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Review

Phyto-miRNAs-based regulation of metabolites biosynthesis in medicinal plants

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ABSTRACT

Medicinal plants, are known to produce a wide range of plant secondary metabolites (PSMs) applied as insecticides, drugs, dyes and toxins in agriculture, medicine, industry and bio-warfare plus bio-terrorism, respectively. However, production of PSMs is usually in small quantities, so we need to find novel ways to increase both quantity and quality of them. Fortunately, biotechnology suggests several options through which secondary metabolism in plants can be engineered in innovative ways to: 1) over-produce the useful metabolites, 2) down-produce the toxic metabolites, 3) produce the new metabolites. Among the ways, RNA interference (RNAi) technology which involves gene-specific regulation by small non-coding RNAs (sncRNAs) have been recently emerged as a promising tool for plant biotechnologist, not only to decipher the function of plant genes, but also for development of the plants with improved and novel traits through manipulation of both desirable and undesirable genes. Among sncRNAs, miRNAs have been recorded various regulatory roles in plants such as development, signal transduction, response to environmental stresses, metabolism. Certainly, the use of miRNAs in metabolic engineering requires identification of miRNAs involved in metabolites biosynthesis, understanding of the biosynthetic pathways, as well as the identification of key points of the pathways in which the miRNAs have their own effect. Thus, we firstly consider these three issues on metabolic engineering of medicinal plants. Our review shows, application of miRNAs can open a novel perspective to metabolic engineering of medicinal plants.

1. Introduction

Medicinal plants are known to produce secondary metabolites which use in phyto-medicine. However, production of these metabolites faces challenges such as small concentration of these compounds in plant parts. Thus, researchers look for a way to increase of their content in medicine plants. In this regard, biotechnology suggests several options through which metabolites biosynthesis can be changed such as RNA interference (RNAi) and counter-silencing technologies. RNAi technology is utilized to silence undesirable genes (Hannon, 2002). As opposed to RNAi, counter-silencing technology can be used to over-express desirable genes. So, RNAi has emerged as one of most powerful approaches for genetic manipulating of medicine plants. RNAi can be inducing by short interfering RNAs (siRNAs) and microRNAs (miRNAs). Among these, miRNAs have been attracted a lot of attention in biotechnology, molecular breeding and genomics. One of the biological functions of miRNA is the regulation of secondary metabolite biosynthesis in plants. There are a number of reports about identification of miRNAs in plants. However, to date, a few researches have reported on regulation of secondary metabolites biosynthesis via miRNAs in medicinal plants (Najafabadi and Naghavi, 2018). So, we firstly reviewed all researches on MiRNAs-based regulation of secondary metabolite biosynthesis in important medicinal plants. Accordingly, we can have a perfect understanding of miRNAs-based metabolic engineering in medicinal plants.

2. Phyto-miRNAs

Biogenesis, structure, functions and applications of miRNAs in plants perfectly reviewed in plants (He and Hannon, 2004; Bartel, 2004;

Abbreviations: Phyto-miRNAs, plant miRNAs; TM, target mimicry; RNAi, RNA interference; sncRNAs, non-coding RNAs; SiRNAs, short interfering RNAs; MiRNAs, microRNAs; Pre-miRNAs, precursor miRNAs; MRE, miRNA response element; PSMs, plant secondary metabolites; BIAs, benzylisoquinoline alkaloids; *QPT*, (*quinolinate phosphoribosyltransferase*); *HMGR*, *3-hydroxy-3-methylglutaryl-coenzyme A reductase*; *FPS*, *farnesyl diphosphate synthase*; *SE*, *squalene epoxidase*; TIAs, terpenoid indole alkaloids; AACT, *Acetyl-CoA acetyltransferase*; PHAT, *Aspartate aminotransferase*; PSO, *premnaspirodiene oxygenase*; RPE, *ribulose-phosphate3-epimerase*; PGM, *phosphoglycerate mutase*; HD-ZIP, *homeobox-leucine* zipper protein; WMD3, Web-based MicroRNA Designer 3; DART, Designer Artificial miRNA Tool

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Chen, 2005; Bartel, 2009; Krol et al., 2010; Zhou and Luo, 2013). The information related to miRNAs, including sequence, structure, function and source, put into the databases such as miRBase. The latest miRBase release (v22, March 2018), contains a total of 38,589 miRNAs have been reported from diverse species, which are publicly available in the miRBase database (PMID: 24275495, Kozomara and Griffiths-Jones, 2014). Of which, 512 and 692 sequences belong to *Arabidopsis thaliana* (a dicot model plant) and *Oryza sativa* (a monocot model plant), respectively.

3. Biotechnology and Phyto-miRNAs

Development of the plants with high production of important PSMs through miRNAs is an attractive goal, actually a dream of plant biotechnologists (Bulgakov and Avramenko, 2015). For this reason, countless efforts have been made to achieve such goals in biotechnology through miRNA technology (Zhang and Wang, 2015).

4. Interactions of Phyto-miRNAs and PSMs

The PSMs like alkaloids and vitamins have been intensively studied and used as important sources for plant-based drugs. The production and accumulation of such PSMs is negatively/positively associated with various miRNAs in plants (Table 1). Interestingly, it has been documented that plant miRNAs can regulate metabolism in human (Xie et al., 2016; Rottiers and Näär, 2012). In addition, it has been reported that PSMs are able to modulate the disease-associated miRNAs level in human (Hu et al., 2013). Thus, PSMs can be used for treatment of diverse cancers in human (Hong et al., 2015).

5. Metabolic engineering and Phyto-miRNAs

Metabolic engineering involves the manipulating of metabolic pathways to increase of useful metabolites, decrease of toxic metabolites, and or produce of novel metabolites in plants (Capell and Christou, 2004; Yuan and Grotewold, 2015). Engineering of complex biosynthetic networks has been intensively limited in due to our poor understanding of the regulatory and biosynthetic pathways underlying the biosynthesis of metabolites. So, we need adequate information on the biosynthetic pathway of PSMs prior to conducting a metabolic engineering process on target plants. Among diverse tools, miRNAs can help us to better understand and manipulate of biosynthetic pathways (Bulgakov and Avramenko, 2015).

Opportunities and challenges on engineering of biosynthetic pathways of PSMs have been widely reviewed (Hughes and Shanks, 2002; Kutchan, 2005; Trethewey, 2004). The first stage to metabolic engineering is recognition target pathways and their constituent genes via bioinformatics and Omics technologies including genomics, transcriptomics, proteomics, and metabolomics. The second stage is selection and engineering the target gene(s) involved in metabolic pathway. In this stage, the intended genes would be either silenced or expressed, and tissue specifically or constitutively. In third stage, the engineered plants would be screened and utilized subsequently for various purposes in agriculture, medicine, industry and possibly in army projects (Tang et al., 2007; Zhou and Luo, 2013) (Fig. 1).

Table 1
MiRNAs and their targets in the important medicinally plants.

miRNA	Target gene	The effected secondary metabolite	Plant	Reference
pso-miR13	*7-OMT*	Morphinan	*Papaver somniferum*	Boke et al. (2015)
pso-miR2161	*4-OMT*			
psomiR408	*Reticuline oxidase-like protein*			
miRX17	*QPT1*	Nicotine	*Nicotiana tabacum*	Li et al. (2015)
miRX27	*QPT2*			
miRX20	*CYP82E4*			
miRX19	*NAC-148*			
miR164	*NtNAC-R1*	Nicotine	*N. tabacum*	Fu et al. (2013)
miR164	*Taxane 13α hydroxylase*	Taxol	*Taxus baccata*	Hao et al. (2012)
miR171	*Taxane 2α-O-benzoyl-transferase*			
miR854e	*Farnesyl diphosphate synthase*	Ginsenosides	*P. ginseng*	Mathiyalagan et al. (2013)
miR854b and miR854c	*Squalene epoxidase (SE)*			
miR1439b and miR1439h	*Beta amyrin sythase*			
miRNA-4995	*One enzyme involved in biosynthesis of terpenoids*	Picroside-I	*Picrorhiza kurroa*	Vashisht et al. (2015)
miR-5021	*Two enzymes involved in biosynthesis of TIAs*	Terpenoid indole alkaloids (TIAs)	*Catharanthus roseus*	Pani and Mahapatra (2013)
miR828a and miR948a	*MYB12 lipoxygenase*	Flavonoids	*Salvia sclarea*	Legrand et al. (2010)
miR2911	*γ-Tocopherol methyl transferase*	α-Tocopherol	*Helianthus annuus*	Barozai et al. (2012)
miR390	*A gene involved in trichome development*	Artemisinin	*Artemisia annua*	Pe'rez-Quintero et al. (2012)
miR7539, miR5021 and miR1134	*Upstream genes of terpenoid pathway*	Terpenoid	*Xanthium strumarium*	Fan et al. (2015)
miR7540	*R-linalool synthase*	Xanthanolide	*Xanthium strumarium*	Fan et al. (2015)
miR5183	*Gibberellin 3-oxidase*			
miR6449	*Ent-kaurene synthase*			
miR5255	*Squalene epoxidase*			
miR5491	*Beta-amyrin synthase*			
miR6435	*Germacrene A oxidase*			
miR-168	*Acetyl-CoA acetyltransferase*	PSMs	*Swertia chirayita*	Padhan et al. (2016)
miR-11320	*Aspartate aminotransferase*			
miR-166a	*Premnaspirodiene oxygenase*			
miR-11071	*Ribulose-phosphate3-epimerase*			
miR-156a	*Phosphoglycerate mutase*			
miR-166b	*A gene encoding homeobox-leucine zipper protein (HD-ZIP)*			
miR1533	*SPL7*	Terpenes	*Ferula gummosa*	Najafabadi and Naghavi (2018)
miR5021	*SPL11*			
miR5658	*ATHB13*			
miR393	*A auxin receptor*	Camalexin	*A. thaliana*	Robert-Seilaniantz et al. (2011)

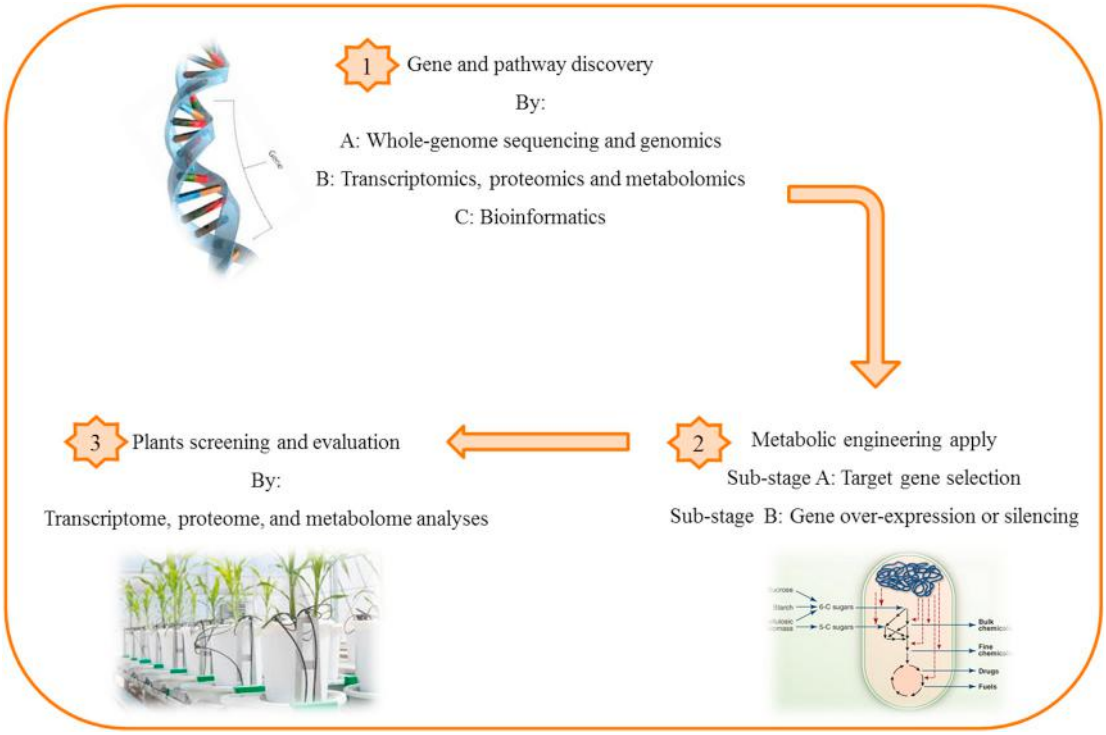

Fig. 1. Steps involved in pathway metabolic engineering.
(Modified from Tang et al., 2007).

6. Phyto-miRNAs-based regulation of metabolites biosynthesis

6.1. Alkaloids biosynthesis in opium poppy

Papaver somniferum (opium poppy), as a medicinal plant, used for production of various morphinan alkaloids, has been widely studied from diverse medicinal and agricultural perspectives (Hashemi and Naghavi, 2016; Ziegler and Facchini, 2008). The poppy synthesizes benzylisoquinoline alkaloids (BIAs) as secondary metabolites in capsule latex (Rezaei et al., 2016a; Rezaei et al., 2016b; Weid et al., 2004). But their content in the capsule is low, so the attention of researchers has been attracted to miRNA-based regulation of alkaloids biosynthesis in poppy. To date, all of 20 miRNAs in poppy were bioinformatically identified, and experimentally validated (Unver et al., 2010). In this regard, Boke et al. (2015) documented that miRNAs such as pso-miR13, pso-miR2161 and psomiR408 involve in BIAs biosynthesis. It was predicted that pso-miR13, pso-miR2161 and psomiR408 target *7-OMT*, *4-OMT* and a reticuline oxidase-like protein, respectively (Fig. 2). Genes of *4-OMT* and *7-OMT* are responsible for conversion (*S*)-3′-hydroxy-*N*-methylcoclaurine to *S*-reticuline as well as *S*-reticuline to *S*-laudanosin, respectively. And also, the role of reticuline oxidase is the conversion of *S*-reticuline to (*S*)-scoulerine in the BIA pathway. The authors suggested these miRNAs can be candidates for manipulation in order to elevate the morphine content.

6.2. Nicotine biosynthesis in tobacco

Nicotiana tabacum (tobacco) is known as a model plant for studying alkaloids biosynthesis. Nicotine, a tobacco-specific alkaloid, is used as a key material of bio-pharmaceuticals, bio-pesticides, and bio-chemicals (Fu et al., 2013). Nicotine biosynthesis pathway has been extensively studied (Dewey and Xie, 2013) but not the miRNA-based regulation of nicotine biosynthesis. Li et al. (2015) firstly studied the potential roles of miRNAs on the regulation of nicotine biosynthetic pathway, and recovered that miRX17, miRX27, miRX20 and miRX19 target *QPT1*, *QPT2*, *CYP82E4*, and *PMT2* genes, respectively (Fig. 3). In the nicotine pathway, these genes namely *QPT1* (quinolinate phosphoribosyltransferase 1), *QPT2*, and *CYP82E4* (a cytochrome P450 monooxygenase) and *PMT2* involved in nicotine biosynthesis (Dewey and Xie, 2013; Sierro et al., 2014). So that, *QPT*, *CYP82E4* and *PMT* have a role in conversion Quinolinic acid to nicotine *N*-demethylase, Putrescine to *N*-methylputrescine, as well as Nicotine to Nornicotine, respectively. In the next study, Fu et al. (2013) studied relationship among miR164 and nicotine content in tobacco. They demonstrated that after topping, the miR164 is down-regulated, and consequently *NtNAC-R1* is up-regulated in tobacco roots. Thus, it was suggested that topping can ultimately resulting in the increase of nicotine content.

6.3. Taxol biosynthesis in Yew

Taxol (generic name paclitaxel) is a successful anticancer drug (Hao et al., 2008) with a total market value of over $1 billion per year (Malik et al., 2011). It was originally isolated from *Taxus baccata* in 1962s, categorized structurally as a member of terpenoids and is approved for chemotherapy of lung, breast and non-small cell cancers. The cost of taxol is high since the drug is present at extremely low concentrations in Yew bark. So, the scientists are continuously trying to find effective ways for increasing taxol content in Yew (Nasiri et al., 2016; Nasiri et al., 2015a; Nasiri et al., 2015b; Naghavi et al., 2015; Howat et al., 2014). The biosynthetic pathway of taxol is intricate and displayed in Fig. 4 (Croteau et al., 2006; Jennewein et al., 2001). In an attempt to identify possible miRNAs affecting Taxol biosynthesis, it was found that two paclitaxel biosynthetic enzymes (i.e., taxane 13*α* hydroxylase and taxane 2*α*-*O*-benzoyl-transferase), are the targets of miR164 and miR171, respectively. Taxane 13α-hydroxylase is similar in sequence and properties to the taxane-10β-hydroxylase and converts taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α,13α-diol (Jennewein et al., 2001). And also, taxane 2α-*O*-benzoyl-transferase carried out an important function in Taxol biosynthesis (Ramírez-Estrada et al., 2016). The authors suggested that miR164 and miR171

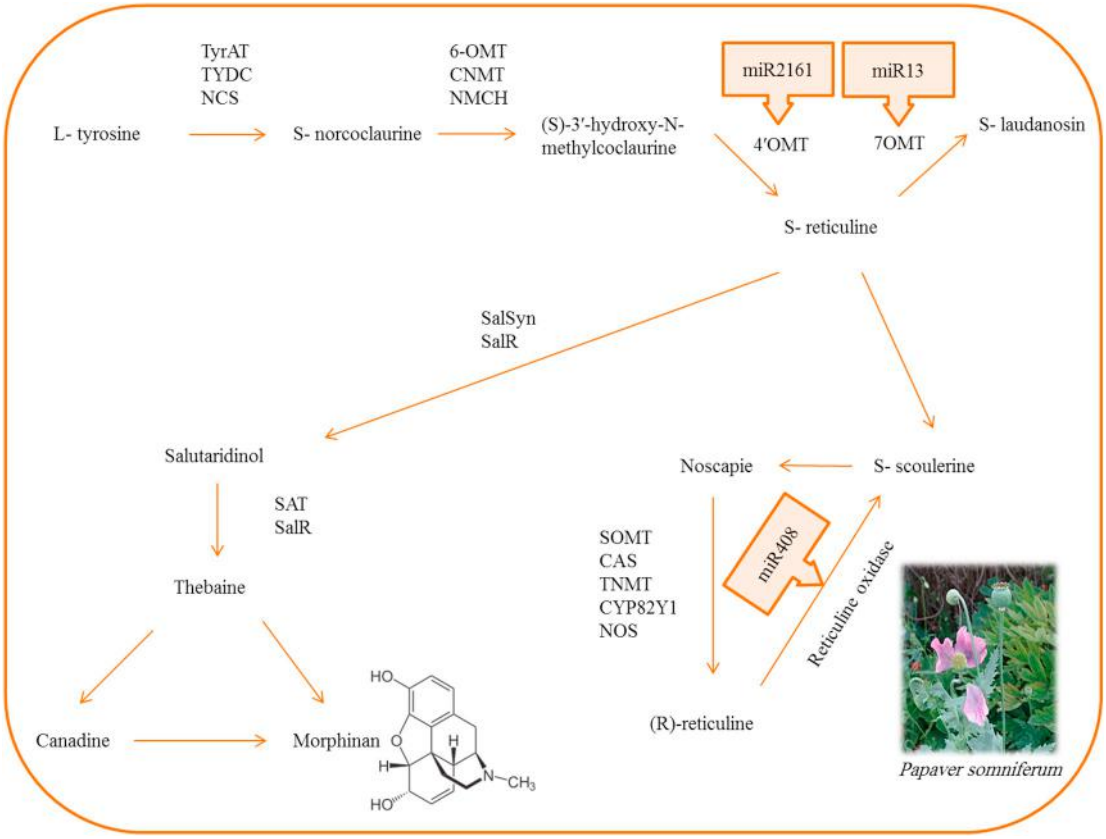

Fig. 2. Biosynthetic pathway of BIAs in opium poppy. Abbreviations: TyrAT, tyrosine aminotransferase; TYDC, tyrosine decarboxylase; NCS, norcoclaurine synthase; 6-OMT, (*S*)-norcoclaurine 6-*O*-methyltransferase; CNMT, (*S*)-coclaurine *N*-methyltransferase; NMCH, (*S*)-*N*-methylcoclaurine-3′-hydroxylase; SalSyn, salutaridine synthase; SalR, salutaridine reductase; SAT, 7(*S*)-salutaridinol 7-*O*-acetyltransferase; CODM, codeine *O*-demethylase; T6ODM, thebaine 6-*O*-demethylase; COR, codeinone reductase; SOMT, scoulerine 9-*O*-methyltransferase; CAS, (*S*)-canadine synthase; TNMT; tetrahydroprotoberberine *N*-methyltransferase; CYP82Y1, *N*-methylcanadine 1-hydroxylase; NOS, noscapine synthase; 4-OMT, 3′-hydroxyl-*N*-methylcoclaurine 4′-*O*-methyltransferase; 7-OMT, (*R*, *S*)-reticuline 7-*O*-methyltransferase.
(Adapted from Beaudoin and Facchini, 2014).

negatively regulate taxol biosynthesis in *T. baccata* (Hao et al., 2012). According to this regulation, the miR164 and miR171 silencing can be a potential tool to increase of paclitaxel in Yew. However, the commercial use of this opportunity requires further study.

6.4. Ginsenosides biosynthesis in ginseng

Panax ginseng (Korean or Chinese ginseng) is a medicinal plant in oriental medicine, where the plant roots are mainly utilized for medicinal purposes. Ginseng contains triterpene ginseng saponins called ginsenosides. Ginsenosides are responsible for different pharmacological activities including anti-stress activities, anti-hyperglycemic activities, anti-inflammatory, anti-oxidant, and anti-cancer effects (Choi, 2008). Biosynthetic pathway of ginsenosides is complicated. The genes including those that code 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), farnesyl diphosphate synthase (FPS), geranyl-diphosphate synthase, squalene synthase, and squalene epoxidase (SE) were reported as the putative genes in this pathway (Wang et al., 2012) (Fig. 5). These genes were predicted as the miRNA targets, especially *SE* is targeted by miR854b and miR854c, *FPS* is targeted by miR854e (Mathiyalagan et al., 2013) and *HMGR* and *FPS* were targeted by diverse miRNAs (Xie et al., 2011). In addition, miR1439b and miR1439h were predicted to target beta amyrin sythase in *P. ginseng*. This enzyme converts 2,3-oxidosqualene to beta-amyrin, leadings to the production of oleanane type ginsenosides (Ro). The ginsenoside Ro is the only oleanane-type pentacyclic triterpene, which is a minor component in *P. ginseng*, and has some pharmacological effects on human. Such information provides us with a promising outlook for boost the ginsenosides content.

6.5. Picrosides biosynthesis in Indian gentian

Picrorhiza kurroa (Indian gentian) has industrial values, actually due to the presence of picroside-I (P-I) and picroside-II (P-II) together with the other metabolites like picroside-III, picroside-IV, apocynin, androsin, catechol, kutkoside, etc. in Fact, the medicinal importance of *P. kurroa* is due to its pharmacological properties like antioxidant, hepatoprotective, antiasthamatic and antiallergic, anticancerous activity and immunomodulatory, etc. (Sood and Chauhan, 2010; Stuppner and Wagner, 1989). In regard to pharmacological important of picrosides, several attempts have been made to understand the biosynthesis of picrosides in *P. kurroa* (Pandit et al., 2013), however, the molecular basis of picrosides content is not yet well-understood. Picrosides biosynthetic pathway is complex (Fig. 6). Despite this complexity, researchers have been seeking to find the picrosides biosynthesis-associated miRNAs. In this way, Vashisht et al. (2015), for the first time, showed that miRNA-4995 has a regulatory role in terpenoids biosynthesis and affects the production of picroside-I in *P. kurroa*. It is because miRNA-4995 targets 3-deoxy-7-phosphoheptulonate synthase, an enzyme involved in the first step of phenylpropanoid pathway. This enzyme contributes to the formation of cinnamic acid which is required for P-I biosynthesis. Being the first enzyme of the pathway, this enzyme holds the key to progress of pathway as its down-regulation could affect the creation of cinnamic acid, thereby affecting P-I content in *P. kurroa*.

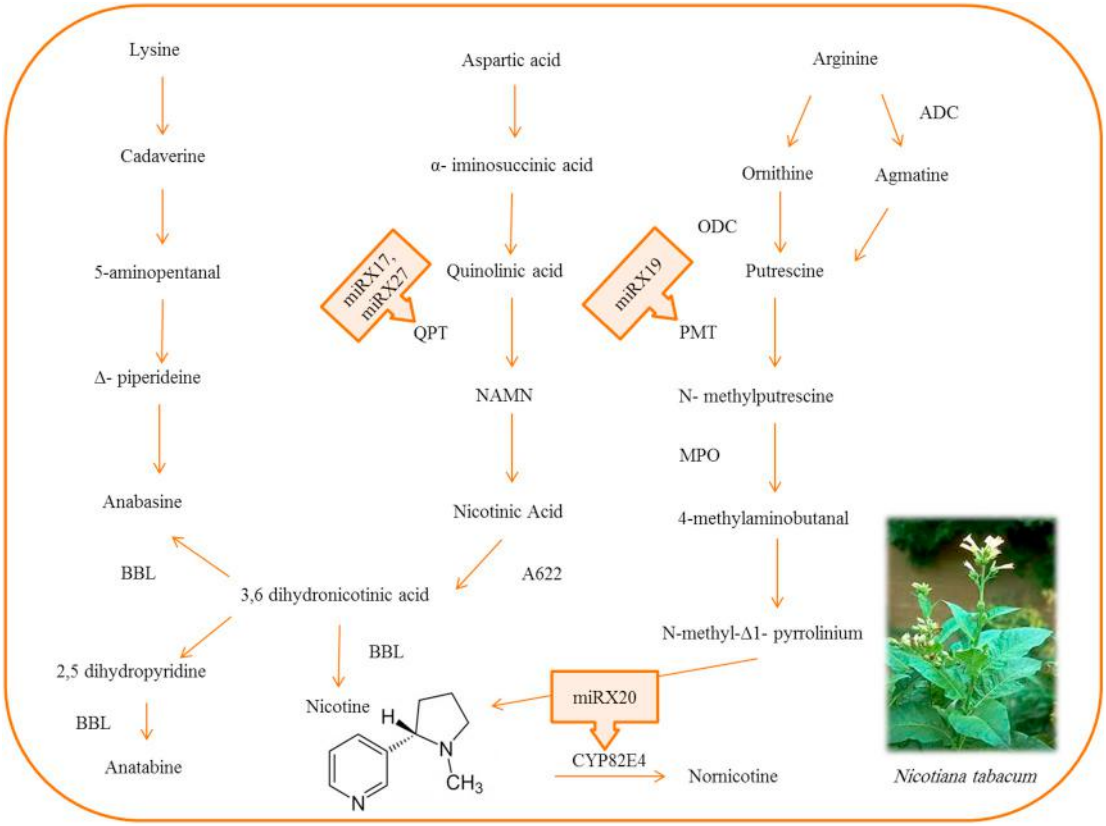

Fig. 3. Nicotine biosynthetic pathway in tobacco. Abbreviations: A622: isoflavone reductase-like protein; ADC: arginine decarboxylase; BBL: berberine bridge enzyme-like; MPO: *N*-methylputrescine oxidase; ODC: ornithine decarboxylase; PMT: putrescine methyltransferase; QPT: quinolinate phosphoribosyltransferase. (Adapted from Dewey and Xie, 2013).

6.6. Terpenoid indole alkaloids biosynthesis in Teresita

Catharanthus roseus (Teresita) is known as pharmaceutical potential, which mainly because of components namely presence terpenoid indole alkaloids (TIAs) (Heijden et al., 2004). In brief, terpenoids are the largest family of natural products in plants with over 30,000 compounds, and it has been established a broad spectrum of biological and physiological actions. For instance, a number of properties have been recorded for terpenoids such as antimicrobial, antifungal, antiparasitic, antiviral, antihyperglycemic, antihypoglycemic, anti-inflammatory and immunomodulatory (Shah et al., 2009). In general, biosynthetic pathway of TIAs in *C. roseus* is a too complicated system (El-Sayed and Verpoorte, 2007). In this pathway, vinblastine and vincristine as the well-known antitumor are produced only in small quantities. So, it is required to better understand the biosynthetic pathway of TIAs in order to improve the quantity of vinblastine and vincristine. In this sense, for the first time, miR-5021 was identified in *C. roseus* which targets two enzymes involved in biosynthesis of TIAs, GCPE protein and terpenoid cyclase (Pani and Mahapatra, 2013). Given that, usage miR-5021 as a metabolic engineering tool, could open a new field in order to get more vinblastine and vincristine in *C. roseus* plants.

6.7. Flavonoid biosynthesis in clary sage

Salvia sclarea (clary sage) is assumed as an important medicinal plant, which produces various phenolic constituents, flavonoids alongside flavonols, and widely utilized as a plant reference with antioxidant activity (Miliauskas et al., 2004). So, realization of flavonoid biosynthetic pathway will help to efficiently use of clary sage for medicinal objectives. In an attempt for the identification of *S. sclarea* miRNAs and their targets in biosynthetic pathway flavonoids, it was recognized that both miR828a and miR948a target the gene related to MYB12 Lipoxygenase which is involved in flavonoid biosynthetic pathway (Legrand et al., 2010). Furthermore, the authors predicted complete terpene and phenolic metabolic pathways to generate secondary metabolites.

6.8. Tocopherols biosynthesis in Sunflower

Helianthus annuus (Sunflower) contains important components such as tocopherol, a non-enzymatic antioxidant known as “vitamin *E*”. Epidemiological evidence showed that low tocopherol in human lead to chronic diseases such as neurological disorders, cardiovascular disease, cancer, cataracts, and age-related macular degeneration. Therefore, decoding the biosynthetic pathway of tocopherols (Fig. 7) will help us to overcome this problem. In an effort, Barozai et al. (2012) identified miRNAs and their targets in tocopherols biosynthesis of Sunflower. It was recovered miR2911 targets γ-tocopherol methyl transferase which is involved in γ-tocopherol production and consequently α-tocopherol. In these results, miR2911 decreased the α-tocopherol level which accounts for more than 95% of the total tocopherol. Anyway, we can see the medicinally importance of metabolic engineering in cases such as “Golden Rice” (including high level of vitamin E) that aided to scientists in order to decrease number of blindness children in Asia countries (Stein et al., 2006). It shows that as our science grows up in metabolism, the boundaries between plant types, such as medicine and crop, become less.

6.9. Artemisinin biosynthesis in Sweet Wormwood

Artemisia annua (Sweet Wormwood) is widely used as therapy against malaria (Ranjbar et al., 2015), which causes approximately

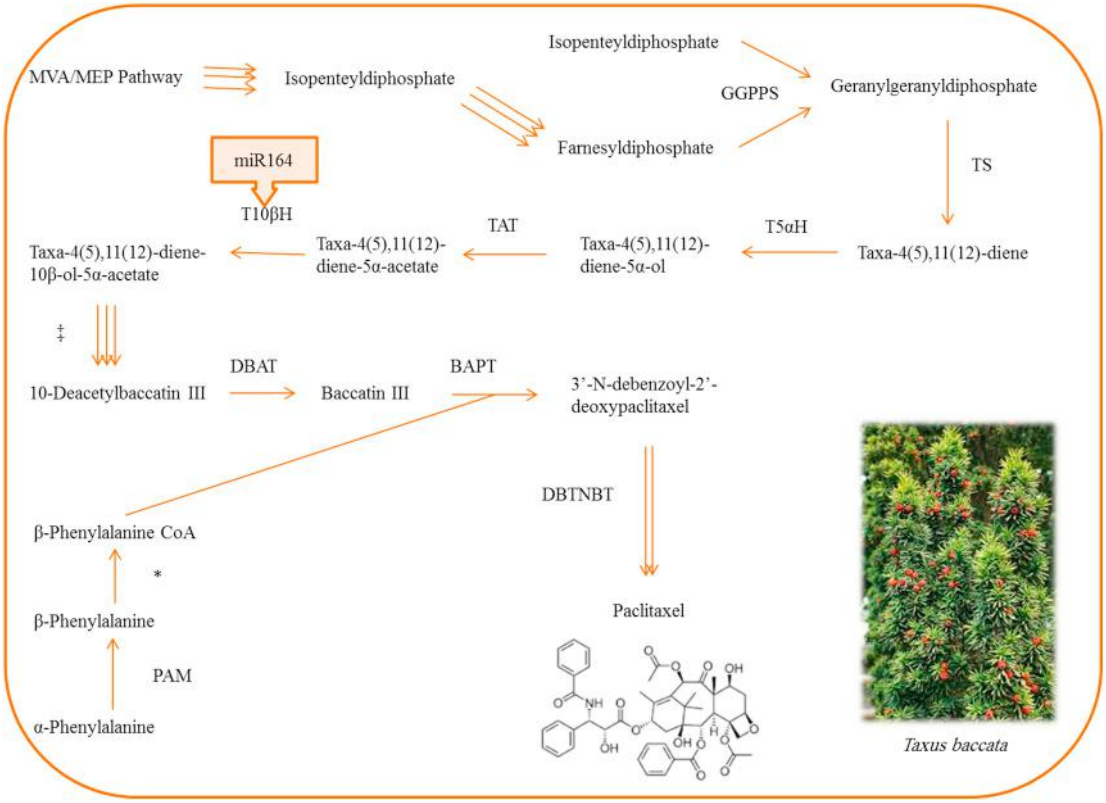

Fig. 4. Taxol biosynthetic pathway in *T. baccata*. Abbreviations: MVA, mevalonic acid; MEP, 2-*C*-methyl-D-erythritol-4-phosphate; GGPPS, geranylgeranyldiphosphate synthase; TS, taxa-4(5),11(12)-diene; T5aH, taxa-4(5),11(12)-diene-5a-hydroxylase; TAT, taxa-4(5),11(12)-diene-5a-ol-*O*-acetyltranseferase; T10bH, taxane-10b-hydroxylase; DBAT, 10-deacetylbaccatin III-*O*-acetyltransferase; BAPT, baccatin III 13-*O*-(3-amino-3-phenylpropanoyl) transferase; DBTNBT, 30-*N*-debenzoyl-20-deoxytaxol-*N*-benzoyltransferase; PAM, phenylalanineaminomutase; *, b-phenylalanine coenzyme A ligase. Multiple arrows imply more than one bio-synthetic step.
(Adapted from Croteau et al., 2006).

584,000 deaths each year (WHO, 2015). However, the relatively low content of artemisinin in *Artemisia* is a limiting factor for its large-scale production (Ghafoori et al., 2013). Artemisinin production (Fig. 8) is normally taken place in the secretory cells of *Artemisia* and its yield is highly associated with glandular trichomes and trichome density in different tissues (Zare-Mehrjerdi et al., 2013). For better understanding of miRNAs-affected artemisinin synthesis in *A. annua*, Pe'rez-Quintero et al. (2012) in their study to identify miRNAs and their potential targets demonstrated that miR390 targets a gene involved in trichome development. Thus, in this way, miR390 indirectly affects artemisinin content.

6.10. Terpenoid biosynthesis in cockleburr

Different tissues of the *Xanthium strumarium* (cockleburr), particularly its leaf, root and fruit tissues, have been used in phytomedicine, actually for the treatment of rhinitis, malaria, tuberculosis, rheumatism, cancer. The pharmacological properties of *X. strumarium* are largely attributed to the presence of xanthanolides, which have been reported to possess antifungal, antibacterial, and cytotoxic activities (Han et al., 2007). Certainly, in order to obtain enough xanthanolides, we need to know more about their biosynthesis. Fan et al. (2015), for the first time, studied the miRNA-based regulation on terpenoid biosynthesis in *X. strumarium*. Their finding suggested that miR7539, miR5021 and miR1134 are involved in regulating terpenoid biosynthesis through targeting upstream genes of terpenoid pathway. Their results also showed that several downstream enzymes in the pathway namely, R-linalool synthase, gibberellin 3-oxidase, ent-kaurene synthase, squalene epoxidase, beta-amyrin synthase, and germacrene A oxidase, were targeted by miR7540, miR5183, miR6449, miR5255, miR5491, and miR6435, respectively. Gene expression data made evident that miR6435 is specifically expressed in glandular trichome. This note is consistent with the feature that glandular trichomes are the primary sites for biosynthesis of xanthanolides. In the end, they hypothesized that miR6435 indirectly plays a key role in modulate of xanthanolide biosynthesis in *X. strumarium* glandular trichomes.

6.11. PSMs biosynthesis in Gentianaceae

Swertia chirayita (Gentianaceae) is a source of many pharmacologically important molecules exhibiting a diverse range of therapeutic values. These molecules belong to different classes of secondary metabolites such as xanthones and their derivatives, terpenoids, flavonoids and secoiridoid glycosides. In an attempt to identify miRNAs effecting on PSMs biosynthesis in Gentianaceae by Padhan et al. (2016), a total of 10 miRNAs were predicted which target 11 potential points having roles in secondary metabolism. Results showed that *miR-168*, *miR-11320*, *miR-166a*, *miR-11071*, *miR-156a* and *miR-166b* target Acetyl-CoA acetyltransferase (AACT), Aspartate aminotransferase (PHAT), premnaspirodiene oxygenase (PSO), ribulose-phosphate3-epimerase (RPE), phosphoglycerate mutase (PGM) and a gene encoding homeobox-leucine zipper protein (HD-ZIP), respectively. As a result, the authors suggested that the miRNAs can be targeted for genetic manipulation in order to enhance secondary metabolites biosynthesis in Gentianaceae.

6.12. Terpenes biosynthesis in Ferula gummosa

Ferula gummosa is a well-known medicinal and industrial plant for its galbanum, an aromatic gum resin (Singh and Sharma, 2015). However,

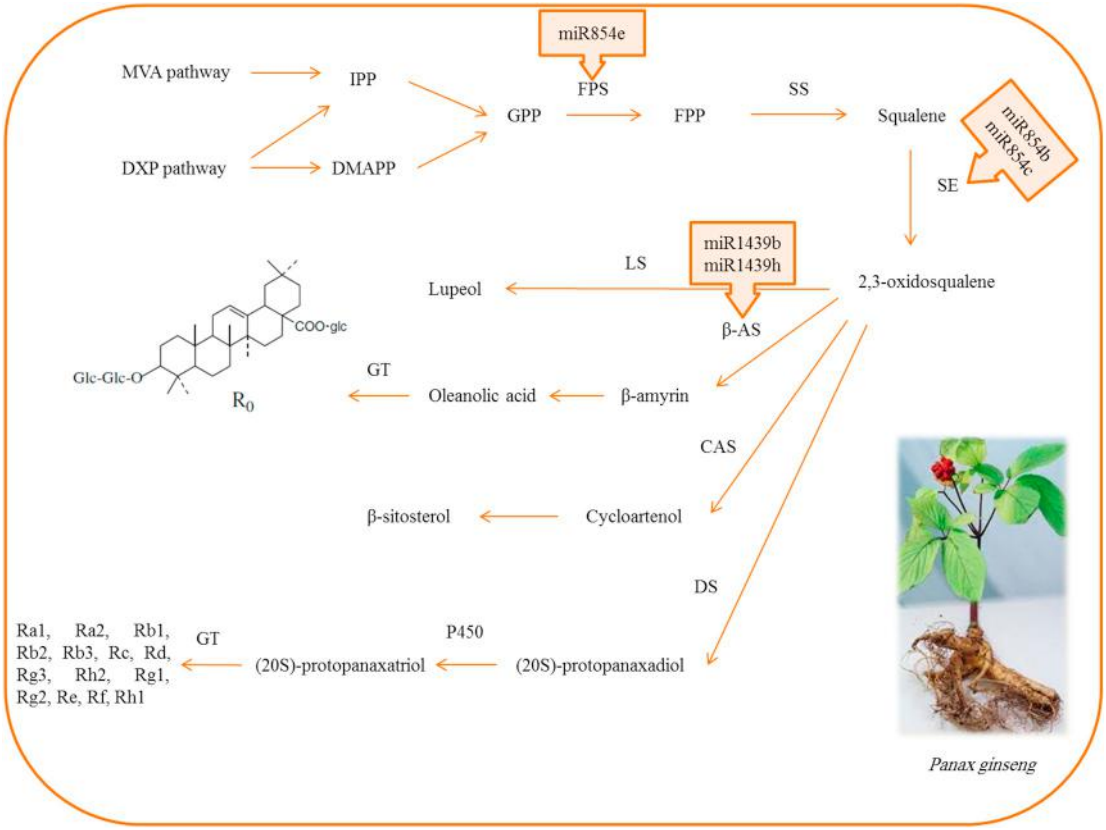

Fig. 5. Ginsenosides biosynthetic pathway in *P. ginseng*. Abbreviations: IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; SE, squalene epoxidase; beta-AS, beta-amyrin synthase.
(Adapted from Wang et al., 2012).

its amount is not high in the plant, and so we need miRNA-based engineering to reach the desired level of galbanum. In this regard, Najafabadi and Naghavi (2018), firstly predict miRNAs and their targets involved in terpene biosynthesis using computational and experimental approaches. They revealed that miR2919, miR5251, miR838, miR5021, and miR5658 are related to the terpene biosynthesis pathway (Fig. 9). In addition, it demonstrated that miR1533, miR5021, and miR5658 putatively target three transcription factors namely SPL7, SPL11, and ATHB13 related to enzymes which convert 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate to 1-hydroxy-2-methyl-2-butenyl-4-diphosphate, isopentenyl-PP to (E, E)-Farnesyl-PP, as well as 3-hydroxy-3-methyl-glutaryl-CoA to mevalonate, respectively (Najafabadi et al., 2017). Differential gene expression showed that the expression levels of these miRNAs are negatively correlated to the expression levels of both TFs and their co-expressed terpene biosynthesis genes. So it can be inferred that miR1533, miR5021, and miR5658 are candidates for metabolic engineering of terpenes quantity.

6.13. Camalexin and glucosinolate biosynthesis in Thale Cress, a model medicinal plant

It seems that *A. thaliana* (Thale Cress), as a model plant in medicine and agriculture, have little impact on advances in medical research, a number of reports indicated that this is a misconception. Interestingly, several important processes in human biology are easily surveyed in *A. thaliana* and also many discoveries relevant to human disease and health have been well illustrated using *A. thaliana* (Jones et al., 2008). For example, scientists through the study of *A. thaliana*, showed that auxin acts like molecular glue and stabilizes interaction between two proteins. Thus, work in *A. thaliana* presented a new sight of protein-protein interactions (Jones et al., 2008). As another example, we can point to relation between miRNAs generation and signaling mechanisms related to various biosynthetic pathways. Robert-Seilaniantz et al. (2011), for instance, displayed that miR393 can repress auxin signaling by targeting the auxin receptors. Besides, they uncovered that overexpression of miR393 lead into increase of glucosinolate levels and decrease of camalexin levels (Robert-Seilaniantz et al., 2011). As a result, the engineered plant was able to re-direct metabolic flow toward the effective anti-microbial compounds (Jones and Dangl, 2006).

7. miRNA and target silencing

Now that we get to know the biosynthetic pathways of secondary metabolites in medicinal plants, it's time to learn how to apply the silencing. In silencing process, three items should considering: design the silencing construct, delivery the construct to plants and finally silence the miRNA (by eTMs or amiRNAs) and or silence its target (by amiRNAs).

7.1. Design and delivery the silencing construct

Silencing constructs containing amiRNAs or eTMs were developed through genetic engineering. The next step is transferring the constructs to plant. Among diverse transformation procedures established so far in production of transgenic plants, agrobacterium-mediated transformation is regarded as one of the main technology (Tzfira and Citovsky, 2006). In fact, agrobacterium-mediated transformation approach has been widely utilized in various plant species such as *Papaver somniferum* (Alagoz et al., 2016), *Nicotiana tabacum* (Marton et al., 1979), *A. thaliana* (Zhang et al., 2006), *Taxus baccata* (Han et al., 1994), *Panax ginseng* (Yoshikawa and Furuya, 1987), *Picrorhiza kurroa* (Mishra et al., 2011), *Catharanthus roseus* (Parr et al., 1988), *Salvia sclarea* (Kuźma

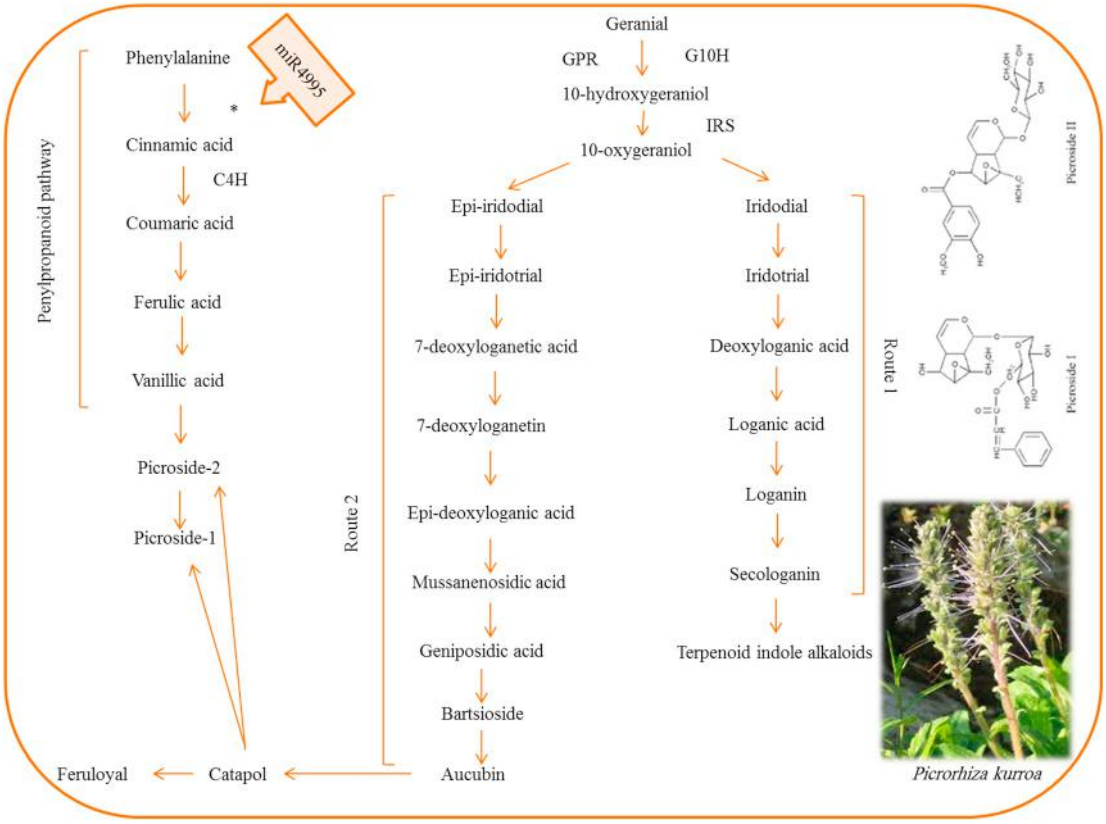

Fig. 6. Picrosides biosynthetic pathway. Abbreviations: CPR: cytochrome P450 reductase; G10H: geraniol 10-hydroxylase; 10 HGO: 10-hydroxygeraniol oxidoreductase; IRS: Iridoid synthase; C4H: cinnamoyl 4-hydroxylase; *: 3-deoxy-7-phosphoheptulonate synthase.
(Adapted from Kumar et al., 2012; Bhat et al., 2013).

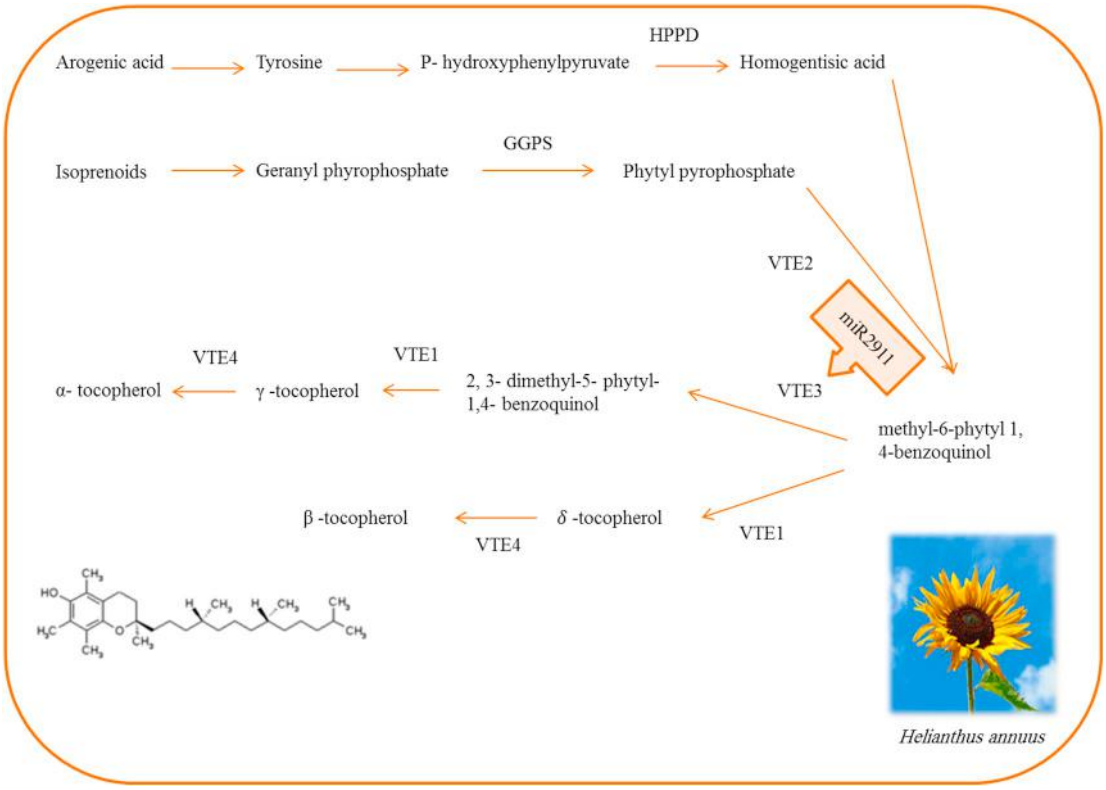

Fig. 7. Biosynthetic pathway of tocopherols. Abbreviations: HPPD, *p*-hydroxyphenylpyruvate dioxygenase; GGPS, geranylgeranyl pyrophosphate synthetase; VTE1, tocopherol cyclase; VTE2, homogenitisate phytyltransferase; VTE3, γ-tocopherol methyl transferase; VTE4, tocopherol methyl-transferase.
(Adapted from Haddadi et al., 2012).

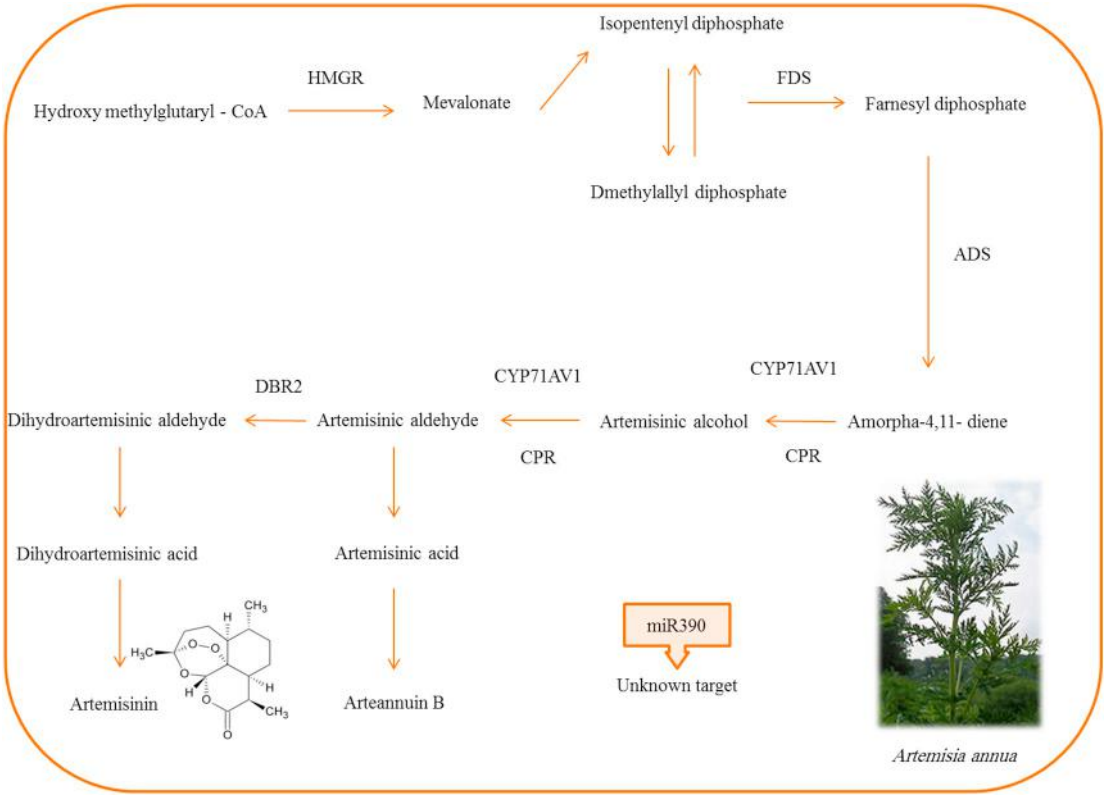

Fig. 8. Biosynthetic pathway of artemisinin in *A. annua*. Abbreviations: HMGR, HMG-CoA reductase; FDS, farnesyl pyrophosphate synthase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; DBR2, artemisinic aldehyde Delta11(13) reductase. (Adapted from Zare-Mehrjerdi et al., 2013).

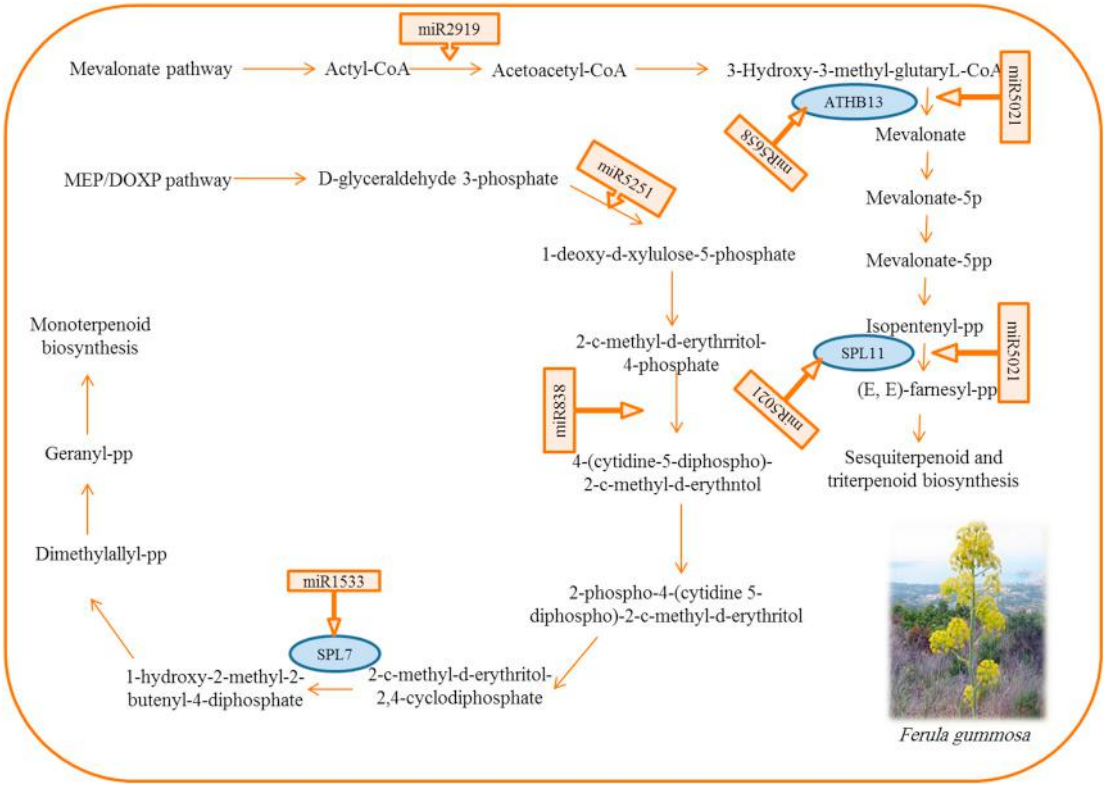

Fig. 9. Biosynthetic pathway of terpenes in *F. gummosa*.
(Adapted from Najafabadi et al., 2017).

et al., 2006), *Helianthus annuus* (Knittel et al., 1994), *Artemisia annua* (Vergauwe et al., 1996). For example, Alagoz et al. (2016) successfully transferred silencing constructs into *Agrobacterium tumefaciens* cells by using an electroporator and then the transfected *A. tumefaciens*, infiltrated into *P. somniferum* leaves by a needleless syringe.

7.2. eTM: silencer of miRNA

Recently, a number of long noncoding RNAs have been identified in plants acting as endogenous target mimics (eTMs) to inhibit the biological action of miRNAs (Franco-Zorrilla et al., 2007; Todesco et al., 2010; Meng et al., 2012; Wu et al., 2013; Li et al., 2015). Discovery methods of TMs are based on the fact that a specific miRNA response element (MRE) is common between TM and the corresponding mRNA targeted by a specific miRNA. So, TMs can be predicted by survey the co-occurrence of MREs in the mRNAs and them. Software called "TraceRNA" has been developed for TM exploration (Gupta, 2015). And also, a database of plant eTMs referred to as "PeTMbase" (available online at http://petmbase.org), has been established with a highly efficient search tool (Karakülah et al., 2016). To date, a limited number of studies have been done on plant eTMs. An example is Reichel and Millar (2015) research in which miR319 and miR159 silenced with *MIM319* and *MIM159* in Arabidopsis. Therefore, understanding and using the mechanism of target mimicry in medicinal plants requires further studies.

7.3. amiRNA: silencer of miRNA and its target

In addition to natural miRNAs, artificial miRNAs (amiRNAs) play fundamental roles in gene silencing studies (Chang et al., 2006). In brief, the amiRNA technology utilizes miRNA precursors to produce sRNAs that guide gene silencing (Ossowski et al., 2008). Today, there is a number of software such Web-based MicroRNA Designer 3 (WMD3) and Designer Artificial miRNA Tool (DART) to design amiRNAs (Gupta, 2015). So far, amiRNAs have been used for various studies such as decoding of *A. thaliana* circadian clock (Kim and Somers, 2010), *A. thaliana* flowering (Yeoh et al., 2011), *A. thaliana* HSPs (Latijnhouwers et al., 2010), *A. thaliana* AGPs (Coimbra et al., 2009), and etc. (Tiwari et al., 2014; Sablok et al., 2011). Thus, amiRNAs are a useful tool for specific gene silencing in plants, especially when several related target genes need to be down-regulated. Of course, it's possible to silence a particular miRNA. For example, Eamens et al. (2010) designed amiRNAs to silence *MIR159* family in a loci-specific manner by aiming to non-shared regions of pre-miRNA in *Arabidopsis*.

8. Conclusions and future perspectives

The global demand for Phyto-pharmaceuticals is steadily growing (Ulrich-Merzenich et al., 2007). As a result, over the past years, the interest in PSMs has been rapidly intensified, actually due to the four reasons: 1) Medicinal plants are a main source for cost-effective production of Phyto-pharmaceuticals (pharmaceutical view). 2) Medicinal plant cultivation usually performed in large areas, so it could be industrially considered as a nice opportunity to make money (industrial view). 3) Majority of PSMs exhibit the inhibitory behavior against various pathogenic agents (agricultural view). 4) PSMs may comprise a number of deleterious toxins either for bio-warfare or bio-terrorism (army view) (Roxas-Duncan and Smith, 2012). On the other hand, it is normally a difficult task to achieve adequate production of PSMs for many medicinal plants. Therefore, we need an efficient procedure to manipulating medicinal plant in order to changes PSMs levels. In this sense, it has been documented that miRNAs play key roles in production of PSMs (Gandhi et al., 2015). Despite numerous efforts about identification of possible miRNAs as regulatory factors involved in the biosynthetic pathways of various PSMs (see above for details), we need extra investigations, actually to acquire more insight toward their regulatory roles in production of PSMs. In this scene, amiRNAs can be used in gene silencing (Chang et al., 2006). AmiRNA appears to be effective and promises great specificity and safety. However, it has been reported that the success rate of amiRNA-based gene silencing could be close to 75% (Ossowski et al., 2008). Of course, perfect complementarity between amiRNA and its target gene could increase the efficiency of gene silencing in plants. As a general conclusion, the authors suggested that the identified miRNAs can be targeted in order to enhance secondary metabolites biosynthesis in medicinal plants. It remains a question that technologies such as amiRNAs and metabolic engineering in plant biotechnology only would have welfare for human or they can be used as a tool for unethical goals such as bio-terrorism?

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