Chapter 9
RNA Interference (RNAi): A Genetic Tool to Manipulate Plant Secondary Metabolite Pathways

Ashutosh R. Pathak, Swati R. Patel, and Aruna G. Joshi

Abstract Plants produce a variety of secondary metabolites which are being used as a source of medicine since the beginning of mankind, albeit most of them are synthesized in low concentrations. The developments in the field of 'omics' techniques help in the identification of genes of these metabolites having complex regulatory networks. Genetic engineering helps in manipulating the pathway which in turn increases the metabolite content and RNA interference (RNAi) is one such tool being used for the same. It is a homology dependent gene silencing technology in which the expression of pathway gene/promoter can be regulated by the introduction of double-stranded RNA (dsRNA) as it degrades the target mRNA. Since its discovery, this tool has been useful in manipulating the biosynthetic flux toward desired metabolite(s) by down-regulation of the competing pathway. In this chapter we discuss about RNAi as a tool to manipulate secondary metabolite pathways in plants.

Keywords Biosynthetic pathway · Medicinal plants · Metabolic engineering · RNA interference (RNAi) · Secondary metabolites

9.1 Introduction

Plants produce around 2,00,000 types of secondary metabolites as a defense response and they are useful sources of drugs, fragrances, pigments, food additives, and pesticides for mankind (Dixon and Strack 2003; Kutchan and Dixon 2005). It is estimated that 70–80% of the people worldwide rely mainly on herbal medicines for

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G. Tang et al. (eds.), *RNA-Based Technologies for Functional Genomics in Plants*, Concepts and Strategies in Plant Sciences, https://doi.org/10.1007/978-3-030-64994-4_9

their primary healthcare (Canter et al. 2005). Reports document that out of 50,000–70,000 plants that are used worldwide for medicinal purposes, nearly 10,000 plants have become endangered (Brouwer et al. 2002; Edward 2004). World Health Organization (WHO) estimated that the market of herbal medicine will grow up to US$5 trillion by the year 2050 with an annual growth of 5–15% (Kumar and Gupta 2008). Due to complex chemical structures of the metabolites, they are difficult to synthesize chemically, and metabolites such as ajmalicine, ajmaline, artemisinin, berberine, colchicines, digoxin, ginsenosides, morphine, quinine, shikonin, taxol, vincristine, vinblastine, etc., are still extracted from plants (Rao and Ravishankar 2002). However plants synthesize metabolites in low concentrations and are restricted to a particular species or genus (Verpoorte et al. 2002). Thus to fulfill the demand, a large number of plants are collected from the wild which depletes the plants from natural habitat. Another problem faced by industries is the requirement of a large quantity of material for extraction of metabolites e.g., 2.5 kg of taxol requires 27,000 tons of *Taxus brevifolia* bark and thus the availability of plants for herbal medicines becomes a major problem (Rates 2001).

Synthesis of metabolites is under the control of different genes that are expressed in a particular tissue or cell type (Pichersky and Gang 2000). The plant genome contains 20,000–60,000 genes of which around 15–25% are involved in the synthesis of secondary metabolites (Bevan et al. 1998; Somerville and Somerville 1999). Metabolic engineering of pathways has key applications in alleviating the demands for limited natural resources (Lau et al. 2014). The secondary metabolite pathways are chain reactions catalyzed by enzymes that convert substrates into products with one or more branched points (Farré et al. 2014). Thus main challenge in manipulating the pathways is their complex nature which involves many regulatory factors (Kooke and Keurentjes 2012). Different strategies like blocking a competitive pathway, over-expressing regulatory genes/transcription factors, or inhibiting the catabolism of molecules can be used for the enhancement of metabolites (Koffas et al. 1999; Gomez-Galera et al. 2007).

9.2 Metabolic Engineering

Metabolic engineering is defined as the ‘directed improvement of product formation or cellular properties through the modification of specific biochemical reactions or the introduction of new genes with the use of recombinant DNA technology’ (Stephanopoulos 1999). The main aim of this technique is to redirect the precursor pool toward the synthesis of the desired compound(s) through alteration in the gene expression, and it is done either in positive (over-expression) or negative (down-regulation) manner (Pickens et al. 2011; Farré et al. 2014). The metabolic flux of the pathways can be regulated by the metabolites themselves, which in turn influences the activity of enzymes, transcription factors, and signaling proteins. The chemical diversity mainly arises through alkaloid, phenylpropanoid, and terpenoid pathways, thus number of studies have been carried out for identification of their regulatory

genes and transcription factors (Wu and Chappell 2008; Nagegowda 2010). High throughput 'omics' technologies like genomics, transcriptomics, proteomics, and metabolomics are being used for elucidation of the pathways (Vemuri and Aristidou 2005; Caspi et al. 2013). In non-model plants where whole genome sequencing is not available, gene identification is done by a comparatively cheaper technique like expressed sequence tags (ESTs) (Joshi and Pathak 2019). Thus, the process of metabolic engineering in medicinal plants research is divided into three steps: (i) selection of plant species and elucidation of the pathways through 'omics' technology, (ii) targeting the gene of interest through genetic engineering tool, and (iii) screening the plants for metabolite content (Lau et al. 2014) (Fig. 9.1).

One of the key ways to reduce the levels of undesirable metabolites is recessive gene disruption and dominant gene silencing (Tang and Galili 2004). But the latter is a more promising approach to decrease the synthesis of undesirable compounds by suppression of branch-point gene which redirects the enzymatic reactions to increase the metabolite(s) of interest (DellaPenna 2001). Silencing the expression of a particular gene can be done in three different ways: (i) transcriptional gene silencing (TGS), (ii) post-transcriptional gene silencing (PTGS), and (iii) translation inhibition (Hamilton and Baulcombe 1999; Mansoor et al. 2006). But the central dogma of life suggests that if mRNA is silenced, further synthesis of secondary metabolites will be stopped (Abdurakhmonov 2016). RNA interference (RNAi) also known as post-transcriptional gene silencing (PTGS) is frequently used for gene down-regulation and thus known as the 'knock-down' method (Tang and Galili 2004).

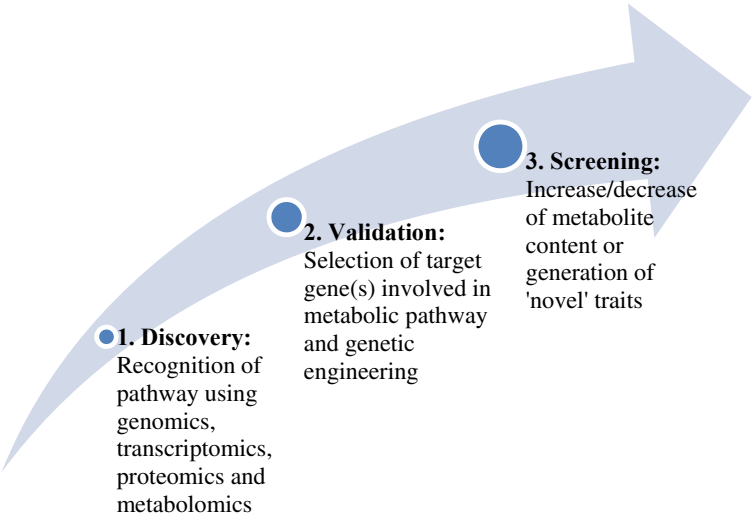

Fig. 9.1 Steps of metabolic engineering

9.3 RNA Interference (RNAi)

RNAi is a quick, easy, and sequence-specific homology-based tool to down-regulate the expression of targeted mRNA (Small 2007). Initially it was thought to function as a part of the defense mechanism against viruses when discovered in plants (Mansoor et al. 2006). The history of RNAi is nearly three decades old where Napoli and co-workers in 1990 transformed petunia plants with *chalcone synthase* (*CHS*) gene and the flower color changed from dark purple to white/chimeric, and this phenomenon was named as co-suppression. After five years, Guo and Kemphues (1995) reported knock-down of *par-1* gene expression in *Caenorhabditis elegans* through both sense and antisense RNA. The reason behind gene silencing remained unknown till Andrew Fire and Craig Mello reported that potent and specific genetic interference can be done by double-stranded RNA (dsRNA) in *C. elegans* which triggered the silencing of genes as it had identical sequences to the mRNA. This type of gene silencing was termed as 'RNA interference (RNAi)' (Fire et al. 1998) and in 2006 Fire and Mello received the Nobel Prize for discovering it (Allen et al. 2004). At the same time similar phenomenon was also reported in plants by Waterhouse et al. (1998) where dsRNA induced gene silencing which was more efficient than either sense or antisense RNA. RNAi technology suppresses the expression of enzymes that are expressed in the number of tissues at different developmental stages, whereas sense or antisense RNA fails to block the activity of enzymes that are encoded by multigene family (Larkin et al. 2007). Wesley et al. (2001) compared the silencing efficiency of hpRNA (dsRNA) and antisense RNA, and reported that hpRNA increases gene silencing by 90–100%. Thus it was confirmed that RNAi became the most promising tool for the suppression of dominant gene expression (Smith et al. 2000). One advantage of this tool is its dominant nature and the silenced gene is passed on in the T1 generation which created new opportunities in agriculture and production of metabolites (Lessard et al. 2002; Verpoorte et al. 2002). Many researchers use *in vitro* cultures to down-regulate the gene as it reduces the risk of contaminating food sources and environment, and provides a platform to test a metabolic engineering strategy that will be utilized for large scale production of metabolites (Wu and Chappell 2008). The main aims of RNAi technology for engineering secondary metabolites synthesis is given in Fig. 9.2.

9.3.1 Mechanism

Micro RNA (miRNA), short interfering RNA (siRNA), and small hairpin RNA (hpRNA) are types of small non-coding RNAs that are mainly involved in RNAi mechanism (Aukerman and Sakai 2003; Palatnik et al. 2003). Artificial microRNA (amiRNA)-based vectors have also proved to be effective for gene silencing since

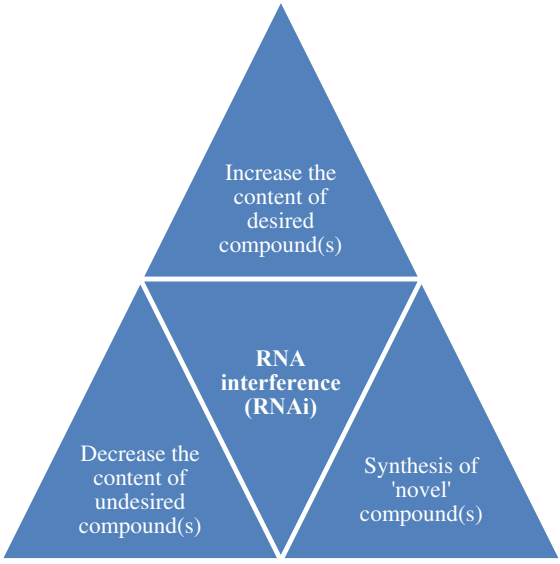

Fig. 9.2 Uses of RNAi technology in manipulating secondary metabolite pathway

the last decade (Warthmann et al. 2008). Smith et al. (2000) suggested that a more feasible approach is to clone both sense and antisense sequences separated by an intron region which forms a hairpin RNA (hpRNA) molecule upon transcription and triggers gene silencing. Aberrant single-stranded RNA (ssRNA) with an intron-hairpin construction triggers the generation of dsRNA by RNA-dependent RNA polymerase (RdRP) and activates the RNAi pathway (Waterhouse et al. 2001). Dicer, a ribonuclease III-type enzyme, is activated by ATP which recognizes dsRNA and cuts them into smaller segments of 21–25 bp. These small RNAs are then incorporated into a nuclease complex known as the 'RNA-induced silencing complex' (RISC) which contains argonaute protein (AGO). Then one of the strands of siRNA (guide strand) becomes stably associated with AGO and the other strand (passenger strand) is degraded. The guide strand then leads RISC to its target mRNA and AGO protein binds the guide strand to the target sequence for complementary base pairing. Successful docking of the RISC-siRNA complex with mRNA will then either block the translation or degrade mRNA using exonucleases (Kusaba 2004). Reports suggest that the directionality of dsRNA processing and the target RNA cleavage sites are predefined, and the sequence complementary to the guide siRNA will be recognized and cleave the target mRNA in the central region which is 10-12 nt from the 5' end of siRNA (Elbashir et al. 2001). Lastly, the siRNA molecules are amplified via RdRp on the target mRNA and these siRNAs will, in turn, induce a secondary RNA interference i.e., transitive RNAi (Denli and Hannon 2003).

9.3.2 Vector and Transformation Methods

Different vectors are used to suppress gene expression in plants and the vector-based RNAi technology was improved by using an intron as the linker (Waterhouse et al. 1998; Smith et al. 2000). These RNAi vectors are specifically designed to generate long dsRNA with the same sequence as the target genes. Similarly, vectors designed to express hairpin RNAs (hpRNAs) are also successfully applied to silence the corresponding target genes (Wesley et al. 2003). Nowadays biotechnology companies are developing specialized vector constructs for RNA interference in plants (see table), which after transformation into host plant converts into dsRNAs and triggers efficient silencing.

One of the major issues in plant genetic transformation is to obtain a stably transformed plant which depends on the transformation methods. The first choice is Gram-negative, soil-borne pathogen *Agrobacterium* spp., which is also known as 'natural genetic engineer' is commonly used to transform numerous dicotyledonous plants (Zupan et al. 2000). But the wild-type Ti plasmid is very large (200 kb) and difficult to manipulate, which was overcome by the development of binary vectors (Bevan 1984). In such a system, the Ti plasmid of *Agrobacterium* has been disarmed by removing the T-DNA and keeping *vir* regions intact. Simultaneously, a separate binary vector is constructed which carries an origin of replication that is compatible with the Ti plasmid of *Agrobacterium*. When the binary vector is introduced into *Agrobacterium* the *vir* genes of Ti plasmid will act *in trans* to transfer the recombinant T-DNA from the binary vector to the host plant cell. As the binary vectors are smaller and comparatively easier to construct than wild-type Ti plasmids, the *Agrobacterium*-mediated transformation is considered as a reliable technique (Lessard et al. 2002).

Transient gene expression in majority of the plant species can be done via particle bombardment and electroporation. These techniques are useful especially when long term expression is not required for e.g., to test the effectiveness of various gene constructs before stable transformation (Lessard et al. 2002). One of the advantages of this method is high transformation frequency, which resulted in the successful transformation of plastids in tobacco and tomato (Maliga 2001). But these methods require the use of tissue culture protocols to regenerate transgenic plants/callus whereas *Agrobacterium*-mediated transformation overcomes this limitation by directly transforming germ-line cells or seeds and is one of the first choice for RNA interference in plants (Tague 2001).

RNAi is a promising way to manipulate the metabolite pathway (Borgio 2009) and it was first used by Mahmoud and Croteau (2001) in *Mentha* x *piperita* to reduce the level of menthofuran through antisense suppression of the *mfs* gene which codes for the cytochrome P450 (+) menthofuran synthase, which in turn increased the content of essential oils in plants. Later on many studies documented that the content of various volatiles can be increased in *Mentha* spp. by silencing different genes or transcription factors (Mahmoud et al. 2004; Wang et al. 2016; Reddy et al. 2017). Since the beginning of this technique, *berberine bridge enzyme (BBE)* is the gene

of interest for RNAi research as many scientists knock-down the expression of this gene to study its effect on the content of different alkaloids, especially benzophenanthridine type in many plant species (Park et al. 2002; Frick et al. 2004; Fujii et al. 2007). Waterhouse et al. (1998) documented that this technology can be useful to alter the flower colors as compared to conventional breeding and genetic transformation. RNAi has been applied to suppress the genes of anthocyanin biosynthesis like *anthocyanidin synthase* (ANS) in *Torenia* spp. which changed the flower color in transgenic plants (Nagira et al. 2006; Nakamura et al. 2006). Similarly, other genes of flavonoid pathways like *isoflavone synthase (IFS)*, *flavone synthase II (FNSII)*, *flavonol synthase (FLS)*, *flavanone 3-hydroxylase (F3H)*, *flavonoid 3'-hydroxylase (F3'H)*, *flavonoid 3',5'-hydroxylase (F3'5'H)*, *flavone 6-hydroxylase (CYP82D1.1)*, *flavone 8-hydroxylase (CYP82D2)*, *chalcone isomerase (CHI)*, *chalcone synthase (CHS),* etc., were silenced and their effect on flavonoids was reported by many workers (Subramanian et al. 2005; Nakatsuka et al. 2007; Seitz et al. 2007; Park et al. 2011; Jiang et al. 2014; Zhang et al. 2015; Zhao et al. 2018). Recently Hu et al. (2020) reported that the down-regulation of one of the flavonoid biosynthetic pathway gene *laccase* gene *(Lac1)* affects the cotton fiber development. Whereas Liu et al. (2002) down-regulated the expression of two fatty acid desaturase genes i.e., *stearoyl-acyl-carrier protein Δ9-desaturase (SAD)* and *oleoyl-phosphatidylcholine ω6-desaturase (FAD)* in cotton seeds, which increased the content of stearic acid and oleic acid for better oil quality. Similarly, the content of different types of ginsenosides was increased or decreased in different species of *Panax* (*P. ginseng*, *P. notoginseng* and *P. quinquefolium*) by RNAi technique to identify the roles of different genes in ginsenoside biosynthetic pathway (Han et al. 2006; Zhao et al. 2015; Wang et al. 2017). This strategy has been used for commercial-scale production of desired plant products e.g., decaffeinated *Coffea arabica* and *Coffea canephora* plants were produced by silencing *theobromine synthase* gene using RNAi (Ogita et al. 2003, 2004). Table 9.1 depicts the plant species in which RNAi has been used to silence the secondary metabolite genes as well as the vector and transformation methods used for the same.

9.4 Conclusion

RNAi is the choice of present-day researchers to manipulate the genes synthesizing secondary metabolites. Since RNAi is a sequence-specific process, this requires the selection of a unique or conserved region of the target gene which ensures that the multiple gene families can be silenced. But the major bottleneck is that the complete information about the genomes of many non-model plants for secondary metabolite synthesis is lacking. The major drawback of RNAi tool is its unintended targets as 21–25 nt homology is required to suppress the gene function, even then it is still being used for identifying the gene functions and to increase the content of the desired metabolite.

Table 9.1 Down-regulation of plant secondary metabolite pathways using RNAi

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Arabidopsis thaliana	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pFGC5941[a]	Decreased content	Hoffmann et al. (2004)
	Flavonols, sinapate esters, and anthocyanins	*DNA-binding with-one-finger (DOF4;2)*	pJawohl8-RNAi[a]	Increased content	Skirycz et al. (2007)
	Putrescine	*ATR7*	pFGC5941[a]	Increased content	Sujeeth et al. (2020)
Artemisia annua	Artemisinin	*Cinnamate-4-hydroxylase (C4H)*	pART 27[a]	Increased content	Kumar et al. (2016)
	Artemisinin	*MYC2*	pHELLSGATE12[a]	Decreased content	Shen et al. (2016)
	Artemisinin	*Squalene synthase (SQS)*	pCAMBIA 2300[a]	Increased content	Ali et al. (2017)
	β-caryophyllene	*Pleiotropic drug resistance transporter (PDR3)*	pHELLSGATE12-iAaDPR3[a]	Decreased content	Fu et al. (2017)
	Artemisinin	*1-Deoxy-d-xylulose-5-phosphate reductoisomerase (DXR)*	pSGRNAi[a]	Decreased content	Wang et al. (2018)
	Artemisinin	*HY5*	pHELLSGATE12[a]	Decreased content	Hao et al. (2019)
Atropa belladonna	Tropane alkaloids (Hyoscyamine, scopolamine, and littorine), phenyllactate and tropine	1. *UDP-glycosyltransferase (UGT1)* and 2. *Littorine synthase (LS)*	pBin19[a]	Increased phenyllactate, tropine, and decreased hyoscyamine, scopolamine, and littorine content	Qiu et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Tropane alkaloid (hyoscyamine, anisodamine, and scopolamine), putrescine, and *N*-methylputrescine	*Ornithine decarboxylase (ODC)*	pBIN19[b]	Decreased content	Zhao et al. (2020)
Betula platyphylla	Betulin	*S-nitrosoglutathione reductase (GSNOR)*	pSGRNAi-GSNOR[a]	Increased content	Ma et al. (2019)
	Betulinic acid and oleanolic acid	1. *Cycloartenol synthase (CAS)* and 2. *β-amyrin synthase (β-AS)*	*pRNAi-GG (for CAS) and pCAMBIA 1303 (for β-AS)*[a]	1. Increased betulinic acid and oleanolic acid content 2. Increased betulinic acid and decreased oleanolic acid content	Yin et al. (2020)
Brassica napus	Pectin and lignin	*MYB43*	pFGC5941M-BnMYB43I[a]	Increased pectin and decreased lignin content	Jiang et al. (2020)
Eschscholzia californica	Benzophenanthridine alkaloids	*Berberine bridge enzyme (BBE)* and *N-methylcoclaurine 3'-hydroxylase (CYP80B1)*	pBI102[a]	Decreased content	Park et al. (2002)
	Benzophenanthridine alkaloid	*Berberine bridge enzyme (BBE)*	pBI102[b]	Decreased content	Park et al. (2003)
	(S)-Reticuline	*Berberine bridge enzyme (BBE)*	pART27[a]	Increased content	Fujii et al. (2007)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Forsythia koreana	Pinoresinol and matairesinol	*Pinoresinol/lariciresinol reductase (PLR)* and *sesamin synthase (CYP81Q1)*	pSPB3104[a]	Increased pinoresinol and decreased matairesinol content. Novel sesamin synthesis	Kim et al. (2009)
Gentiana straminea	Oleanolic acid	*β-amyrin synthase (βAS)*	pK7GWIWG2D(II)[c]	Decreased content	Liu et al. (2016)
Glycine max	Isoflavone	*Isoflavone synthase (IFS)*	CAM-sUbi:GFP[b]	Decreased content	Subramanian et al. (2005)
	Saponins	*β-amyrin synthase (βAS)*	pUHR:7S-IR[c]	Decreased content	Takagi et al. (2011)
	Isoflavone	*Flavone synthase II (FNSII)* and *flavanone 3-hydroxylase (F3H)*	pCAMBIA3300[b]	Increased content	Jiang et al. (2014)
Gossypium hirsutum	Stearic acid and oleic acid	*Stearoyl-acyl-carrier protein Δ9-desaturase (SAD-1)* and *oleoyl-phosphatidylcholine ω6-desaturase (FAD2-1)*	pBI-Lec[a]	Increased content	Liu et al. (2002)
	Gossypol and pigmentation	*CGP1*	pHellsgate 4[a]	Decreased content	Gao et al. (2020)
	Flavonoids	*Lac1*	pHellsgate 4[a]	Increased content	Hu et al. (2020)
Isatis indigotica	Lignan (Conifer alcohol, lariciresinol, pinoresinol, secoisolariciresinol and its diglucoside and pinoresinol 4-O-glucopuranoside)	*WRKY34*	pCAMBIA1300-IiWRKY34[a]	Decreased content	Xiao et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Lavandula × *intermedia*	Essential oils	*1,8-cineole synthase (CINS)*	pBI121[a]	Change in essential oil composition/altered fragrance	Tsuro et al. (2019)
Linum usitatissimum	Lignin and phenolic acids	*Cinnamyl alcohol dehydrogenase (CAD)*	pHellsgate2[a]	Decreased content	Wróbel-Kwiatkowska et al. (2007)
Lithospermum erythrorhizon	Shikonin	*Ethylene insensitive3 like protein (EIL-1)*	pBI121[b]	Decreased content	Fang et al. (2016)
	Shikonin	*Multidrug resistance associated protein (MRP)*	pBI121[b]	Decreased content	Zhu et al. (2018)
Manihot esculenta	Cyanogenic glucoside	*Valine N-monooxygenase (CYP79D1* and *CYP79D2)*	pCAMBIA 2301[a]	Decreased content	Jørgensen et al. (2005)
Medicago truncatula	Apocarotenoids	*Carotenoid cleavage dioxygenases (CCD1)*	pRedRoot[b]	Decreased content	Floss et al. (2008)
Mentha × *piperita*	Essential oil	*Menthofuran synthase (MFS)*	pGAdekG[a]	Increased content	Mahmoud and Croteau (2001)
	Limonene	*Limonene-3-hydroxylase (LH)*	pGALS and pGALH[a]	Increased content	Mahmoud et al. (2004)
Mentha spicata	Monoterpenes	*YABBY5*	pK7WG2D[a]	Increased content	Wang et al. (2016)
	Monoterpenes	*MYB*	pK7WG2D[a]	Increased content	Reddy et al. (2017)
Mimulus lewisii and *M. verbenaceus*	Carotenoid	*Reduced carotenoid pigmentation2 (RCP2)*	pFGC5941[a]	Decreased content	Stanley et al. (2020)
Nicotiana benthamiana	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pTV00[d]	Decreased content	Hoffmann et al. (2004)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Nicotine	*bHLH1* and *bHLH2*	pK7GWIWGII[a]	Decreased content	Todd et al. (2010)
	(+)-Valencene	*5-Epi-aristolochene synthase (EAS)* and *squalene synthase (SQS)*	pK7GWIWG2(II)[a]	Increased content	Cankar et al. (2015)
Nicotiana glauca	Nicotine and anabasine	*Ornithine decarboxylase (ODC)*	ODC-RNAi[b]	Decreased content	DeBoer et al. (2013)
Nicotiana tabacum	Anatabine	*Putrescine N-methyltransferase (PMT)*	pYC3JR[b]	Increased content	Chintapakorn and Hamill (2003)
	Diterpenes	*Cembratriene-ol cyclise (CYC-1)* and *CYP71D16*	pKYLX71–35S2[a]	Decreased content	Wang and Wagner (2003)
	Anthocyanins	*Chalcone isomerise (CHI)*	pEBisHR-35SintNtCHIir[a]	Decreased content	Nishihara et al. (2005)
	Nornicotine	*Nicotine N-demethylase (CYP82E4)*	pKYLX71[a]	Decreased content	Gavilano et al. (2006)
	Pelargonidin	*Flavonol synthase (FLS)* and *flavonoid 3'- hydroxylase (F3'H)*	pEBisBR[a]	Increased content	Nakatsuka et al. (2007)
	Nicotine	*Ornithine decarboxylase, (ODC), arginine decarboxylase (ADC) aspartate oxidase (AO), S-adenosylmethionine synthetase (SAMS), agmatine deiminase (AIC) arginase (ARG)*	p45-2-7-1[a]	Decreased content	Martinez et al. (2020)
	Flavonols, chlorogenic acid, and anthocyanins	*HDG2*	pHellsgate 2[a]	Decreased content	Wang et al. (2020)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Ocimum basilicum	Eugenol and coniferyl alcohol	*Coniferyl alcohol acyltransferase1 (CAAT1)*	pK7WG2D[a]	Decreased content	Dhar et al. (2020)
Panax ginseng	Ginsenoside	*Dammarenediol synthase (DDS)*	pK7GWIWG2(I)[a]	Decreased content	Han et al. (2006)
	Ginsenoside and phytosterol	*Squalene epoxidase1 (SQE1)*	pB7GWIWG2(II)[a]	Increased phytosterol and decreased ginsenoside content	Han et al. (2010)
	Ginsenoside Ro (Oleanane type)	*β-amyrin 28-oxidase (CYP716A52v2)*	pB7GWIWG2(II)[a]	Decreased content	Han et al. (2013)
	Ginsenoside (Protopanaxadiol and protopanaxatriol type)	*Protopanaxadiol synthase (CYP716A47)*	pBI121[b]	Decreased content	Sun et al. (2013)
	Ginsenoside and β- amyrin	*β-amyrin synthase (βAS)*	pK7GWIWG2(II)[b]	Increased dammarane-type ginsenoside and decreased β-amyrin and oleanane type ginsenoside	Zhao et al. (2015)
	Ginsenoside (Protopanaxadiol and protopanaxatriol type)	*Protopanaxadiol 6-hydroxylase (CYP716A53v2)*	pB7GWIWG2(II)[a]	Increased protopanaxadiol and decreased protopanaxatriol ginsenoside content	Park et al. (2016)
	Ginsenoside (Total, Rd and protopanaxadiol type)	*UDP-glycosyltransferase (3-O-UGT2* and *UGT94Q2)*	pBI121[b]	Decreased content	Lu et al. (2017b)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Panax notoginseng	Triterpene and ginsenosides (Rb1, Rg1, Rh1, Re, and Rd)	*Cycloartenol synthase (CAS)*	pHellsgate2[a]	Increased content	Yang et al. (2017)
Panax quinquefolium	Ginsenoside	*Protopanaxadiol synthase (D12H)*	pBI121[b]	Decreased content	Sun et al. (2013)
	Ginsenosides (protopanaxadiol and protopanaxatriol type)	*Protopanaxatriol synthase (CYP6H)*	pBI121[b]	Increased protopanaxadiol and decreased protopanaxatriol ginsenoside content	Wang et al. (2014)
	Ginsenoside (Total, Rd and protopanaxadiol type)	*UDP-glycosyltransferase (3-O-UGT2* and *UGT94Q2)*	pBI121[b]	Decreased content	Lu et al. (2017b)
	Ginsenoside Rh2	*UDP-glucosyltransferase (3-O-UGT1)*	pBI121[b]	Decreased content	Lu et al. (2017a)
Panicum virgatum	Lignin	*Ferulate 5-hydroxylase (F5H) and caffeic acid O-methyltransferase (COMT)*	pANIC8D (for F5H) and pANIC8B (for COMT)[a]	Decreased content	Wu et al. (2019)
Papaver somniferum	*(S)*-Reticuline	*Codeinone reductase (COR)*	pPLEX X002i[a]	Increased content	Allen et al. (2004)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Alkaloids	*Berberine bridge enzyme (BBE)*	pPOP19[a]	Increased laudanine, reticuline, laudanosine, salutaridine, dehydroreticuline, scoulerine and decreased oripavine content in latex No change in benzophenanthridine alkaloids but decreased oripavine and salutaridine content	Frick et al. (2004)
	Salutaradine	*Salutaridinol 7-O-acetyltransferase (SalAT)*	pPLEX X002i[a]	Increased content	Allen et al. (2008)
	Morphine	*Salutaridinol 7-O-acetyltransferase (SalAT)*	pART27[a]	Increased salutaridine and salutaridinol and no change in morphine content	Kempe et al. (2009)
Petunia hybrida	Methylbenzoate	*Benzoic acid/salicylic acid carboxyl methyltransferase1* and *2 (BSMT1* and *2)*	pHANNIBAL[a]	Decreased content	Underwood et al. (2005)
	Volatile benzenoids	*ODORANT1 (ODO1)*	pK7GWIWG2(I)[a]	Decreased content	Verdonk et al. (2005)
	Phenylacetaldehyde and 2-phenylethanol	*Phenylacetaldehyde synthase (PAAS)*	pART27[a]	Decreased content	Kaminaga et al. (2006)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Benzoic acid, methylbenzoate, benzyl alcohol, and benzylaldehyde	*Benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase (BPBT)*	pCAMBIA 1303[a]	Increased benzyl alcohol and benzylaldehyde but decrease benzoic acid and methylbenzoate content	Orlova et al. (2006)
	Isoeugenol and other volatiles	*Coniferyl alcohol acyltransferase (CFAT)*	pFMV[a]	Decreased content	Dexter et al. (2007)
Pinus radiata	Lignin	*Hydroxycinnamoyl transferase (HCT)*	pAHC25[c]	Decreased content	Wagner et al. (2007)
Populus grandidentata × *P. alba*	Lignin	*p-coumaroyl-CoA 3-hydroxylase (C3′H)*	pART27[a]	Decreased content	Coleman et al. (2008)
Populus tremula × *P. alba*	Condensed tannins and salicinoids	*Tonoplast sucrose proton symporter (SUT4)*	pGSA1285[a]	No change	Harding et al. (2020)
Rehmannia glutinosa	Phenolic acids (Ferulic acid, caffeic acid, and chlorogenic acid)	*p-coumarate-3-hydroxylase (C3H)*	pRNAi-GG[a]	Decreased content	Yang et al. (2020)
Rosa hybrida	Delphinidin	*Dihydroflavonol 4-reductase (DFR)*	pSPB919[a]	Decreased content	Katsumoto et al. (2007)
Salvia miltiorrhiza	Rosmarinic acid and salvianolic acid B	*Phenylalanine ammonia lyase (PAL)*	pPAL1[a]	Decreased content	Song and Wang (2011)
	Danshensu and salvianolic acid B	*Cinnamoyl CoA-reductase (CCR)*	pART27-CCRi[a]	Increased content	Wang et al. (2012)
	Rosmarinic acid	*R2R3 MYB*	pART27[a]	Increased content	Zhang et al. (2013)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Tanshinone	*Copalyldiphosphate synthase (CPS)*	pK7GWIWG2D (II)-SmCPS[b]	Decreased content	Cheng et al. (2014)
	Salvianolic acid B	*Cinnamoyl-coA reductase (CCR)* and *caffeic acid O-methyltransferase (COMT)*	pAtPAP1-SmCCRi-SmCOMTi[a]	Increased content	Zhang et al. (2014)
	Phenolic acids	*Chalcone synthase (CHS)*	pART27[b]	Increased content	Zhang et al. (2015)
	Tanshinone	*CYP76AH1*	pK7GWIWG2D-AH1[b]	Decreased content	Ma et al. (2016)
	Rosmarinic acid and salvianolic acid B	*Caffeoyl-CoA o-methyltransferase* (CCoAOMT)	pART27-CCoAOMTi[a]	Decreased content	Wang et al. (2017)
	Rosmarinic acid and salvianolic acid B	*Myeloblastosis (MYB)* and *transparent testa glabra1 (TTG1)*	p*SmTTG1*–RNAi and pMDC123SB-AtMIR390a-B/c[a]	Decreased content	Li et al. (2018)
	Phenolic acid	*bHLH51*	pSmbHLH51–RNAi[a]	Increased content	Wu et al. (2018)
	Tanshinone	*Ethylene response factor (ERF6)*	pCMBIA1304[b]	Decreased content	Bai et al. (2018)
	Tanshinone	*APETALA2/ethylene-responsive factor (AP2/ERF) (ERF128)*	pK7GWIWG2D (II)-GFP-SmERF128i and pK7WG2D-GFP-SmERF128OX[b]	Increased content	Zhang et al. (2019b)
	Tanshinone	*MYB98b*	pART27[b]	Decreased content	Liu et al. (2020)
	Phenolic acid and tanshinone	*bHLH92*	pK7GWIWG2D (II)[b]	Increased content	Zhang et al. (2020)
Scutellaria baicalensis	Baicalin, baicalein, and wogonin	*Chalcone isomerase (CHI)*	pK7GWIWG2[b]	Increased baicalein and wogonin, and decreased baicalin content	Park et al. (2011)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Baicalin, baicalein, wogonin, and wogonoside	*Flavone synthase II* [(1) *FNS II-1,* (2) *FNS II-2* and (3) *cinnamic acid–specific coA ligase (CLL-7)*]	pK7WGIGW2R[b]	1. No effect 2. Decreased content and novel compound 3. Decreased content	Zhao et al. (2016)
	Baicalin, baicalein, wogonoside, and wogonin	1. *Flavone 6-hydroxylase (CYP82D1.1)* and 2. *Flavone 8-hydroxylase (CYP82D2)*	pK7WGIGW2R[b]	1. Increased wogonoside and wogonin, and decreased baicalin and baicalein content 2. Increased baicalin and decreased wogonoside and wogonin content No change in baicalein content	Zhao et al. (2018)
	Wogonin and wogonoside	*O-Methyltransferases (OMTs)*	pK7WGIGW2R[b]	Decreased content	Zhao et al. (2019)
Symphytum officinale	Homospermidine and pyrrolizidine alkaloids	*Homospermidine synthase (HSS)*	pH7GWIWG2(II)[b]	Decreased content	Kruse et al. (2019)
Taraxacum antungense	Chlorogenic acid and caffeic acid	*Hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase (HQT)*	pCAMBIA1300[a]	Decreased content	Liu et al. (2018)
	5-Caffeoylquinic acid	*Hydroxycinnamoyl-coA quinate hydroxycinnamoyl transferase (HQT)*	pCAMBIA1300[a]	Increased content	Liu et al. (2019)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
Taraxacum koksaghyz	Rubber, poly(cis-1,4-isoprene), dolichols, inulin, and pentacyclic triterpenes	*cis-prenyltransferase-like 1 protein like subunit* 1. *CPTL1* 2. *CPTL2*	pLab12.5-pREF-TbRTA-RNAi (for *CPTL1*) and pFGC5951-TkCPTL2-RNAi (for *CPTL2*)[a]	1. Increased inulin and pentacyclic triterpenes but decreased rubber and poly(cis-1,4-isoprene) content 2. Decreased rubber but no change in poly(cis-1,4-isoprene), inulin and pentacyclic triterpenes content No change in dolichols content	Niephaus et al. (2019)
Torenia fournieri	Anthocyanin and chlorophyll	*Glutathione S-transferase (GST1* and *2), ubiquitin conjugating enzyme (UBC), anthocyanidin synthase (ANS), putative cullin, putative flowering-time, glucose 6-phosphate/phosphate translocator (GPT), glutathione conjugate transporter*	pANDA35HK[a]	Decreased anthocyanin and variation in chlorophyll content	Nagira et al. (2006)
Torenia hybrida	Anthocyanidins	*Anthocyanidin synthase (ANS)*	pSPB[a]	Decreased content	Nakamura et al. (2006)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Aurone	*Aureusidin synthase 1 (AS1), flavanone 3-hydroxylase (F3H), chalcone 4-O-glucosyltransferase (4CGT), dihydroflavonol 4-reductase (DFR)*	pSFL308[a]	Novel color	Ono et al. (2006)
Tripterygium wilfordii	Triptolide	*Copalyl diphosphate synthases (TPS7v2* and *TPS9v2)* and *miltiradiene synthase (TPS27v2)*	pK7GWIWG2D[c]	Decreased content	Su et al. (2018)
	Triptolide and celastrol	*1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR)*	pK7GWIWG2D[c]	Decreased content	Zhang et al. (2018)
	Triptolide and wilforine	*3-Hydroxy-3-methylglutaryl- coA reductase (HMGR)*	pK7GWIWG2_II-RedRoot[b]	Decreased content	Zhang et al. (2019a)
	Triptolide, wilforgine and wilforine	*TGA1*	pYBA1132-RNAi[a]	Decreased content	Han et al. (2020)
Withania somnifera	Withanolide and sterol	*1-Deoxy-D-xylulose 5-phosphate reductoisomerase2 (DXR2)* and *3-hydroxy-3-methylglutaryl coA reductase2 (HMGR2)*	pART27[a]	Decreased content	Singh et al. (2014)
	Glycowithanolide, withaferin A and glycosylated sterols	*Sterol glycosyltransferases (SGTs)*	pFGC1008[a]	Increased withaferin A and decreased glycowithanolide content	Saema et al. (2015)
	Withanolide	*Cycloartenol synthase (CAS)*	pGSA1131[a]	Decreased content	Mishra et al. (2016)

(continued)

Table 9.1 (continued)

Plant	Target metabolite(s)/ pathway	Target gene(s)/transcription factor	Vector*	Effect	Reference
	Campesterol, sitosterol, stigmasterol, and cholesterol	*Sterol methyltransferase1 (SMT1)*	pART27[a]	Increased cholesterol and decreased of campesterol, sitosterol, and stigmasterol content	Pal et al. (2019)

*Transformation method-
[a]*Agrobacterium tumefaciens*
[b]*Agrobacterium rhizogenes*
[c]Particle bombardment/Gene gun
[d]Virus

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