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AaMYB1, and its orthologue AtMYB61, affect terpene metabolism and trichome development in *Artemisia annua* and *Arabidopsis thaliana*

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SUMMARY

The effective anti-malarial drug artemisinin (AN) isolated from *Artemisia annua* is relatively expensive due to the low content in the plant as it is only synthesized within the glandular trichomes. Therefore, genetic engineering of *A. annua* is one of the most promising approaches to improve AN yield. In this work, *AaMYB1* transcription factor has been identified and characterized. When *AaMYB1* is overexpressed in *A. annua*, either exclusively in trichomes or in the whole plant, essential AN biosynthetic genes are also overexpressed and consequently AN amount significantly increased. *Artemisia AaMYB1* constitutively overexpressing plants displayed a higher number of trichomes. In order to study this role on trichome development and others possibly connected biological processes, *AaMYB1* was overexpressed in *Arabidopsis thaliana*. For supporting our findings in *A. thaliana*, *AaMYB1* orthologue from this model plant *AtMYB61* was identified and *atmyb61* mutants characterized. Both *AaMYB1* and *AtMYB61* affected trichome initiation, root development and stomatal aperture in *A. thaliana*. Molecular analyses indicated that two crucial trichome activator genes are misexpressed in *atmyb61* mutant plants and in plants overexpressing *AaMYB1*. Furthermore, *AaMYB1* and *AtMYB61* are also essential for gibberellin (GA) biosynthesis and degradation in both species by positively affecting the expression of the enzymes that convert GA₉ into the bioactive GA₄ as well as the enzymes involved in the degradation of GA₄. Overall, these results identify *AaMYB1/AtMYB61* as a key component of the molecular network that connect important biosynthetic processes, and reveal its potential value for AN production through genetic engineering.

INTRODUCTION

Artemisinin based combinatory therapies (ACTs) are today the most widely used treatment of uncomplicated malaria (WHO, 2015). The number of ACT courses delivered in 2013 reached 337 million (WHO, 2015). Recently, AN derivatives have also been used for treatment of various cancers, autoimmune diseases and viral infections (Efferth et al., 2002; Chaturvedi et al., 2010; Graham et al., 2010; Crespo-Ortiz et al., 2012). Despite great progress in semisynthetic AN microbes (Zeng et al., 2008; Paddon and Keasling, 2014), A.

annua is still the main resource for AN. However, the plant extraction based AN industry (WHO, 2015) is hindered by the fact that the content of AN in *A. annua* is extremely low (0.1-10 mg/g dry weight), which makes the price still too high for a higher number of malarial patients (Abdin et al., 2003; Zhang et al., 2008). Therefore, it is critical to improve the AN yield *in planta* (Pulice et al., 2016); but for reaching that, it is first necessary to understand the biosynthesis and its regulation.

Many studies have been carried out to investigate the AN biosynthetic pathway. Two molecules of isopentenyl diphosphate (IDP) and one molecule of dimethylallyl diphosphate (DMADP) are condensed by farnesyl diphosphate synthase (FDS) to farnesyl diphosphate (FDP). FDP is subsequently cyclized into amorpha-4,11-diene by amorpha-4,11-diene synthase (ADS), which is the first unique intermediate of artemisinin biosynthesis (Bouwmeester et al., 1999; Mercke et al., 2000). Amorpha-4,11-diene is then oxidized by amorpha-4,11-diene 12-hydroxylase (CYP71AV1), reduced by artemisinic aldehyde $\Delta^{11}(13)$ reductase (DBR2) to dihydroartemisinic aldehyde and again oxidized by aldehyde dehydrogenase 1 (ALDH1) into the direct precursor of AN, dihydroartemisinic acid (DHAA) (Teoh et al., 2006; Zhang et al., 2008; Teoh et al., 2009). The final production step is considered to be a non-enzymatic reaction (Brown, 2010; Covello et al., 2008). However, recent results have proposed that a peroxidase enzyme or an alternative series of oxidations, occurring exclusively *in planta*, may in fact catalyse the crucial last reaction that converts the precursor into the valuable AN molecule (Bryant et al., 2015).

Despite AN biosynthetic pathway is relatively well elucidated; only a few studies have been carried out recently regarding the genetic regulation of the pathway. These include studies on AaWRKY1 (Ma et al., 2009; Han et al., 2014), AabHLH (Ji et al., 2014), AabZIP1 (Zhang et al., 2015) and different AaAP2/ERF transcription factors (TFs) such as AaERF1, AaERF2, AaORA and AaTAR1 (Yu et al., 2011; Lu et al., 2013; Tan et al., 2015), which all have been shown to affect the transcription of AN biosynthetic genes. However, other TFs families might also be essential for this important pathway. Among them, one of the major families involved in plant secondary metabolism is MYB family. MYB superfamily has selectively expanded during evolution, particularly R2R3-MYB subfamily, which main characteristic is that it has two DNA-binding MYB domain repeats (Dubos et al., 2010). Different R2R3-MYB members play key roles in several secondary metabolism processes in different plant species (Martin and Paz-Ares, 1997; Dubos et al., 2010); among them are the flavonols, anthocyanin or proanthocyanins biosynthesis (Lepiniec et al., 2006; Stracke et al., 2001;

Gonzalez et al., 2008; Gonzalez et al., 2009). Interestingly, diverse putative MYB binding-motifs have been found in most of the regulatory regions of AN biosynthetic genes (Table S1). Therefore, it could be hypothesized that R2R3-MYB members may also interfere in the AN metabolism regulation that occurs exclusively in the glandular secretory trichomes (GSTs) of *A. annua*.

Plant trichomes are specialized defensive epidermal protrusions on the surfaces of leaves and other aerial organs of many plants. Trichomes are present in most of the vascular plant surfaces, and their main role is to defend plants against insect herbivores, virus, UV light and/or excessive water loss (Traw and Bergelson, 2003). A very important characteristic of plant trichomes, especially in GSTs, is that they are able to synthesize and store large amounts of unique metabolites (Schilmiller et al., 2008), as AN in *A. annua*. *A. thaliana* is the model plant that has been used for decades to address crucial questions regarding trichome development (Hülkamp, 2004; Traw and Bergelson, 2003; Gilding and Marks, 2010). *A. thaliana* leaf non-glandular trichomes are epidermal unicellular structures with a stalk and three branches (Marks et al., 2009). Interestingly, R2R3-MYB members, which includes GLABROUS1 (GL1/MYB0), MYB23 and MYB66, as well as some R3-MYB as CAPRICE (CPC) and TRYPTYCHON (TRY) have been described to be involved in the positive and negative control of trichome morphogenesis, respectively (Schellmann et al., 2002; Kirik et al., 2004; Kang et al., 2009).

A better understanding of the molecular mechanisms that control the biosynthesis of special metabolites in trichomes will be important for future commercial biopharming applications (Webster et al., 2004; Murphy, 2007; Ahmad et al., 2012; Pulice et al., 2016). In our study, we have identified *AaMYB1*, a R2R3-MYB gene isolated from a trichome specific EST library from *A. annua* plants and its orthologue *AtMYB61* in *A. thaliana*. Our results show that *AaMYB1* positively affect not only AN biosynthesis but also affect other connected processes such as GA biosynthesis and trichome initiation.

RESULTS AND DISCUSSION

AaMYB1 gene isolation from *Artemisia annua*

So far, 625 putative TFs have been identified in *A. annua* and classified in 49 families as listed in the Plant TFs Database (<http://planttfdb.cbi.pku.edu.cn/index.php?sp=Aan>), 39 of those belong to R2R3-MYB family. In order to further identify any TF transcription factor responsible for AN biosynthesis regulation, we used an *A. annua* trichome specific EST

library. This library contains EST sequences found in *A. annua* GSTs, where AN biosynthesis exclusively takes place (Olofsson et al., 2012). According to this library, one gene encoding two MYB domain repeats was found to be expressed in GSTs and named as *AaMYB1*.

AaMYB1 was first cloned by using primers based on conserved nucleotide sequences of putative MYB genes. After 3'- and 5'-RACE-PCR an open reading frame of 993bp encoding a protein of 330 amino acids was obtained. To understand its potential function, *AaMYB1* expression pattern was first analyzed using quantitative real-time PCR in *A. annua* main tissues: root, stem, juvenile leaf, adult leaf and flower bud (Figure 1A). AN content in *A. annua* differs within tissues and a number of studies suggest that it keeps increasing at the vegetative growth stage and becomes highest in leave bracts just before entering into the flowering stage (Liersch et al., 1986; Ferreira et al., 1995). Analysis of different wild-type *A. annua* tissues reveals relative high AN content in leaves just during blooming stage and in flower buds (Ferreira et al., 1995). Interestingly, *AaMYB1* expression was found to start increasing when plant leaves reached adult stage, and keep increasing until flower buds arose (Figure 1A). This expression pattern was similar to the expression profile of important AN biosynthesis enzymes such as *ADS* and *CYP71AV1* as well as some TFs reported to activate AN biosynthesis (Yu et al., 2011; Zhang et al., 2015). Surprisingly, high *AaMYB1* expression was also found in roots (Figure 1A). Neither roots nor main stems contain any significant amount of AN (Woerdenbag et al., 1991; Ferreira et al., 1995). However, roots produce trichome-like structures (hairs) that have been used as *in vitro* bioreactor for AN production in different plant species (Souret et al., 2003; Patra & Srivastava 2015).

***AaMYB1* controls the expression levels of the artemisinin biosynthesis pathway genes**

In *A. annua* leaves, flower buds and flowers, AN as well as some other sesquiterpenes are synthesized and stored exclusively in the GSTs of those organs (Duke et al., 1994). Unlike trichomes of *A. thaliana* that are not glandular structures, GSTs from *A. annua* are formed by ten cells in five pairs (Ferreira et al., 1995). The entire main AN biosynthesis enzymes, including *CYP71AV1*, are almost exclusively expressed in the GSTs (Olsson et al., 2009). Indeed, GUS assays showed that the 1,148 bp *CYP71AV1* promoter region was exclusively active in the GSTs of leaves and flower buds but not in other tissues/cells of the plant, including the T-shaped trichomes (TST) (Wang et al., 2013). These results obtained by Wang et al., 2013, led us to also use the *CYP71AV1* promoter as a proper tool for overexpressing *AaMYB1* specifically in GSTs.

Diverse independent *pCYP71AV1::AaMYB1* and *35S::AaMYB1* transgenic *A. annua* lines were generated and selected in order to characterize the possible involvement of AaMYB1 in AN production in comparison to control wild-type plants. The expression level of AaMYB1 of these transgenic lines in comparison with *Anamed* wild-type plants was at least 50- and 200-fold higher in *pCYP71AV1::AaMYB1* and *35S::AaMYB1* plants, respectively (Figure 1B). Once confirmed the overexpression of *AaMYB1*, the relative expression ratio for the genes encoding AN biosynthesis enzymes in three independent *pCYP71AV1::AaMYB1*, three independent *35S::AaMYB1* transgenic lines and wild-type plants were determined. Indeed, the genes of the AN biosynthetic pathway were clearly up-regulated in all these overexpressing *AaMYB1* lines (Figure 1C-G). The *CYP71AV1* and *ADS* transcript levels were highly increased in all these transgenic plants, while *FDS*, *DBR2* and *ALDH1* genes were modestly up-regulated in comparison to control wild-type plants (Figure 1C-G). Indeed, these results are similar to those obtained when overexpressing other TFs as AaERF1 and AaTAR1 reported to activate AN biosynthesis (Yu et al., 2011; Zhang et al., 2015). Taken together, these results show that AaMYB1 significantly activate the transcription of several key genes of the AN biosynthetic pathway, specially *ADS* and *CYP71AV1*, and consequently may result in an increased amount of AN in these transgenic plants.

AaMYB1 increases artemisinin content and trichome proliferation in *Artemisia annua*.

Following this rationale, we measured the AN and DHAA content in plants from three independent *pCYP71AV1::AaMYB1* lines, as well as, from three independent *35S::AaMYB1* lines and compared with control plants (Figure 2). According to our data, all positive transgenic plants tested accumulated more AN and DHAA than wild-type plants (Figure 2). Indeed, the leaves of *35S::AaMYB1* plants produced almost doubled AN amounts compared to the *Anamed* wild-type variety plants, a typical high artemisinin producer (HAP) yielding more than 1 % (w/w) AN (Figure 2A). These plants produced 3- to 4-fold more DHAA than the wild-type plants (Figure 2B). The *35S::AaMYB1* plants produce higher amounts of AN and DHAA than the *pCYP71AV1::AaMYB1* plants (Figure 2). These plants also show a somewhat higher expression of the AN biosynthetic genes (Figure 1C-G). The results obtained may be due to the fact that the *A. annua 35S::AaMYB1* plants showed higher GST number and density when compared with *Anamed* wild-type variety plants (Figure 3A and B); suggesting that the AN and DHAA increase in these plants may be partially due to the AaMYB1 positive role on trichome initiation. From the above results, we can conclude that when *AaMYB1* is over-expressed either in the whole plant or exclusively in the trichomes,

the expression levels of AN biosynthetic genes are clearly overexpressed (Figure 1). As a result, the AN and DHAA accumulation significantly increases in adult leaves (Figure 2). Considering that the dry weight and biomass of these transgenic leaves are similar to wild-type leaves, overexpression of *AaMYB1* seems an ideal strategy to develop high artemisinin producer transgenic *A. annua* plants.

AaMYB1 possible orthologues from *Arabidopsis thaliana*

For confirming AaMYB1 role on trichome initiation, *A. thaliana* AaMYB1 orthologues were searched. The phylogenetic relationship between MYB TFs of these two distant plant species was deduced using the amino acid sequence of AaMYB1 and the possible *A. thaliana* orthologues (Figure 4A). Although MYB proteins belong to a very large family of TFs (Dubos et al., 2010), bioinformatics and phylogenetic analyses clearly revealed that AaMYB1 is closely related with the *A. thaliana* cluster *S13* of the MYB TFs family (Figure 4B). The *S13* cluster is a R2R3-MYB subclass that includes four genes, *AtMYB50*, *AtMYB55*, *AtMYB61* and *AtMYB86* (Dubos et al., 2010). Based on deep phylogenetic analyses, *AtMYB61* was identified as the closest related, showing 73% identity, and therefore becoming a putative *AtMYB61* orthologue candidate.

To gain additional insight into the phylogenetic relationship among AaMYB1 and *A. thaliana* *S13* cluster, bioinformatic analyses using publicly available RNA-seq collections and microarray data revealed again that *AtMYB61* is the closest relative as it showed similar expression pattern in the *A. thaliana* main tissues to AaMYB1 in *A. annua*. Similarly to *AaMYB1*, *AtMYB61* is highly expressed in roots, flower buds and both expressed in juvenile and adult leaves (Figure 4B); while the other members of *S13* subgroup *MYB50*, *MYB55* and *MYB86* do not completely share this expression pattern (Figure 4B). Moreover, when the deduced amino acid sequence of AaMYB1 was used to search for similar sequences from other phylogenetically distant species, a large number of R2R3-MYB TFs were retrieved, as it observed in the alignment of the N-terminal sequences carrying the two R2 and R3 DNA-binding domains (Figure 4C). A high homology is still observed and most of the retrieved sequences belong to the subgroup *13* of the MYB protein family (Stracke et al., 2001). Again in this alignment both AaMYB1 and *AtMYB61* proteins clearly clustered together. Based on these results, *AtMYB61* seems to be the clearest AaMYB1 orthologue.

AaMYB1 and AtMYB61 induce trichome initiation and branching in *Arabidopsis*

In order to corroborate AaMYB1 role on trichome initiation in *A. thaliana* as well as to unravel other possible AaMYB1 biological roles that might be shared and/or connected with AaMYB1 function on trichome proliferation and AN biosynthesis, *AaMYB1* was also overexpressed in the model plant *A. thaliana*. In this case, and as *A. thaliana* trichomes are non-glandular, we decided to transform *A. thaliana* only with the *35S::AaMYB1* vector previously used in *A. annua*. Results show that the expression level of *AaMYB1* on these *A. thaliana* transgenic lines in comparison with wild-type *A. annua* variety *Anamed* plants was at least 9-fold higher (Figure S1). AtMYB61 has been characterized in *A. thaliana* and found to be involved in stomatal aperture (Liang et al., 2005), root lateral formation (Romano et al., 2012) and seed mucilage (Penfield et al., 2001). However, we also observed a trichome development phenotype that has not been previously described. Therefore, we decided to phenotypically study in deep this *A. thaliana* *35S::AaMYB1* plants together with *Atmyb61-2*, a transposon loss-of-function insertion, mutant previously described (Liang et al., 2005).

Phenotypical analyses reveal that *A. thaliana* plants overexpressing *AaMYB1* displayed rosette leaves with a higher trichome density in comparison to wild-type plants (Figure 3C and D); a similar phenotype obtained in *35S::AaMYB1* *A. annua* plants (Figure 3A and B). Indeed, both trichome numbers and density is on average one third higher in *35S::AaMYB1* plants, while *Atmyb61-2* mutants showed a slight decreased of trichomes in comparison to wild-types (Figure 3C and D; Table S2). Additionally, *Atmyb61-2* mutant plants overexpressing *AaMYB1* (*myb61-2 35S::AaMYB1*) developed a similar number of trichomes as wild-type plants (Figure 3C; Table S2); indicating that *AaMYB1* expression rescued the *Atmyb61-2* mutant phenotype.

As a consequence of the endoreduplication, trichomes with mainly three branches, and rarely four branches, are formed on *A. thaliana* rosette leaves (Marks et al., 2009; Balkunde et al., 2010). In comparison to wild-type plants, *A. thaliana* *35S::AaMYB1* plants developed multi-branched rosette leaf trichomes, bearing mostly four and sometimes five branches (Table S2 and Figure S2). These results suggest an involvement of *AaMYB1* not only in trichome initiation but also, even more dramatically, in later processes of trichome development such as trichome branching. Indeed, the number of four and five branched trichomes in wild-type plants is very limited, while such trichomes are dominating in plants overexpressing *AaMYB1* (Table S2). On the other hand, *Atmyb61-2* mutants showed trichomes with fewer

branches than wild-type plants, mostly two and three branches, while *myb61-2* 35S::*AaMYB1* plants showed a ratio of branched trichomes similar to wild type plants (Figure S2). This indicates that expression of *AaMYB1* rescues the *Atmyb61* mutant phenotype and suggests that the endogenous *AtMYB61* may be involved both in trichome initiation and trichome branching. The fact that the single *Atmyb61-2* mutant plants did not reveal a phenotype as strong as that of 35S::*AaMYB1* in trichome initiation and branching might be explained by redundancy effect with other members of the S13 cluster. Similar to many other TFs families in *A. thaliana*, many MYB genes play redundant roles in several biological processes (Dubos et al., 2010). Consequently, *AtMYB61* may control trichome initiation and branching in a redundant manner with the closely related genes of its cluster, *AtMYB86*, *AtMYB50* and *AtMYB55*. In fact, bioinformatic analyses showed that *AtMYB86* and *AtMYB50* genes are also very highly expressed, and almost exclusively, in trichomes (Figure S3). Unfortunately, mutant lines for creating double or multiple knock-out lines with these putative redundant genes were not available.

AaMYB1 and AtMYB61 control the expression of essential trichome genes

Trichome initiation, development and branching are the result of a complex transcriptional network that converges in the activation of two main genes *GL1* and *GLABRA2* (*GL2*) (Figure 3E) (Payne et al., 2000; Zhang et al., 2003). *GL1*, also a R2R3-MYB gene, forms part of a multimeric complex known as the *trichome activation complex* (Zhang, et al., 2003) that has a role at different steps of trichome development. It positively affects trichome initiation and later developmental steps, because mutations in these genes show a significant loss of trichomes and also result in smaller and less branched trichomes (Payne et al., 2000).

To further identify the molecular mechanism underlying the trichome-activating effect of *AaMYB1* and *AtMYB61*, expression analyses of the *GL1* gene in *Atmyb61-2*, wild-type and 35S::*AaMYB1* lines were conducted. qPCR results strongly suggest that both *AaMYB1* and *AtMYB61* transcriptionally activate the expression of this trichome activator complex member gene, as *GL1* expression is clearly increased in 35S::*AaMYB1* plant lines and slightly reduced in *Atmyb61-2* (Figure 3E).

In *A. thaliana*, all the different regulatory pathways of trichome epidermal cell differentiation converge on the activation of the unique gene *GL2* (Szymanski et al., 1998). *GL2* is considered to be the universal promoter of trichome initiation (Szymanski et al.,

1998; Lin et al., 2012). In fact, in *gl2-1* mutant plants show both un-branched and abortive epidermal cells as well as a significant reduction in trichome number (Schellmann, 2002; Zhao, 2008). We analyzed the expression levels of *GL2* gene in *Atmyb61-2* mutant, wild-type and *35S::AaMYB1* backgrounds. Similarly as *GL1* expression, *GL2* levels were significantly increased in *35S::AaMYB1* plants and reduced in *Atmyb61-2* compared with wild type plants (Figure 3E). Thus, both *AaMYB1* and *AtMYB61* are able to positively regulate the expression of *GL2*, the universal trichome initiator. In conclusion, these results provide molecular evidences that both *AaMYB1* and *AtMYB61* are able to control trichome initiation in *Arabidopsis* likely through the activation of essential trichome genes such as *GL1* and *GL2*.

AtMYB61 and AaMYB1 are involved in diverse biological processes in *A. thaliana*

As mentioned, different *AtMYB61* functions have been previously characterized in *A. thaliana*. *AtMYB61* is involved in the control of stomatal aperture in the epidermis as well as in the control of xylem cell differentiation, seed size or root development (Penfield et al., 2001; Liang et al., 2005; Romano et al, 2012). In order to elucidate if *AaMYB1* can act as *AtMYB61* in *A. thaliana* further phenotypic analyses were conducted, such as root development and stomatal aperture.

Root length was measured in the lines overexpressing *AaMYB1* in wild-type (*35S::AaMYB1*) and *Atmyb61-2* backgrounds (*myb61-2 35S::AaMYB1*). As published, *Atmyb61-2* lines showed a shorter root development (Figure 5A and B; Romano et al., 2012); and as expected, *A. thaliana 35S::AaMYB1* root length was longer than in wild-type plants (Figure 5A and B). In addition, stomatal aperture of *myb61-2* lines, wild-type and *35S::AaMYB1 A. thaliana* rosettes were measured at different ABA concentrations. As expected, stomatal opening was significantly reduced in *35S::AaMYB1* lines compared to wild-type plants (Figure 5C and D), while it was greater in *myb61-2* lines as published (Liang et al., 2005). These results certainly corroborate that *AaMYB1* is the real orthologue of the well-described *AtMYB61*.

AaMYB1 and AtMYB61 affect other connected MEP-derived biosynthetic pathways

Gibberellins (GAs) are phytohormones required throughout plant development (Zhang et al., 2003). GAs regulate different plant growth and developmental processes including trichome formation (Smyth et al., 1990; Chien and Sussex, 1996; Perazza et al., 1998; Dill and Sun,

2001; Davis et al., 2009). The GA biosynthetic pathway has been extensively characterized, and most of the genes encoding for the biosynthetic enzymes have been well studied (Olszewski et al., 2002). The GA biosynthetic pathway follows a complex regulatory network, which leads to the final production of the bioactive form of GA, GA₄ (Mitchum et al., 2006). *GIBBERELLIN 3 OXIDASE1* (*GA3ox1*) and *GA3ox2* are enzymes responsible for transforming GA₉ to the bioactive GA₄ (Mitchum et al., 2006). Indeed, *ga3ox1-3* and *ga3ox2-1* single and double mutants are not able to produce bioactive GA and consequently produce defective plants (Mitchum et al., 2006).

External GA applications increase trichome density in leaves and stems of *A. thaliana* (Perazza et al., 1998; Gan et al., 2006). Moreover, it has been recorded that AN production increase significantly in *A. annua* plant after exogenous GA treatment (Paniego and Giulietti, 1996; Zhang et al., 2005). An excess of bioactive GA might result in carbon diverting into AN production in *A. annua* (Zhang et al., 2005). This assumption is supported by the fact that the transcript levels of *FDS*, *ADS* and *CYP71AV1* were increased after treatment with 20 mg/l GA₃ by soil drenching (Banyai et al. 2011; Maes et al. 2011). The GARE motif ([T/A]AACA[G/A]A) is involved in GA-responsiveness (Leitner-Dagan et al., 2006). Indeed, bioinformatic tools reveals the presence of some of the described putative GARE-motifs in the promoters of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* (Table S1).

Our results suggest that overexpression of *AaMYB1* may possibly contribute to AN increase either by down-regulating its competitive pathways or by up-regulating pathways that are indirectly helpful, such as the GA metabolism pathway. To further investigate whether this hypothesis might be possible, we analyzed the expression levels of the enzymes responsible of GA₉ transformation into the bioactive GA₄ in both species (Figure 6). Our results showed that *GA3ox2*, and in a lesser extent *GA3ox1* were activated by both *AaMYB1* and *AtMYB61* in *A. thaliana* (Figure 6A). Interestingly, the relative expression of three putative *A. annua* *GA3ox* orthologues, *AaGA3ox1*, *AaGA3ox2* and *AaGA3ox3* (Table S3) were similarly increased in *A. annua* 35S::*AaMYB1* plants (Figure 6B). Therefore, *AaMYB1* and *AtMYB61* activate the expression of *GA3ox1* and *GA3ox2* genes, which in turn may result in an increase of GA₄ content. This fact would then have clear consequences. On one side, an increase of the GA₄ may activate different GA-dependent pathways such as the trichome initiation genetic pathway and, thus, would result in the above described increase of *GL1* and

GL2 expression that will promote trichome proliferation. On the other side, this GA₄ biosynthesis activation by AaMYB1 found in *A. thaliana* and *A. annua* not only corroborate that their function is well conserved in both species but also may explain the increase of AN content in both *pCYP71AV1::AaMYB1* and *35S::AaMYB1* transgenic *A. annua* plants.

As described, GAs are required throughout development and promote other developmental processes such as flowering induction. Flowering time, an essential plant physiological process, is controlled by a complex network of interdependent genetic pathways that monitor and respond to endogenous and environmental signals (King et al., 2003; Fornara et al., 2010). Plants flower earlier in response to endogenous GA (King et al., 2003; Turck et al., 2008; Yant et al., 2010; Osnato et al., 2012). Mutants defective in the biosynthesis of GA, as *ga3ox1* and *ga3ox2*, exhibit delays in the timing to flower (Mithchum et al., 2006; Osnato et al., 2012). Therefore, we also measured the flowering time in our different plant mutant and over-expression lines. Flowering time was measured as mean value of the number of rosette leaves produced by each genotype plant just before flower blooming. As expected, *A. thaliana 35S::AaMYB1* plants showed a significant early flowering phenotype in comparison with wild-type plants, but without affecting juvenility-adult transition (Figure S4). On the contrary, *Atmyb61-2* plants showed a slightly late flowering phenotype (Figure S4). These new phenotypic data strongly support our results about the action of AaMYB1 and AtMYB61 on the activation of the GA biosynthetic pathway.

Not only GA biosynthesis but also GA degradation may imply physiological consequences. Therefore, a proper balance between the biosynthetic and catabolic enzymes is essential for keeping a correct amount of GA. *GIBBERELLIN 2 OXIDASE (GA2ox)* enzymes are responsible for the degradation of the excess of the bioactive GA₄ into inactive GA₃₄ (Mithchum et al., 2006; Yamaguchi, 2008). To further characterize whether AaMYB1 action may affect both GA processes, expression of two of these GA catabolic enzymes, *GA2ox2* and *GA2ox4* were analyzed in the different *A. thaliana* backgrounds. Surprisingly, GA catabolism genes, *GA2ox4* and in a lesser extent *GA2ox2*, were also positively altered in *A. thaliana* plants overexpressing *AaMYB1* (Figure 6C). Similarly, the relative expression of two *GA2ox* possible *A. annua* orthologues, *AaGA2ox1* and *AaGA2ox2* (Table S3) were identically affected in *A. annua 35S::AaMYB1* lines (Figure 6D). Likely, the excess of produced GA₄ in *35S::AaMYB1* plants, once has affected trichome development and flowering time, might go directly to degradation due to the fact that a hormonal excess could

be harmful for the plant (Nemhauser et al., 2006). In *A. thaliana*, this degradation is a natural defense mechanism to equilibrate diverse phytohormone levels.

In conclusion, our findings indicate that AaMYB1 function is well conserved among distant species such as *A. annua* and *A. thaliana*. Results obtained indicate a clear functional conservation between AaMYB1 and AtMYB61 in both plant species as AaMYB1 can be interchanged with AtMYB61 in *A. thaliana*. Although these genes have adapted and diversified acquiring novel and specialized functions in their respective plant species as AaMYB1 in AN biosynthesis. In addition, molecular evidences demonstrate that AaMYB1 and its orthologue AtMYB61, transcriptionally activate essential genes of the trichome initiation and branching pathways as well as up-regulate other crucial genes of GA biosynthesis and degradation. However, AaMYB1 does not transcriptionally affect other *A. annua* TFs involved in AN biosynthesis such as AaWRKY1 (Figure S5). This transcriptional alteration of GA metabolic genes is reflected not only in the altered number and branching of trichomes, but also affects other GA-dependent processes such as floral induction in *A. thaliana*. Moreover, it has also been shown that AaMYB1 seems to positively control the expression of the enzymes of the AN biosynthesis, especially *CYP71AV1* and *ADS*. Altogether, all these results provide insights into the mechanism by which AaMYB1 regulates and integrate information from very different genetic, but connected, developmental pathways; and may also explain why AN content is significantly increased in both *A. annua* pCYP71:AaMYB1 and 35S::AaMYB1 plants. Indeed, the increase in the synthesis and degradation of the excess of GA that happens in 35S::AaMYB1 plants may have important biological implications in *A. annua*. As mentioned, in *A. annua* both GA and AN metabolic pathways share common intermediates at the early stages of biosynthesis. Two or three molecules of IDP that can be transported between the plastid and cytosol and one molecule of DMADP, all derived from both the plastidial methyl-D-erythritol 4-phosphate pathway (MEP) and the cytosolic mevalonate pathway, are condensed to FDP in the cytosol and GGDP in the plastid, respectively. From these intermediates, AN and GA biosynthesis, among other processes, will take place (Figure 7) and may be somehow interconnected. An excess of bioactive GA may result in carbon diverting into AN production due to a feedback mechanism (Zhang et al., 2005). This assumption is supported by the fact that the levels of transcripts of *FDS*, *ADS* and *CYP71AV1* increase after GA treatment (Banyai et al. 2011; Maes et al. 2011). Therefore, there is a proper balance between these two biosynthetic pathways that share a common core and both of these

pathways have been shown to be transcriptionally regulated by AaMYB1 in different plant models (Figure 7). The regulation of other metabolic pathways by AaMYB1 contribute to AN increase by either down-expressing its competitive pathways or up-expressing pathways that are indirectly beneficial. Finally, both AaMYB1 and AtMYB61 are essential for trichome proliferation where interestingly AN biosynthesis take place. Consequently, it may be speculated that both genes induce different biological processes that are somehow interconnected and that combined may synergistically increase AN content and probably other metabolites of interest. Indeed, a better understanding of the molecular mechanisms that control the biosynthesis of special metabolites in trichomes of different plant species should be taken into consideration as an important tool for future commercial biopharming applications.

EXPERIMENTAL PROCEDURES

Plant Materials

Seeds of *Artemisia annua* L. varieties Anamed (www.anamed.net) and Chongqing (Professor K. Tang, Shanghai Jiao Tong University) were used in the experiments. Seeds were grown in soil under controlled conditions at 22°C (16 h light/8 h dark). The plants were left to grow for 22 days before they were used for stable transformation.

Arabidopsis thaliana seeds were stratified for 3 days at 4°C, and plants were grown in soil under controlled conditions. Transgenic, mutant and control plants used for phenotypic analyses were grown together in the growth chamber. The Columbia (Col-0) ecotype of *A. thaliana* was used as the wild-type in all the experiments and also for plant transformation with 35S::AaMYB1. *Atmyb61-2* is a transposon loss of function stable insertion of MYB61 (SM_3.15947) that was obtained from the Nottingham Arabidopsis Stock Centre (NASC). At least ten independent lines for preliminary characterization were generated for each *Arabidopsis* transgenic line, and data from three T2 independent lines having a stable phenotype are shown.

Cloning of AaMYB1 and transformation of *Artemisia annua* and *Arabidopsis thaliana*

Young leaves of the Chongqing variety were cut off and total RNA was extracted using Purelink™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). Single strand cDNA was synthesized by RevertAid™ H Minus-MuLV reverse transcriptase (Fermentas) protocol. Two primers were designed based on a putative MYB-like EST sequence. 3'RACE and 5'RACE were performed using SMARTer™ RACE cDNA Amplification Kit. A sequence of

993 bp was assembled (GenBank no. KC118530). The gene specific primers GSP1 and GSP2 (Table S2) were used for 5'RACE and 3'RACE, respectively.

The *CYP71AV1* promoter and 35S promoter on frame *AaMYB1* gene were cloned into a modified pCAMBIA1381z and pALLIGATOR2 vector respectively (Bensmihen et al., 2004). The constructs were transferred into *Agrobacterium* strain EHA105 for *Artemisia* transformation protocol (Zhang et al., 2009) and PGV2260 for electroporation in *Arabidopsis* by floral dip (Clough et al., 1998; Karimi et al., 2002). Primers from Table S4 were used for the cloning. At least 10 T1 transgenic lines for each construct were selected.

Expression analyses of *Artemisia annua* and *Arabidopsis thaliana*

Young leaves of both transgenic and wild type *Artemisia* and *Arabidopsis* plants were collected just before flower induction, after several days in flower induction conditions, in order to analyze the correlation between the expression levels of genes encoding biosynthetic enzymes and *AaMYB1*. cDNA synthesis was carried out as described (Rieu and Powers, 2009). The primers for each gene are listed in Table S2: *DBR2*, *CYP71AV1*, *ALDH1*, *ADS*, *FDS*, β -*ACTIN* and *AaMYB1*. Data were normalized using the β -*ACTIN* gene as reference. For *AaMYB1* expression analyses, root, stem, juvenile leaves and adult leaves were collected from wild-type *Anamed* plants when floral induction had not still occurred; while flower buds were collected several days after plant blooming.

For RT-qPCR reactions in *Arabidopsis*, plants were grown in soil under long days and samples were collected at 21 days after germination. The expression levels of genes of interest were monitored by qPCR using SYBR Green I Master Mix and Light Cycler 480 (Roche) using the primers 23 to 40 listed in Table S2. Data were normalized using the *UBQ10* gene as reference. PCR efficiency was calculated and determined as previously described (Talke et al., 2006).

Artemisinin and dihydroartemisinic acid content analysis of *Artemisia annua* L.

The amount of AN and DHAA in wild-type and transgenic plants was determined by extraction and analysis on a Waters Alliance 2695 HPLC system coupled with a Waters 2420 ELSD detector as previously described (Jiang et al., 2016).

Phenotypic analyses for *Arabidopsis thaliana* and *Artemisia annua*

For phenotypic analysis, all the experiments were repeated at least twice. Unless otherwise specified, a minimum of 20 plants was used for trichome analysis for each developmental stage and genotype combination.

Trichome initiation was monitored using an Olympus DP71 microscope by counting all trichomes on the adaxial surface of individual and fully developed rosette leaves (Gan et al., 2007; Yu et al., 2010). Diverse pairs of rosette leaf trichomes were counted independently given that these leaves showed different trichome production. Leaves were chosen at 21 days due to the fact that at this stage they are fully developed. In addition, trichome branching in all these leaves was also analyzed in the diverse genotypes. Data are reported for the 5th-6th rosette leaves as mean value and standard deviation of the number of trichomes for each genotype. Trichomes SEM images were obtained as previously described by Sanchez-Chardi et al, 2010.

For *root length* analysis, sterile seeds were sown on semi-solid MS medium on square Petri plates oriented horizontally. After 21 days in long-day conditions, roots were imaged with a Olympus DP71 microscope-digital camera. Primary root length and total lateral root length were measured using ImageJ 1.38x (Romano et al., 2012). To measure ABA induced *stomatal aperture*, rosette leaves 21 days old grown at short-day conditions were pre-incubated with stomatal opening solution (50mM KCl, 10mM MES, pH6.15) for two hours at the short-day chamber. ABA (1 and 5μM) were added to the opening solution and further incubated for two more hours (Fu et al., 2014). Stomatal opening was calculated by measuring the width and length of a minimum of 50 stoma per genotype in each genotype combination. For *flowering time* in *Arabidopsis*, data are reported as mean value of the number of rosette leaves of 20 plants, each genotype with standard deviation.

ACCESSION NUMBERS

Artemisia AaMYB1 Gene Bank number is KC118530; while sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases.

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SHORT SUPPORTING INFORMATION LEGENDS

Figure S1. Relative expression of *AaMYB1* in three independent *A. thaliana* plant lines overexpressing *AaMYB1* (*35S::AaMYB1-1*, *35S::AaMYB1-2*, *35S::AaMYB1-3*), *A. annua* wild-type variety *Anamed* and *A. thaliana* wild-type ecotype Columbia (Col-0).

Figure S2. A. Total trichome average number on the adaxial surface of 5th-6th rosette leaves of different independent *A. thaliana* plants lines overexpressing *AaMYB1* in wild-type (*35S::AaMYB1*) and *Atmyb61-2* backgrounds (*myb61-2 X 35S::AaMYB1*) as well as wild-type and *myb61-2* lines used as controls. **B.** Average number of 2-, 3-, 4- and 5-branched trichomes on 5th-6th rosette leaves of different independent *A. thaliana* plants lines overexpressing *AaMYB1* in different backgrounds, wild-type and *Atmyb61-2*. **C-E.** Scanning electron micrographs of twenty-one DAG Arabidopsis rosette leaves grown under LD.

Figure S3. Tissue-specific relative expression of *AtMYB86* and *AtMYB50*.

Figure S4. A. Flowering time of different independent *A. thaliana* plant lines overexpressing *AaMYB1* in wild-type (*35S::AaMYB1*) and *Atmyb61-2* backgrounds (*myb61-2 X 35S::AaMYB1*) as well as in wild-type and *myb61-2* lines. **B.** Genetic interaction of *AaMYB1* with juvenile-to-adult transition.

Figure S5. Relative expression of *AaWRKY1* in three independent *Arabidopsis* plant lines overexpressing *AaMYB1* (*35S::AaMYB1-1*, *35S::AaMYB1-2*, *35S::AaMYB1-3*), and *Artemisia annua* wild-type variety *Anamed*.

Table S1. Putative MYB binding sites on the regulatory regions of AN biosynthetic genes (ADS, CYP71AV1, DBR2 and ALDH1)

Table S2. Average number of the total number and 2-, 3-, 4- and 5- branched trichomes of different independent *A. thaliana* plants lines overexpressing *AaMYB1*, wild-type and *Atmyb61-2*.

Table S3. *GA3ox* and *GA2ox* *A. annua* orthologues sequences.

Table S4. Primers used in this study.

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FIGURE LEGENDS

Figure 1. AN enzymes expression levels are positively affected by AaMYB1. **A.** Expression levels of *AaMYB1* in *A. annua* root, stem, juvenile leaf, adult leaf and flower bud. **B-F.** Relative expression levels of *AaMYB1*(**B**), *FDS* (**C**), *ADS* (**D**), *CYP71AV1* (**E**), *DBR2* (**F**) and *ALDH1* (**G**) transcripts in three independent *A. annua* plants overexpressing *AaMYB1* exclusively in trichomes (pCYP71::AaMYB1) and whole plants (35S::AaMYB1). Expression levels of wild-type plants are normalized to 1. All transcript levels are relative to the transcript levels of β -actin. Error bars indicate s.d.

Figure 2. AN and DHAA contents are positively affected by AaMYB1. Amount of artemisinin (AN) and dihydroartemisinic acid (DHAA), measured using HPLC analysis, in wild-type and three independent T1 transgenic plants overexpressing *AaMYB1* exclusively in trichomes (pCYP71::AaMYB1) and whole plants (35S::AaMYB1).

Figure 3. AaMYB and AtMYB61 regulate trichome initiation. **A.** Leaves of *A. annua* wild type and overexpressing *AaMYB1* plants. Scale bars represent 499 μ m. **B-C.** Trichome density measurements of different *A. annua* plants (**B**) and *A. thaliana* (**C**) lines overexpressing *AaMYB1* (35S::AaMYB1) compared to wild-type plants. Trichome density was measured per mm² on the abaxial surface of adult leaves (**B**) and per cm² on the adaxial surface of 5th-6th rosette leaves (**C**). **D.** *A. thaliana* rosette leaves overexpressing *AaMYB1* compared to wild-

type and *Atmyb61-2* mutant leaves. Scale bars represent 200 μm . **E.** Relative expression of *GL1* and *GL2* in three independent *A. thaliana* plants lines overexpressing *AaMYB1* (*35S::AaMYB1*), wild-type and *Atmyb61-2* mutant plants. One representative of three biological replicates is shown with error bars of three qPCR replicates. NS: not significant. Asterisks indicate statistically significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 4. Phylogenetic analyses reveals AtMYB61 as the AaMYB1 closest relative gene in A. thaliana. **A.** Alignment of the protein sequence of AaMYB1 with its closest relative MYB proteins from *A. thaliana* (Subgroup S13). *CLUSTAL 2.1* was used for the alignment. **B.** Tree showing phylogeny among AaMYB1 and their closer members in *A. thaliana*: AtMYB50, AtMYB55, AtMYB61 and AtMYB86. The phylogram tree was constructed aligning the *aminoacid multiple sequence* and using clustering method *Neighbour joining* available at *CLUSTAL 2.1*. Additionally, high through-put expression data, showing the expression of expression genes in the main plant tissues, was obtained using *Genevestigator* and *eFP browser 2.0*. R, root; S, stem; J, juvenile leaf; A, adult leaf and F, flower bud. **C.** Alignment of the N-terminal sequence of a number of MYB transcription factors from diverse plant species. The DNA-binding domains R2 and R3 are in colored boxes.

Figure 5. Root length and stomatal aperture is affected by AaMYB and AtMYB61. **A-B.** Root length (**A**) and root images (**B**) of twenty-one DAG *myb61-2*, wild-type, *35S::AaMYB1* and *myb61-2 35S::AaMYB1* *A. thaliana* plants. **C-D.** Stomatal aperture measurement (**C**) and stomata images (**D**) of *myb61-2*, wild-type and *35S::AaMYB1* *A. thaliana* rosette leaves. Scale bars represent 15 μm . Error bars indicate s.d. NS: not significant. Asterisks indicate statistically significant differences (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Figure 6. AaMYB and AtMYB61 regulate GA biosynthesis. **A-B.** Relative expression of *A. thaliana* *GA3ox1* and *GA3ox2* (**A**) and three putative *A. annua* *GA3ox* orthologues (**B**) in plants overexpressing *AaMYB1*, wild-type and *Atmyb61-2* plants. **C-D.** Relative expression of *A. thaliana* *GA2ox2* and *GA2ox4* (**C**) and two putative *A. annua* *GA2ox* orthologues (**D**) in *35S::AaMYB1*, wild-type and *Atmyb61-2* plants. Expression levels of wild-type plants are normalized to 1. All transcript levels are relative to the transcript levels of β -actin.

Figure 7. Diagram showing GA and AN biosynthetic pathways and their control by AaMYB1 and its A. thaliana orthologue AtMYB61. Dashed arrows mean more than one step.







