008). The biopolymer lignin, critical for plant rigidity, also deters herbivory (Wuyts et al., 2006b) and defends against microbes (Xu et al., 2011). Collectively, specialized metabolites regulated by WRKY TFs contribute to overall plant fitness by enhancing tolerance to various abiotic and biotic stresses.

THE FUTURE: WHERE TO GO FROM HERE?

Significant progress has been made in understanding the role of WRKY TFs over the last two decades; however, much more waits to be explored. Clearly, the regulation of specialized metabolism by WRKY TFs is



▲ WRKY Outgroup Sequence
◆ WRKY Regulating Specialized Metabolism

0.1

Genus species	WRKY Transcription Factor Group								Total
	I	IIa	IIb	IIc	IId	IIe	III		
■ <i>Arabidopsis thaliana</i>	14	3	8	18	7	8	14	72	
■ <i>Azadirachta indica</i>	13	5	13	16	9	7	6	69	
▲ <i>Coffea canephora</i>	10	3	6	15	4	6	5	49	
● <i>Eucalyptus grandis</i>	17	6	10	19	5	8	11	76	
● <i>Fragaria vesca</i>	11	3	8	10	5	5	12	54	
● <i>Linum usitatissimum</i>	30	4	11	18	11	12	15	101	
▲ <i>Picea abies</i>	5	0	19	19	6	9	2	60	

Figure 2. Phylogenetic analysis of *Arabidopsis* (green squares), coffee (brown triangles), eucalyptus (purple circles), flax (blue circles), neem (red squares), spruce (teal triangles), and strawberry (gold circles) WRKY families alongside those regulating specialized metabolism in other species (top) and the number of TFs present in each group (bottom). Human GCMA and FLYWCH plus *G. lamblia*, *D. discoideum*, and *C. reinhardtii* WRKYs were used to form an outgroup to root the phylogenetic tree (gray triangles). WRKY TFs regulating specialized metabolites are denoted with black diamonds. The neighbor-joining tree was constructed using the proportion of amino acids differing between sequences (i.e. the p-distance method) and 1,000 bootstrap replications using MEGA 6.0. Sequence alignment was performed using the ClustalW algorithm. The phylogenetic tree has been deposited into TreeBase under identification number 16440.

highly integrated into plant defense. Understanding WRKY regulation will unravel the little-known but highly intricate regulatory network that governs plant health and metabolic diversity. In the future, we envision several major areas of research that will contribute significantly to understanding WRKY involvement in specialized metabolism.

(1) No study has specifically focused on determining the number of TFs involved in specialized metabolism. Evidence from the more thoroughly characterized R2R3-MYB and bHLH families suggests that approximately 23% and 12% of the subgroups, respectively,

regulate specialized metabolism (Feller et al., 2011). By contrast, we determined up to 43% of the WRKY ortholog clusters involved in the biosynthesis of specialized metabolites (Supplemental Table S1). Future efforts should focus on determining how many different WRKY TFs within a given species regulate specialized metabolite biosynthesis, although this could be complicated by the type and size of the pathway of interest. Many plants, such as *Catharanthus* spp., possess complex specialized metabolic pathways that produce structurally similar compounds. In such plants, it will be interesting to explore whether

expressing WRKY genes from foreign species can differentially modulate biosynthetic genes, leading to the synthesis of novel specialized metabolites with pharmaceutical potential. Identification of all the WRKYs involved in specialized metabolism is essential to unraveling the interwoven networks connecting the syntheses of natural products.

(2) Understanding how the numerous WRKYs of a family differentially regulate unique sets of target genes has long been a major enigma and is just now starting to be deciphered. Previously, only the C-terminal domain of group I WRKY TFs was known to bind DNA; however, recently, this function was extended to the N-terminal WRKY domain (Brand et al., 2013). How dual WRKY domains affect DNA binding and what role each plays in gene regulation necessitate further study. Furthermore, over the last several years, WRKYs have been found to recognize less stringent cis-elements than were suspected previously (Brand et al., 2013). WRKY interactions with VQ proteins, calmodulin, histone deacetylases, and phosphorylation (Kim et al., 2008; Mao et al., 2011; Chi et al., 2013) indicate posttranslational modifications in regulating WRKY TF activity. Many other proteins are anticipated to be involved in the interactions and regulation of WRKY TFs. Identification of these factors and their functions will help further elucidate where WRKYs function in the grand signaling hierarchy of plant-specialized metabolism.

(3) In the future, the ability to use phylogenetic information to predict the functions of WRKYs, especially those in nonmodel plants, and to identify those with novel regulatory roles will be beneficial to crop genetic manipulation for the production of specialized metabolites. With ease of access to sequenced plant genomes, WRKY families have been identified in many plants (Supplemental Table S3). To date, studies involving the complementation of WRKY function between species have been mainly restricted to model plants. The conserved function of WRKY genes and the activation of similar target genes between species are known (Proietti et al., 2011) but only beginning to be understood. Similarly, although functional redundancy is well known in the WRKY family (Jiang and Deyholos, 2009), predicting which TFs perform overlapping roles is still primarily conjecture. Identifying potentially functionally redundant WRKYs may be improved by utilizing programs that categorize groups of orthologous sequences (e.g. OrthoMCL). Understanding and accurately predicting each WRKY's function will aid the identification of orthologous factors that will improve the engineering of less characterized medicinal species.

(4) Many key features of the evolution of the WRKY family still need to be resolved. For example, why do certain species undergo increased expansion of one specific subgroup compared with other WRKY groups? While WRKY TFs have been shown to arise from tandem and segmental gene duplication events in rice (Wu et al., 2005), differential expression, not

sequence divergence, probably led to most WRKY family diversification (Babu et al., 2006). WRKYs have been proposed to play a role in preventing stress as plants emerged from aquatic environments (Wen et al., 2014), but more studies are hastily needed to determine which WRKY members were involved and what functions they possess. Equally important is to identify which core set of these genes were present in ancestral plants and the function of WRKYs in algae. The sole group I WRKY identified from *C. reinhardtii* is orthologous to Arabidopsis WRKY33, which functions in diverse hormone, stress, and metabolic pathways (Zheng et al., 2006; Jiang and Deyholos, 2009; Li et al., 2011, 2012; Birkenbihl et al., 2012; Logemann et al., 2013; Wang et al., 2013a; Schlutterhofer et al., 2014). *C. reinhardtii* WRKY1, therefore, may be an early WRKY mediating cross-talk between pathways in the alga. Further supporting the hypothesis that WRKYs played an ancient role in modulating metabolism, a group I-like WRKY from *G. lamblia* functions downstream of a MAPK cascade to regulate cyst cell wall formation (Pan et al., 2009). Understanding WRKY family evolution will reveal the extent, when, and how WRKYs specifically contributed to the rise of plant-specialized metabolism.

(5) While the number of WRKY TFs known to regulate specialized metabolism has increased appreciably over the past several years, much remains to be learned about how they function with other TFs in regulatory networks. Currently, minimal information is available on where WRKYs fall in the regulatory hierarchy in specialized metabolism. CrWRKY1 regulates the expression of several key TFs involved in TIA biosynthesis (Suttipanta et al., 2011). Additionally, AtWRKY33, NbWRKY8, and OsWRKY45 are targets of MAPK cascades (Andreasson et al., 2005; Qiu et al., 2008b; Mao et al., 2011; Ueno et al., 2013), further suggesting that some WRKYs may function as top-level regulators of specialized metabolism. AtWRKY23 functions downstream of auxin-response factors to synthesize flavonols that are induced to abate auxin signaling (Grunewald et al., 2012). Furthermore, OsWRKY13 regulates over 500 genes, including 39 TFs, and binds the promoters of other WRKY TFs (Qiu et al., 2009). While exceptions probably exist, WRKY TFs, thus far, appear to be key top-tier factors directly regulating the biosynthesis of specialized metabolism.

CONCLUSION

The year 2014 marks two decades since the discovery of the first WRKY, a major milestone in TF research. Over the last score of years, various research groups have identified many WRKY TF families (Supplemental Table S3), and we have learned a considerable amount about how WRKYs function. In the past, most studies have been focused on WRKY TFs in plant defense against abiotic and biotic stresses; however, a neglected role of this family is in regulating

specialized metabolism. Future work will entail defining which WRKYs do what task as well as elucidating the mechanisms that govern each TF, which will likely vary between species. As WRKY TFs are essential to plant-specialized metabolism, understanding their functions will provide ways to successfully reengineer medicinal species to provide continuous and higher yielding materials for drug synthesis. Elucidating how WRKYs regulate metabolism thus is critical not only for improving crop stress tolerance but also for increasing the production of valuable plant-derived natural products.

Supplemental Data

The following supplemental materials are available.

Supplemental Table S1. Orthologs of WRKY TFs involved in specialized metabolism.

Supplemental Table S2. WRKY TF sequences from species producing valuable metabolites.

Supplemental Table S3. List of reported WRKY TF families.

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