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Review Article

RNAi Silencing and the Modification of Plant Natural Product Pathways

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Abstract

Plant secondary metabolites have important effects on medicine, agriculture, and ecological relationships. They are essential for many biological processes. As RNAi technology has advanced, it has made it possible to study gene regulation and control the production of secondary metabolites in plants. Here we aimed to examine the function of RNAi technology in controlling secondary metabolite biosynthesis in plants. In this review, the use of RNAi technology in riboregulating secondary metabolites and its manipulation in plants were studied. The experimental strategies used to understand the role of RNAi technology on natural product levels and the variety of RNAi techniques utilized for these tasks along with pinpointing any potential future uses were investigated to decipher the intricacies of plant secondary metabolite pathways and methods of post transcriptional gene silencing by various types of non coding RNAs. This study also reviewed the evolution of RNAi technology over time, tracking its beginnings to its application in plant systems. These findings could be successfully applied to regulate secondary metabolite biosynthesis in plants which illustrated the capability of RNAi in regulating the synthesis of useful secondary metabolites for various applications. A thorough and comprehensive literature review through various biological databases like PubMed, Springer Nature, ResearchGate, Google Scholar, Web of Science, ScienceDirect etc. was done for this work. We used keywords such as PTGS (Post Transcriptional Gene Silencing), RNAi (RNA interference), riboregulation, secondary metabolite biosynthesis etc. The future potential of RNAi in controlling the secondary metabolites in plants was discussed in the current review. This work also explored how machine learning algorithms might improve target identification, delivery effectiveness, and precision in RNAi-based techniques by incorporating artificial intelligence (AI) driven strategies. It highlighted the promise of RNAi as a game-changing tool in plant research to envision a bright future which will change secondary metabolite management for numerous practical applications.

Keywords: Plant Secondary Metabolites; RNA Interference (RNAi) Technology; Secondary Metabolite Biosynthesis; Riboregulation; Experimental Strategies; Post-Transcriptional Gene Silencing; Non-coding RNAs

Introduction

Phytochemicals are bioactive compounds present in plants with significant medicinal and nutritive benefits [1]. They protect the plants against infectious diseases, and damage while improving the plant's flavor, color, and scent. These phytochemicals act as a plant's line of defense against ecological threats such as stress due to abiotic factors, pollutants and activity of pathogens [2,3]. Additionally, they give plants a number of qualities like defence, development, cell signaling, ability to reproduce, and allelochemicals to deter herbivores [4,5]. Numerous scrutinizations have shown their significant contribution to human health when consumed in a balanced diet [6,7]. Phytochemicals are generally present in leaves, stems, roots, fruit, flowers, and seeds of plants, as well as in vegetables, fruits, whole grains, seeds, and nuts [8]. The outer layers of plant portions contain higher amounts of several phytochemicals, particularly pigment compounds like anthocyanins and flavonoids. The sort of plants that grow depends on the climatic conditions. Depending on the plant's environment, growth stage, and phytochemical levels, variations may occur [9]. Their pharmacological effects include the stimulation of the immune system, antioxidant activity, antibacterial action, anticancer property, modification and manipulation of hormone metabolism pathways, and reduction of agglomeration of platelets [10]. Surprisingly, the existence of phytochemical substances in medicinal plants results in the production of abundant bioresource components for use in a variety of systems, including medications, intermediates for use in medicine, food supplements, dietary supplements, as well as chemical components for synthetic and semi-synthetic therapies [11]. Because of their immediate relevance to human health, phytochemical substances with nutraceutical properties that are found in food are particularly significant. The phytochemicals in plants have demonstrated their ability to lower the risk of infectious diseases like bacterial infections, ulcers, inflammation, and hypertension. There are also non-communicable illnesses including diabetes, cancer, cardiovascular disease, and respiratory problems. [12]. Generally speaking, primary metabolites and secondary metabolites are two categories for phytochemicals according to how they contribute to plant metabolism. Secondary metabolites include curcumin, lignans, phenolics, alkaloids, flavonoids, terpenes, saponins, and glucosides [13,14]. Below is a table listing some important secondary metabolites (Table 1).

Secondary metabolite	Plant source	Use	Reference
Aconitine	*Aconitum chasmanthum* (common name- Indian Napellus)	Sciatica, neuralgia, and rheumatism	[15]
Atropine	*Atropa belladonna* (common name-Deadly Nightshade)	Medication that is cycloplegic, anti-Parkinson's, and antispasmodic	[16]
Caffeine	*Coffea Arabica* (common name- Arabian Coffee)	Apnea in infancy and atopic dermatitis	[17]
Colchicine	*Colchicum autumnale* (common name- Autumn Crocus)	Acute gout therapy and amyloidosis treatment	[18]
Cocaine	*Erythroxylum coca* (common name- Cocaine)	A regional anaesthetic	[19]
Morphine	*Papaver somniferum* (common name- Opium Poppy)	Diarrhoea and pain alleviation	[20]

Taxol	*Taxus brevifolia* (common name- Western Yew)	Anti-cancer	[21]
Berberine	*Berberis vulgaris* (common name- Common Barberry)	AIDS, hepatitis, and eye irritation cure	[22]
Nicotine	*Nicotiana tabacum* (common name- Tobacco)	Anti-smoking	[23]
Ergotamine	*Claviceps purpurea* (common name- Ergot)	Postpartum/postabortion bleeding	[24]

Table 1: Uses of a few significant secondarily metabolites from various plants.

There are three major classes of phytochemicals, namely terpenes, alkaloids and phenolic compounds. There are two processes for producing terpenes: the methylerythritol phosphate approach and the mevalonate pathway. The cytosol and the plastid, which are two distinct organelles, respectively, are where these two processes are finished. Mono-, di-, and tetraterpenes are produced through "methylerythritol 4-phosphate (MEP) pathway", whereas sesquiterpenes and triterpenes are produced by the "MVA system". Prenyltransferase is an enzyme that produces the complex terpenes DMAPP (dimethylallyl diphosphate) and IPP (isopentyl diphosphate) which have high molecular weights and act as fundamental building blocks of these two activities [25]. The second largest class of phytochemicals is alkaloids. They consist of more than 15,000 water soluble secondary metabolites with biological activities, having at least one structural nitrogen atom [26]. L-phenylalanine and L-tyrosine are the precursors of the shikimate pathway, which is used to synthesise the third main group, the phenolic chemicals [27]. The principal phenolic compounds which are aromatic, such as aromatic polypeptides, lignans, coumarins, flavonoids etc. are produced by the aforementioned aromatic amino acids [28]. Figure 1 shows the production of major types of phytochemicals.

Plants may now be genetically modified more quickly and accurately thanks to biotechnological techniques and applications. Specifically necessary for both reproduction and genetically upgrading medicinal plants, are biotechnological technologies including genetic transformation and in vitro regeneration [29].

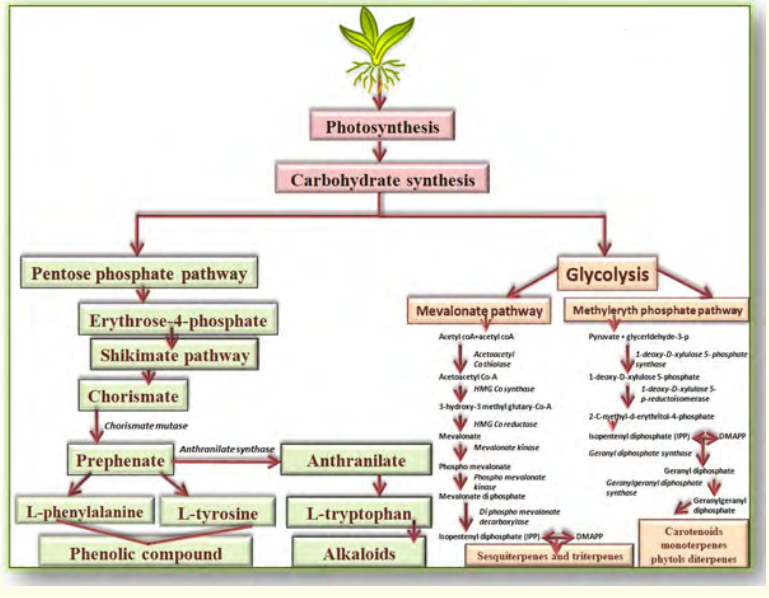

Figure 1: Diagram of the biosynthesis of major types of phytochemicals [25,30].

One such illustration of this is the technology known as RNA interference (RNAi) or posttranscriptional gene silencing (PTGS), which controls the expression of genes. The modification of natural products from herbs that are medicinally important—which can be utilized as colours, medicines, scents, insecticides, and even food additives—has also been greatly impacted by this. Currently, medicinal plants are regarded as being extremely important as life-saving medications. The dependency of world population on medicinal plants has been incread in recent days leading to a considerable augmentation in the utilization of goods and products derived from these plants, additionally, several instances of the gene knockdown strategy, which is frequently employed as a practical way to improve the manufacturing of secondary metabolites inside medicinal plants have become significant in recent days [31].

A biological response known as RNAi or PTGS to "dsRNA" may be explained as the destruction of the "mRNA" corresponding to "dsRNA". A high level of selectivity is present in this dsRNA-mediated gene silencing pathway [32]. The extensive application of this technology has enhanced as well as increased the commercial value of medicinal plants along with their usage in the production of pharmaceuticals and flavourings. In 1998, a specific gene's mRNA degradation mechanism was identified [33]. The RNAi mechanism is triggered by the existence of molecules of RNA in pairs of double strands within a cell. The intricate biochemical mechanism that promotes dismantling of the molecules of mRNA carrying double-stranded RNA that is genetically indistinguishable is triggered by the activation of dsRNA during this process. As soon as mRNA molecules vanish, the accompanying genes shut down, eradicating any form of protein they were encoding. Numerous eukaryotes, including fungi, plants, and mammals, have this conserved mechanism [34].

RNAi Machinary

RNA-induced silencing complex (RISC)

This particular endonuclease picks out and destroys cellular mRNAs that the strand of siRNA complements. It is a blend of proteins and siRNA. The targeted RNA is then separated by the RNAse enzyme when the RISC locates it [35]. Translation levels are lowered by RISC, which is often affiliated with 20–23 bp siRNA [36]. Therefore, it may be said that this protein complex acts as an accelerator to cut one phosphodiester link from mRNA. A ribonuclease protein known as Dicer aids the process of breakdown of dsRNA into uniform, short fragments of ds-RNA (siRNA). It has helicase domain, which splits dsRNA separated by 21–25 base pairs, and creates 2-nt overhanged siRNA with 50 phosphorylated ends [37]. The RNAi pathway's initial step uses this component as a catalyst. Argonaute, an accelerator component of dicer, with the capability to degrade mRNA into the strand which acts as "guide siRNA" is also crucial part of this multiprotein complex [38].

RNA-Dependent RNA polymerase (RdRP)

RdRp can be defined as a RNA enzyme with multiple functional capabilities that aids in genome replication and supports the transcriptional activity of single-stranded (ss) RNA into double-stranded (ds) RNA. Despite the variation in sequences, the structure's fundamental characteristics have remained constant and mimic a right palm which is cupped [39].

siRNA (Primary and secondary)

The dsRNA impetus yields diverse range of siRNAs (primary), which serves as determinant of specificity of RISC. This can be challenging to recognize why microinjecting 400–500 bp dsRNA results in a significant RNA degradation response when amplified dsRNA is not present [40]. Numerous researches have examined the possibility of RNAi targeting RNA sequences beyond the dsRNA-inducer molecule. The unc-22 gene of endogenous origin is also muted in the event of transitive silencing. This implies that the expansion of target sequences for silencing is caused by interaction with the unc-22::GFP transcript [41]. Numerous subsequent RNase protection tests have supported the aggregation of siRNAs with sequence similarity to unc-22 [41,42]. It was determined that the RdRP rrf-1 mutant is necessary for the synthesis of "secondary" siRNAs. Due of this, a model machinery that shows the mechanism of induction of primary siRNAs was proposed from the direct cleavage of newly inducted, ds RNA molecules which are homologous mRNA-pairable. In addition to synthesising a sizable population of secondary siRNAs, this leads to the generation of additional dsRNAs. These siRNAs can sometimes be amplified and extend beyond the original dsRNA trigger, which makes them extremely important for PTGS. Figure 2 depicts mechanism of RNAi gene silencing.

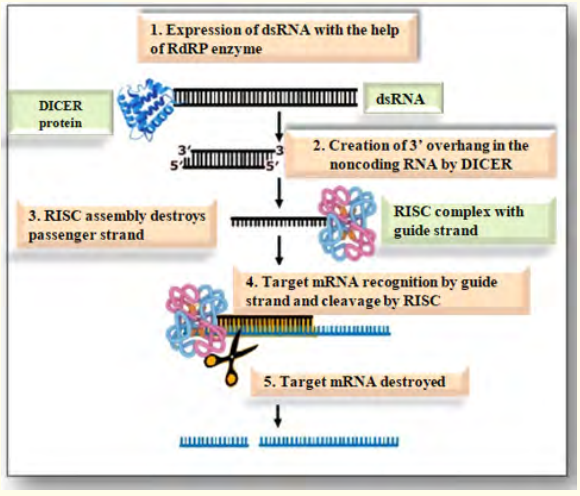

Figure 2: Mechanism of RNAi gene silencing.

However, plants appeared to have a comparable biochemical machinery to that of transitive silencing that produced massive quantities of dsRNA from the transgenes that were incorporated. The intensification effect where transportable molecule which can transmit information from nearby PTGS starting location which initiates the production of dsRNA de novo in disconected tissues using homologous mRNA templates which may contribute to systemic silencing in plants [42].

The mechanism underlying RNAi's effects

RNAi technique promotes synthesis of "aberrant ssRNA" from a transgene. The biosynthesis of double stranded RNA with the aid of RdRP is inclusive of this process. Along with initiating the RNAi pathway, this in turn starts the process of RdRP producing dsRNA [43]. The machinery also incorporates miRNA (microRNA), which controls gene expression in both plants and animals [44, 45]. A tiny hpRNA (hairpin RNA) known as a precursor of miRNA (pre-miRNA) has "bulges" in the stem. Every pre- ds-, hp- miRNA can be converted by Dicer producing "RNA duplexes" of 21–25 nt length. As dsRNA reaches plant cell, the Dicer enzyme targets it. When Dicer is triggered by ATP, it splits the dsRNA into 21–25 siRNAs. These are then assembled into the RISC-designated nuclease complex. These integrated siRNAs are unravelled in the following stage [46]. The noncoding strand that is still present in the multiprotein complex (RISC), which additionally destroys mRNA, is what allows for complex activation. The siRNA complements the cleaved mRNA. In order to create a novel chemical in plants, heterologous genes are typically inserted. By using RNAi technology, many of the stages in the manufacture of these plant molecules can be controlled to lower levels of undesired substances. Listed below is a summary of the noncoding RNAs and their regulatory effects on the production of particular phytochemicals (Table 2).

Secondary metabolite	Plant source	Use	Reference
Aconitine	*Aconitum chasmanthum* (common name- Indian Napellus)	Sciatica, neuralgia, and rheumatism	[15]
Atropine	*Atropa belladonna* (common name-Deadly Nightshade)	Medication that is cycloplegic, anti-Parkinson's, and antispasmodic	[16]
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Nicotine	*Nicotiana tabacum* (common name- Tobacco)	Anti-smoking	[23]
Ergotamine	*Claviceps purpurea* (common name- Ergot)	Postpartum/postabortion bleeding	[24]

Table 2: Noncoding RNAs and how they regulate the synthesis of certain phytochemicals.

RNAi applications in plants with therapeutic significance

Centella asiatica (Apiaceae) (common name-Asiatic Pennywort)

According to investigations by Sharma., *et al*. 2020, the genes HMGR and DXR were silenced in *Centella asiatica* by the introduction of the CaHMGR-RNAi and RNAi-DXR and constructs. In the MVA and MEP pathways, respectively, the DXR and HMGR are enzymes of regulatory importance. This study therefore contributed to the understanding that these two genes are necessary for the manufacture of centelloids, which are terpenoid saponins found in *Centella asiatica* [75,76]. Figure 3 illustrates how RNAi silencing of the DXR and HGMR genes reduced biosynthesis of centelloids in *Centella asiatica*.

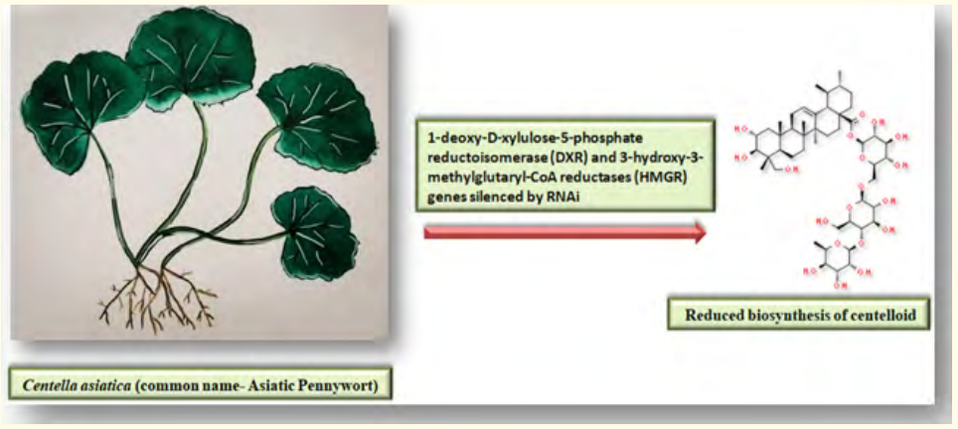


Figure 3: RNAi silencing of the DXR and HGMR genes reduced biosynthesis of centelloids in *Centella asiatica*.

Artemisia annua (Asteraceae) (common name- Annual Absinthe)

Artemisinin, a sesquiterpenoid endoperoxide molecule that is extremely effective against malaria, is isolated from this plant. With the help of RNA interference (RNAi) technology, the downregulation of the CH4 gene was made possible, and salicylic acid (SA) and artemisinin levels both increased [77]. The DXR gene's critical function in the manufacture of artemisinin in *A. annua* was discovered thanks to RNA interference (RNAi)-mediated silencing of the gene [78]. In order to enhance the medicinal characteristics of *Artemisia annua*, RNAi technology has been widely applied. The AaPDR3 gene was knocked out to create AaPDR3-RNAi transgenic *Artemisia* plants, which had low levels of -caryophyllene and higher levels of artemisinin [79]. Similar to this, boosting artemisinin by decreasing the expression of the SQS gene was made possible by RNAi technology [80]. Additionally, it was discovered that the transcription factor AaHY5 positively regulates the manufacture of artemisinin, and this was proved by experimentation owing to the effect of RNAi silencing of the AaHY5 gene [81]. Figure 4 depicts RNAi mediated downregulation of CH4 gene causing enhanced biological synthesis of artemisinin in *Artemisia annua*.

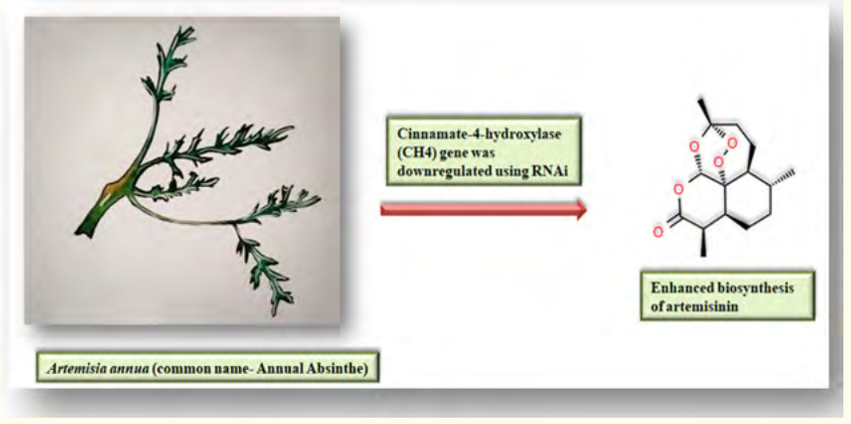


Figure 4: RNAi mediated post transcriptional silencing of the CH4 gene causing enhanced biological synthesis of artemisinin in *Artemisia annua.*

Panax notoginseng (Araliaceae) (common name-Tienchi Ginseng)

China makes substantial use of this well-known medicinal herb. By using RNAi technology, the biological synnthesis of triterpene in this plant was improved. Farnesyl pyrophosphate synthase (FPS) gene transformation was followed by RNAi-mediated silencing of the CAS (cycloartenol synthase) gene. The observation showed that these transgenic *Panax notoginseng* lines produced more saponins than usual [82]. Figure 5 shows post transcriptional gene silencing of CAS gene in *Panax notoginseng* by RNAi for enhanced saponin biosynthesis.

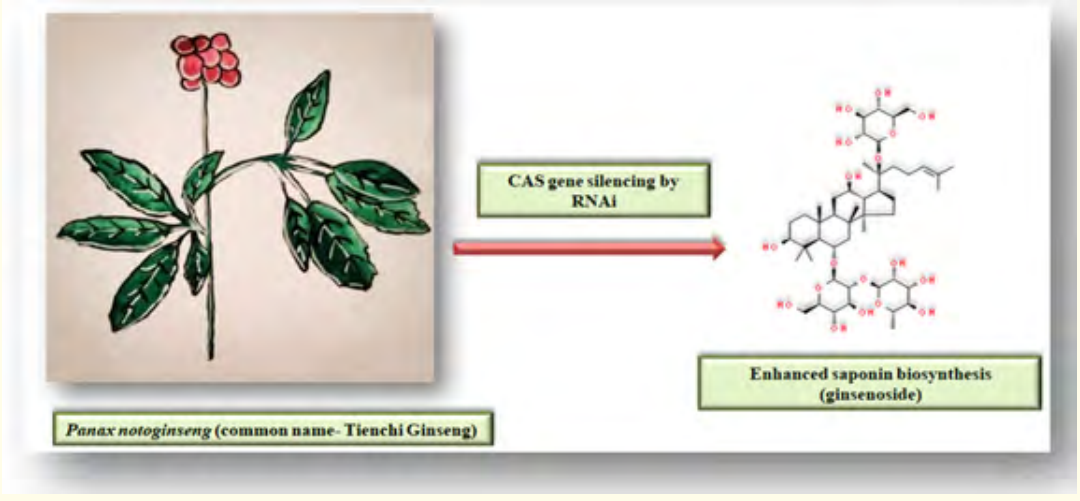


Figure 5: Post transcriptional gene silencing of CAS gene in *Panax notoginseng* by RNAi for enhanced saponin biosynthesis.

Rehmannia glutinosa (Plantaginaceae) (common name-Chinese Foxglove)

Rehmannia glutinosa, member of the Scrophulariaceae family is a popular herb of therapeutic importance in China. Several different pharmacologically effective chemicals can be found in its roots. However, the discovery that the C3H gene in this plant is in charge of producing phenolic acid/phenylpropanoid was made possible by using RNAi suppression technology.

Allelopathic phenolic production is downregulated in the roots of *R. glutinosa* as a result of P-coumarate-3- hydroxylase (C3H) being suppressed by RNAi [83]. Figure 6 shows P-coumarate-3-hydroxylase (C3H) gene being suppressed by RNAi technology which downregulated phenolic synthesis in *Rehmannia glutinosa*.

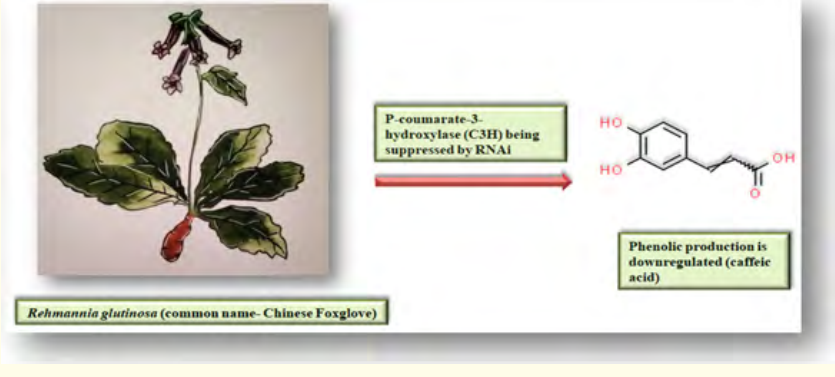


Figure 6: C3H gene being suppressed by RNAi technology which downregulated phenolic synthesis in *Rehmannia glutinosa*.

Isatis indigotica (Brassicaceae) (common name-Woad)

64 IiWRKY genes were found in this plant's transcriptome. Additionally, it was discovered that the expression of IiWRKY34 in tetraploids was much higher than in diploids, which is favourably linked with aggregation of lariciresinol. IiWRKY34 regulates production of the compound lariciresinol. Experimentations using overexpressed genes and RNA interference have shown that this gene also stimulates root growth and salt and drought stress tolerance [84]. Figure 7 shows IiWRKY34 gene silenced by RNAi which caused a reduction in lariciresinol biosynthesis in *Isatis indigotica*.

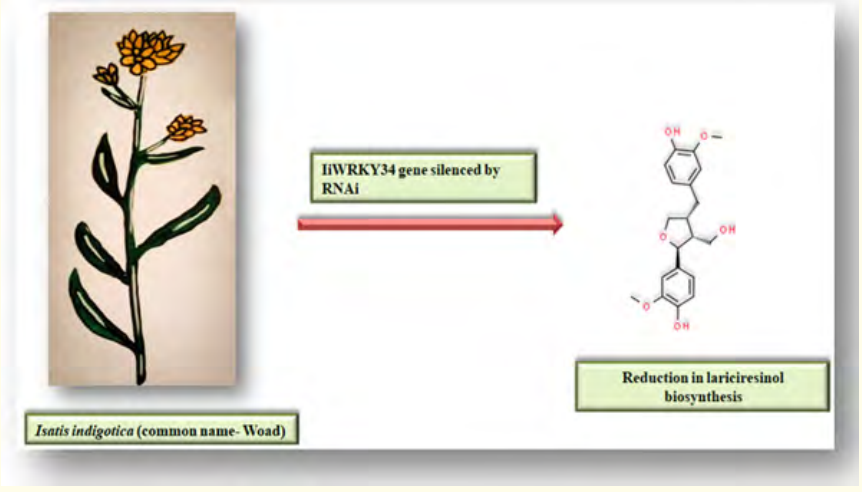


Figure 7: IiWRKY34 gene silenced by RNAi which caused a reduction in lariciresinol biosynthesis in *Isatis indigotica*.

Brassica napus (Brassicaceae) (common name-Rapeseed)

Rapeseed growth and development have been shown to be reduced by RNAi reduction of the expression of BnMYB43 gene family [85]. Additionally, it reduces yield and weakens lodging resistance. This restriction does, however, enhance defences towards *Sclerotinia sclerotiorum*. These results show that BnMYB43, a crucial component which triggers activation of defense mechanisms at the expense of growth suspension, positively regulates vascular lignifications, shape of the plant, and potential of yeild while negatively influencing immunity against *S. sclerotiorum* [85].

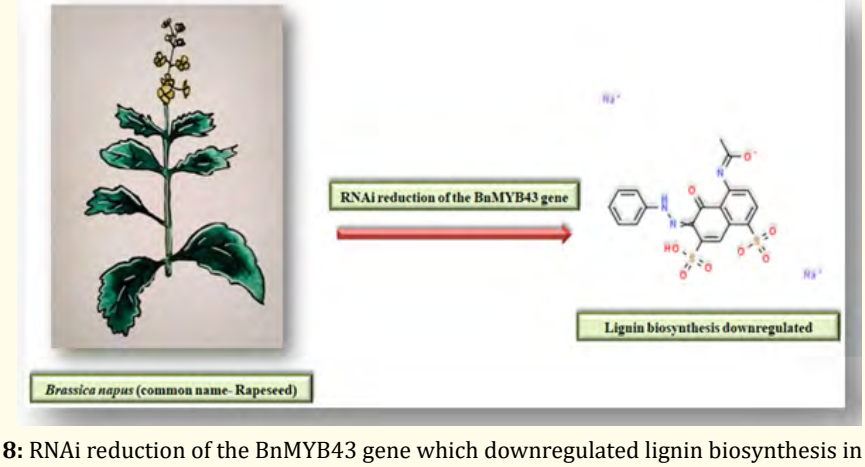


Figure 8: RNAi reduction of the BnMYB43 gene which downregulated lignin biosynthesis in *Brassica napus*.

Figure 8 depicts RNAi reduction of the BnMYB43 gene which downregulated lignin biosynthesis in *Brassica napus*.

Panicum virgatum (Poaceae) (common name- Switchgrass)

Coniferaldehyde and coniferyl alcohol are hydroxylated in angiosperms to produce syringyl (S) lignin by ferulate 5-hydroxylase (F5H).

Caffeic acid O-methyltransferaseRNAi (COMT-RNAi) transgenic *Panicum virgatum* (switchgrass) plants show that the F5H downregulation inhibited the formation of S lignin, which further led to an increase in G (guaiacyl) units and a decrease in 5-OH (G units). However, when F5H was overexpressed in COMT-RNAi plants (transgenic), concentration of 5-OH units was elevated and G units were lowered at that time.

Relying on the level of COMT silencing in *Panicum virgatum*, the loss in S lignin biosynthesis was either fully or partially compensated [86]. Figure 9 shows F5H gene downregulated by RNAi which caused a reduction hydroxylation of coniferyl alcohol in *Panicum virgatum*.

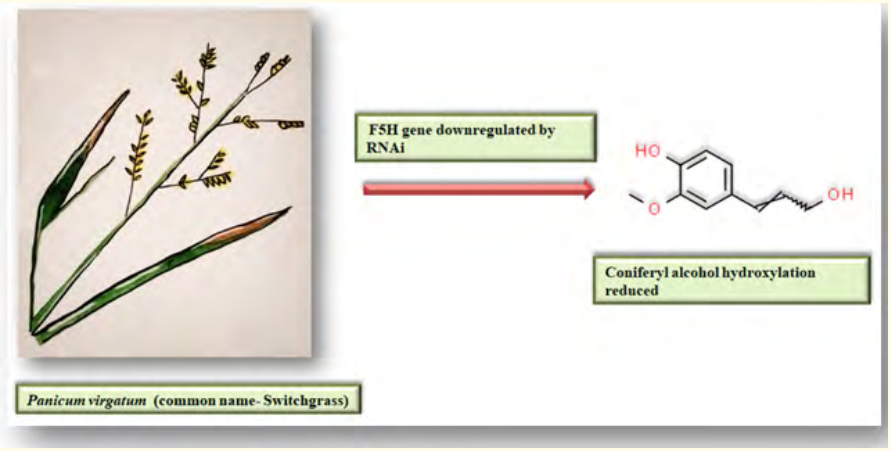


Figure 9: F5H gene downregulated by RNAi which caused a reduction hydroxylation of coniferyl alcohol in *Panicum virgatum*.

Papaver somniferum (Papaveraceae) (Common Name- Opium Poppy)

Using RNA interference (RNAi), it has been possible to silence the codeinone reductase-encoding gene COR in this plant. Transgenic plants aggregated the alkaloid (S)-reticuline after mRNA cleavage at the expense of other chemical compounds. This occurs seven enzymatic steps prior to codeinone reductase. The unexpected accumulation of (S)-reticuline suggests the existence of a regulating system that blocks intermediates of biological synthesis of benzylisoquinoline from approaching the branch which is morphine-specific. Upstream and downstream of (S)-reticuline, there are seven more enzymes in the route which had unchanged transcript levels. Therefore, RNAi-mediated substitution of reticuline, a non-narcotic alkaloid, along with morphine in *Papaver somniferum* may be feasible [87]. Figure 10 depicts reduction of the codeinone reductase-encoding gene COR by RNAi which enhanced biosynthesis of (S)-reticuline in Opium Poppy.

Populus sp. (Salicaceae) (common name- Poplar)

Identifying a certain gene's role namely MYB134 was made possible through RNAi-mediated silencing. Reduced aggregation of "CT" (Condensed Tannin) occurs when the expression is shut down via RNA interference (RNAi). Because of this, MYB134 is in charge of the biosynthesis of CT. The buildup of CT in the roots was unaffected in the transgenic *Populus sp*., indicating the existence of extra CT controller in the roots. This highlights the intricacy of CT directive in *Populus sp*. The leaves of control and the experimental MYB134-RNAi were uncovered to the RO species

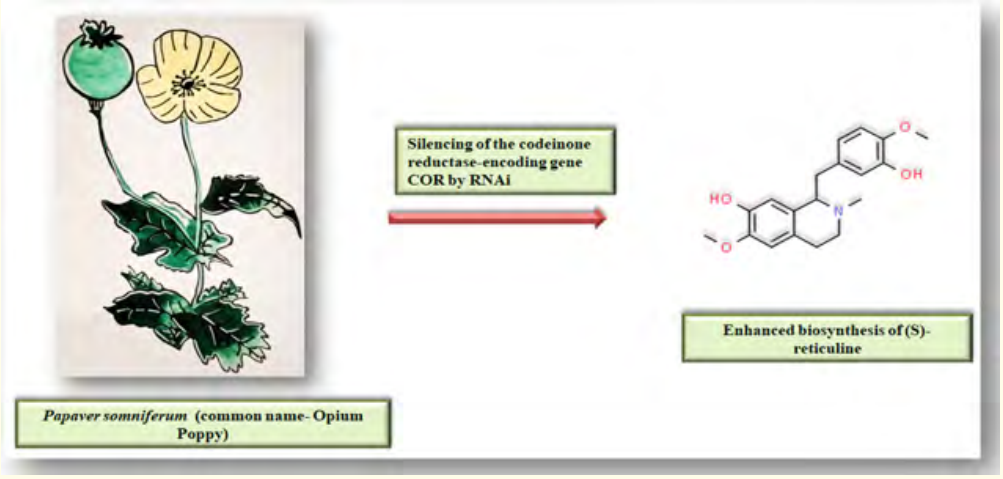


Figure 10: Reduction of the codeinone reductase-encoding gene COR by RNAi which enhanced biosynthesis of (S)-reticuline in Opium Poppy.

production (methyl viologen) in order to assess the impact of CT downregulation during resilience shown to oxidative stress. Lower chlorophyll fluorescence was found in MYB134-RNAi plants compared to wild-type leaves, indicating much more photosystem II damage. Compared to wild-type leaves, MYB134-RNAi plants had concentrations of H_2O_2 (hydrogen peroxide) which was much higher. H_2O_2 is another type of ROS or reactive oxygen species. This suggests that "Condensed Tannin" can act as an antioxidant and moreover shield plants from stress due to oxidation [88]. Figure 11 shows MYB134 gene silenced by RNAi which caused accumulation of a higher concentration of hydrogen peroxide in *Populus nigra*.

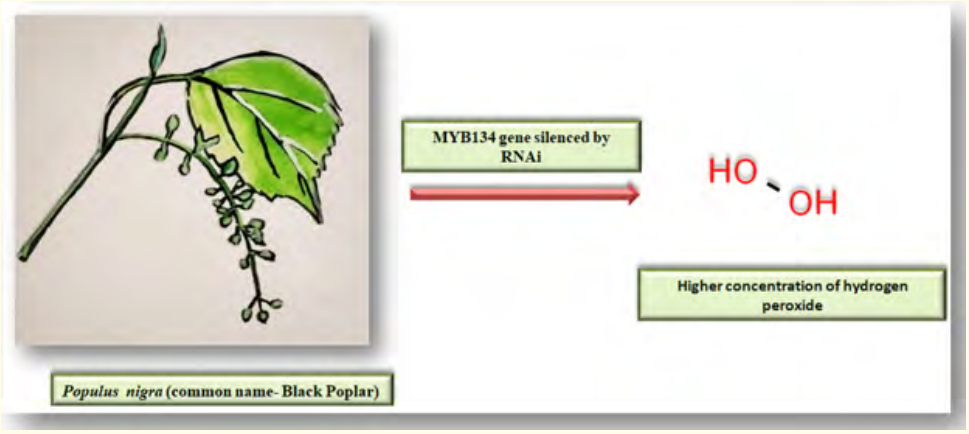


Figure 11: MYB134 gene silenced by RNAi which caused accumulation of a higher concentration of hydrogen peroxide in *Populus nigra*.

Betula platyphylla (Betulaceae) (common name- Asian White Birch)

This plant underwent post transcriptional silencing of the gene named GSNOR, which codes for S-nitrosoglutathione reductase. When compared to the wild-type plant, this causes the production of betulin in transgenic lines to increase by a factor of at least two. Furthermore, lupeol synthase (LUS), which is encoded by a different gene, was expressed more frequently in GSNOR-RNAi transgenic plants. The essential enzyme LUS is necessary for the production of betulin. These results demonstrated that betulin production is mediated by GSNORRNAi suppression both genetically and pharmacologically [89].

Research showed that post transcriptional silencing of cycloartenol synthase and -amyrin synthase significantly decreased the manifestation of genes responsible for biological synthesis of triterpenoid. In BpCAS gene silencing birch, BpY and BpW gene function was increased. For Bp-AS silencing in *Betula* tree, BpY gene function was significantly reduced but BpW expression was increased. The amount of betulinic acid in BpCAS-silenced birch was significantly increased. In addition, BpCAS silencing birch had significantly higher levels of betulinic acid, oleanolic acid, total triterpenoids, and soluble sugars [90]. Figure 12 shows RNAi-mediated silencing of the GSNOR gene which caused an increase in the biosynthesis of betulin in *Betula platyphylla*.

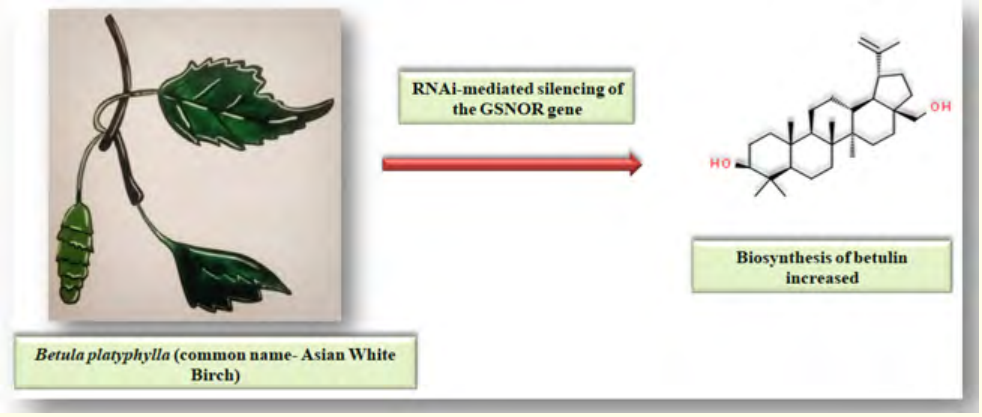


Figure 12: RNAi-mediated silencing of the GSNOR gene which caused an increase in the biosynthesis of betulin in *Betula platyphylla*.

Nicotiana tabacum (Solanaceae) (common name-Tobacco)

It was observed reduced nicotine levels in the leaves of certain plants when they downregulated three enzymes - "ornithine decarboxylase," "aspartate oxidase," and "arginine decarboxylase." The most significant reduction in nicotine concentration was seen in transgenic plants where "aspartate oxidase" was silenced using RNA interference. Additionally, it was found that putrescine, a primary polyamine involved in nicotine biosynthesis, displayed a good relationship with nicotine concentration in tobacco (transgenic) created through "RNAi-silencing" of "ornithine decarboxylase", "arginine decarboxylase" [91].

A research was conducted research on NtHDG2-RNAi transgenic lines, employing RNAi-mediated silencing to target the HD-ZIP family member class IV gene, "NtHDG2." Interestingly, it was discovered that the NtHDG2-RNAi plants exhibited a reduced content of flavonols compared to wild-type plants, ranging from 20.9% to 52.7%. Further investigations revealed that the gene "NtMYB12," having regulatory function with 3 genes (structural) - "NtF30 H," "NtPAL", "NtF3GT," which are responsible for flavonoid formation, were expressed in such a way that led to increased flavonol accumulation in Nicotiana leaves, seemingly driven by NtHDG2 [92]. Figure 13 illustrates the impact of RNAi-induced silencing of "aspartate oxidase," resulting in the lowest level of nicotine biosynthesis in *Nicotiana tabacum*.

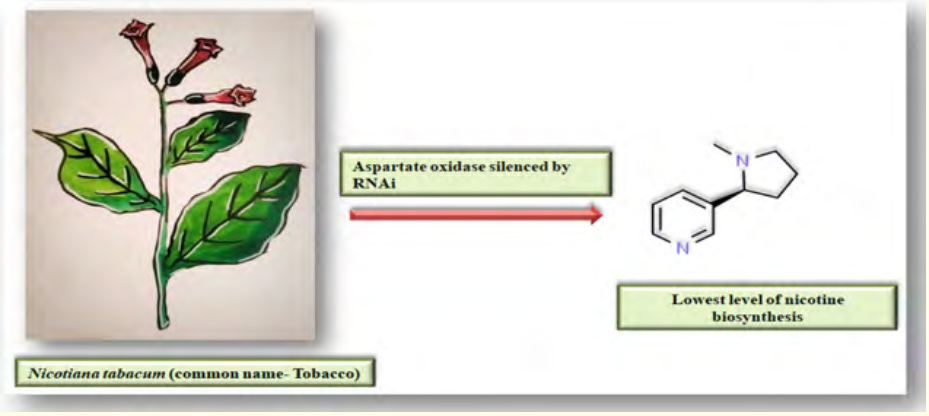


Figure 13: Aspartate oxidase silenced by RNAi which caused lowest level of nicotine biosynthesis in *Nicotiana tabacum*.

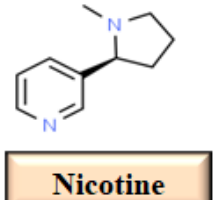
Table 3 enlists the application of RNAi to mediate silencing in various medicinal plants.

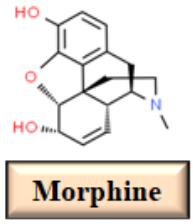
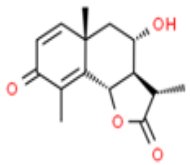
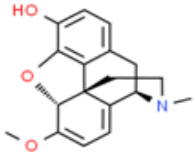
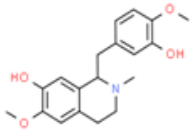
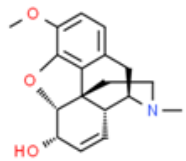
Species	RNAi silences the target gene or transcription factor	Effect observed	Reference
Artemisia annua (Asteraceae) (common name- Annual Absinthe)	Cinnamate-4-hydroxylase (CH4)	This gene's downregulation results in an increase in the amount of artemisinin	[77]
	DXR gene	This gene silencing helps our understanding towards the artemisinin pathway	[78]
	AaPDR3 gene	A decrease in the amount of -caryophyllene and a progressive increase in the amount of artemisinin	[79]
	Squalene synthase SQS	Turning down this gene boosts artemisinin	[80]
	AaHY5	AaHY5 positively controls artemisinin biosynthesis	[81]
Centella asiatica (Apiaceae) (common name- Asiatic Pennywort)	DXR gene	Production of centelloids is reduced when these two genes are silenced	[75]
Panax notoginseng (Araliaceae) (common name- Tienchi Ginseng)	Cycloartenol synthase (CAS)	Increased saponin concentration	[82]
Rehmannia glutinosa (Plantaginaceae) (common name- Chinese Foxglove)	P-coumarate-3-hydroxylase (C3H)	Allelopathic phenolic production is downregulated in the roots of *R. glutinosa* when this gene is silenced	[83]
Isatis indigotica (Brassicaceae) (common name- Woad)	IiWRKY34	Lariciresinol's production is adversely regulated by RNAi silencing of this gene	[84]

Brassica napus (Brassicaceae) (common name- Rapeseed)	BnMYB43	Oilseed rape's growth and development are reduced by RNAi suppression of this gene, but it increases the plant's resistance to *Sclerotinia sclerotiorum*	[85]
Panicum virgatum (Poaceae) (common name- Switchgrass)	Caffeic acid O-methyltransferase (COMT)	Downregulation of Ferulate 5-hydroxylase (F5H) reduces S lignin biosynthesis while increasing Guaiacyl (G) units.	[86]
Papaver somniferum (Papaveraceae) (common name- Opium Poppy)	Codeinone reductase (COR)	Codeine, morphine, thebaine, and oripavine all reduced, but (S)-reticuline increased	[87]
Populus sp. (Salicaceae) (common name- Poplar)	MYB134	Condensed tannin (CT) buildup is decreased when this gene is inhibited by RNA interference (RNAi)	[88]
Betula platyphylla (Betulaceae) (common name- Asian White Birch)	S-nitrosoglutathione reductase (GSNOR)	Increased betulin biosynthesis is caused by this gene's inhibition, which also increases the expression of the lupeol synthase (LUS) gene	[89]
	BpCAS (Cycloartenol synthase	Enhancement of BpY and BpW gene function	[90]
Nicotiana tabacum (Solanaceae) (common name- Tobacco)	Genes encoding ornithine decarboxylase, aspartate oxidase, and arginine decarboxylase	Putrescine concentration is also regulated by these genes, and their silencing by RNAi lowers nicotine levels	[91]
	NtHDG2	This gene's silencing results in a 20.9% reduction in flavonol concentration	[92]

Table 3: Application of RNAi-mediated silencing in many medicinal plants.

The use of RNAi technology to increase the production of certain phytochemicals and lowering levels of undesired substances in some plants [93].

Species	Phytochemical name and chemical structure	Molecular formula	Average mass	Monoisotopic mass
Nicotiana tabacum (common name- Tobacco)	 Nicotine	$C_{10}H_{14}N_2$	162.232 Da	162.115692 Da

Papaver somniferum (common name- Opium Poppy)	 Morphine	$C_{17}H_{19}NO_3$	285.338 Da	285.136505 Da
Artemisia annua (common name-Sweet Wormwood)	 Artemisinin	$C_{15}H_{18}O_4$	262.301 Da	262.120514 Da
Papaver somniferum (common name- Opium Poppy)	 Oripavine	$C_{18}H_{19}NO_3$	297.348 Da	297.136505 Da
Papaver somniferum (common name- Opium Poppy)	 Reticuline	$C_{19}H_{23}NO_4$	329.390 Da	329.162720 Da
Papaver somniferum (common name- Opium Poppy)	 Codeine	$C_{18}H_{21}NO_3$	299.364 Da	299.152130 Da

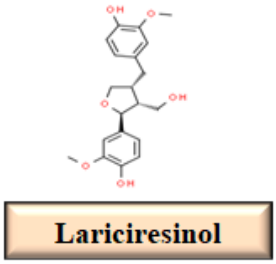
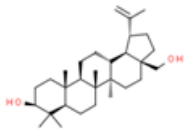
Sambucus williamsii (common name- North China Red Elder)	 Lariciresinol	$C_{20}H_{24}O_6$	360.401 Da	360.157288 Da
Betula platyphylla (common name- Asian White Birch)	 Betulin	$C_{30}H_{50}O_2$	442.717 Da	442.381073 Da

Table 4: Enhanced Production of Certain Compounds Using RNAi.

(Chemical data and structures were taken from- https://www.chemspider.com/).

Applications involving "gene silencing" can benefit significantly from "RNA interference" (RNAi) as a potent tool. Ever since its discovery RNAi has found widespread use in the scientific community [40]. However, in the realm of therapeutic plants, RNAi is still in its early developmental stages. It is worth noting that RNAi and gene disruption strategies differ conceptually, each presenting a unique set of advantages and disadvantages.

The utilization of RNAi proves beneficial in facilitating the production of crucial pharmacological compounds within medicinal plants. This technology serves as a valuable asset for research purposes, accelerating processes and inspiring novel applications. Numerous instances demonstrate RNAi technology's effective employment in identifying genes govening diverse pharmacological traits or studying outcomes of specific gene silencing events.

To harness the potential of RNAi fully, it becomes imperative to comprehend its effects on medicinal plants and design an inducible RNAi system, involving a proficient inducer and an appropriate promoter. As it was expound, the comprehension of RNAi technology's impact on medicinal plants is gradually unraveling, hinting at its capacity to enhance their properties [93].

Plant regulatory noncoding RNAs

As per a study, plants showcase a diverse range of noncoding RNAs that actively regulate gene expression, function at the time of transcription and post-transcription processes, influencing many developmental events of plant growth and its response towards environmental stressors and other signals [94]. "MicroRNAs," a notable group of regulatory RNAs, are renowned for their multifaceted regulation [95].

These RNAs (endogenous), consisting of 18 to 24 nt length, play crucial roles in development and stree response of plants through gene expression and function at post-transcriptional level. The miRNA target gene regulation depends on the complementary sequence of miRNAs and its target gene. Interaction between miRNAs and their targets can lead to mRNA cleavage, translation inhibition, or the production of secondary small interfering RNAs. Recent insights have identified miRNAs as potential bioactive agents capable of cross-species and cross-kingdom gene expression regulation [96].

Additionally, "small interfering RNAs" (siRNAs) have emerged as crucial controllers of plant responses which may be activated by miRNA [97]. These "phased secondary siRNAs," often around

21 or 24 nucleotides in length, exhibit a unique phased expression pattern. Among these siRNAs a subset is referred to as "trans-acting siRNAs" [98].

Apart from miRNAs and siRNAs, "long noncoding RNAs" (lncRNAs) are another significant category of noncoding RNA in plants. These lncRNAs, typically longer than 200 base pairs and incapable of encoding complete proteins, act as essential regulators of plant genes. It was seen that lncRNAs can function as scaffolds assembling various proteins into functional complexes, decoys mimicking specific regions of target proteins, guides for other small RNA complexes to reach target sites, or enhancers generating downstream regulatory RNAs [99-101].

Recent studies have shed light on "circular RNA", a prevalent type of noncoding (nc) RNA. CircRNAs have drawn significant attention due to their potential role as miRNA decoys in plants [102-105]. Genomic analyses in *Salvia miltiorrhiza* explored many circRNAs containing miRNA-attaching region, some of that regulate genes responsible for producing growth regulators like brassinosteroids and gibberellins, as well as secondary metabolites such as terpenes. Notably, these regulatory noncoding RNAs have garnered considerable interest for their impact on secondary metabolite production, stress responses, and various developmental stages.

Secondary metabolite biosynthesis is regulated by miRNA

The process of miRNA biogenesis and mRNA silencing in plants follows a specific pathway. Initially, "MIR genes" undergo translation by Pol II, resulting in the formation of "pri-miRNAs" that folds create hairpin-like structures. Nuclear splicing following subsequent process require the cooperative actions of "HYL1," "TGH," and "SE." DCL1 then successively processes "pre-miRNAs" and "pri-miRNAs", generating 1 or many "phased miRNA/miRNA* duplexes". After that, methylation occurred by "HEN1" and transported in cytoplasm by "HST1." Once inside the cell, the selected miRNA is incorporated into a specific "RISC" containing "AGO1," which directs it to either cleave the target mRNA transcript or inhibit its translation. Figure 14 visually represents the miRNA biogenesis process in plants.

Recent revelations have acknowledged the role of miRNAs as "riboregulators" influencing the biosynthesis and accumulation of secondary metabolites in plants [106,107]. Additionally, studies have demonstrated the involvement of miRNAs in regulating various metabolites across different plant species, including flavonoids, alkaloids, terpenoids, and lignin [108]. Flavonoids, known for their critical functiong as signaling molecules, phytohormones, facilitators of plant and microbe relationships, and agents involved in responses against stress, represent a class of metabolites (secondary) composed of phenylpropanoids. These are widely distributed in the plant kingdom [109-112]. The core phenylpropanoid pathway gives rise to various flavonoid metabolites, with multiple enzymes and substrates being shared among them. Several miRNAs were identified as the regulator of flavonoid production.

A previous computational analysis using 323,318 ESTs from *Helianthus*, researchers discovered miRNAs from the miR2911 family that are directly involved in controlling tocopherol production in the plant [74].

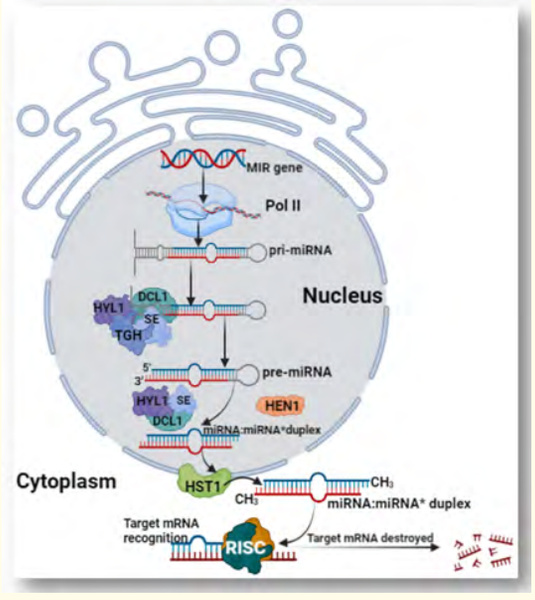


Figure 14: miRNA biogenesis in plants and targeted mRNA degradation.

"Anthocyanins", a significant subclass of flavonoids, are subject to stringent regulation by "miRNAs" across various plant species. In *Arabidopsis thaliana's* stem section, the accumulation of anthocyanins was found to be closely linked to the post-transcriptional control of "SPL" genes by "miR156." The upregulation of "miR156" resulted increased anthocyanin accumulation through targeting of SPL9 gene. Interestingly, they also identified SPL9 as a down-regulator of anthocyanin level due to its role in destruction of "MYB-bHLH-WD40 protein complex" [73]. "Figure 15" depicts a diagrammatic pathway illustrating how miR156 regulates SPL9 to enhance anthocyanin biosynthesis by destabilizing the MYB-bHLH-WD40 protein complex.

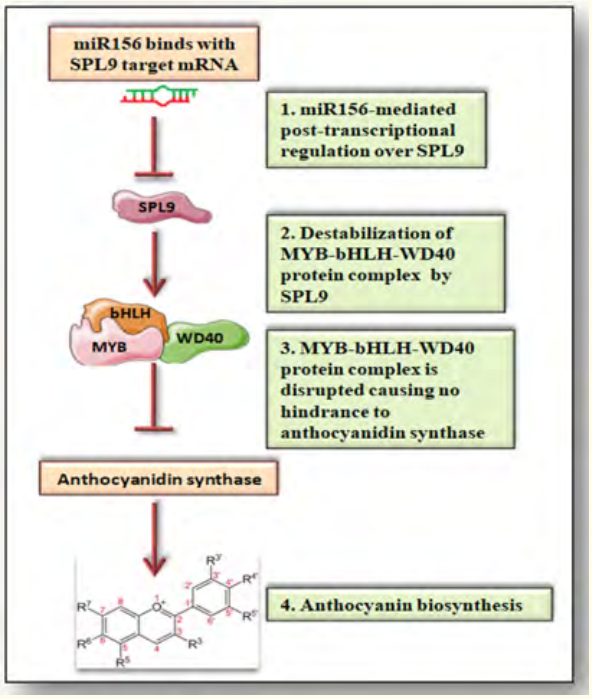


Figure 15: miR156 regulation of anthocyanin biosynthesis.

In a recent analysis of the persimmon ("*Diospyros kaki*") fruit transcriptome, it was observed that the accumulation of proanthocyanidins or tannins correlated with the influence of several distinctively expressed miRNAs. Among the identified miRNAs, "miR858" and "miR156" exhibited opposing activities in the regulation of genes responsible for proanthocyanidin synthesis. According to a research, "miR858" exhibited a positive regulatory role in the process of proanthocyanidin synthesis, whereas "miR156" played a negative regulatory role [67]. Additionally, investigations in *A. thaliana* unveiled that "miR858a" was responsible for controlling the MYB transcription factors associated with flavonoid production. Through studies involving overexpression and mutations, it was further demonstrated that specific miRNAs are crucial controllers of flavonoid synthesis [113].

Additionally, "*Osmanthus fragrans*" demonstrated the crucial role of "miR858a" in regulating flavonoid biosynthesis, which was modulated by its negative correlation with key genes involved in flavonoid synthesis, such as "MYB1," "CHI" (chalcone isomerase), "CHS" (chalcone synthase), and "FLS" (flavonol synthase) [72].

Furthermore, a recent transcriptome analysis of the Himalayan mayapple ("*Podophyllum hexandrum*") unveiled several miRNAs likely to regulate various pathways for the production of polypropanoid and flavonoid compounds. Among these, pathways associated with biosynthesis many natural products were found to be under the control of the gene "caffeoyl-CoA O-methyltransferase." In addition, "miR1873", "miR5532," were also seemed to regulate "2-hydroxyisoflavanone dehydratase" and "dihydroflavonol 4-reductase C," respectively, both of which are associated with pathways that produce flavonoids [69].

Podophyllotoxin, a highly valuable secondary metabolite known for its anticancer properties, was the subject of a different study on the same species. In this study, the miRNAs and targets, along with metabolic pathways (downstream) relevant for producing "podophyllotoxin" were investigated. The study highlighted "miR396b," "miR2673a," "miR828b," and "miR2910" as potential molecules for increasing concentration of *P. hexandrum* podophyllin content. These recognized miRNAs targeted transcripts associated with "WRKY 37" and "MYBF1" transcription factors, "flavonol synthase," "glyceraldehyde 3-phosphate dehydrogenase," "peroxidase," "malate dehydrogenase," "phosphoenolpyruvate carboxylase," and the "shikimic acid pathway," all of which are involved in podophyllotoxin production [114].

Recently, it has been discovered that the production pathway genes for podophyllotoxin are regulated by newly identified miRNAs that respond to methyl jasmonate. This study identified 66 novel miRNAs with targets such as "cytochrome p450," "flavonol synthase/flavanone 3-hydroxylase," "4-coumarate: ligase," and "phenylalanine ammonia-lyase." These miRNAs were found to be connected to the biosynthesis of several important natura products [115].

According to research on miRNA-mediated regulation of flavonoid pathways under salt stress, these pathways play a pivotal role in reprogramming metabolic flux during challenging circumstances. The study observed that under salinity exposure, "*Halostachys caspica*" exhibited differentially expressed miRNAs and their corresponding targets. These targets were associated with salt stress-related pathways, such as the "calcium signalling pathway," the "MAPK signalling pathway," "plant hormone signal transduction," and "flavonoid biosynthesis" [116].

A study on the coexpression of miRNAs in "*Camellia sinensis*" revealed the intricate regulation of gallated catechin, a well-known flavanol. Specifically, "miR156" exerted an adverse control on gallated catechin, while "miR166" and "miR172" positively enhanced its production. This research shed light on the potent influence of miRNAs on the synthesis of flavor compounds in the tea plant "*C. sinensis*" [71].

Terpenoids constitute the largest family of volatile chemicals synthesized by plants from C5 precursors [117]. Through in silico research across numerous plant species, several miRNAs with potential roles in controlling terpene production and their biological activities have been discovered. An early transcriptome investigation of "*Salvia sclarea*" identified miRNAs and their targets associated with the terpenoid and phenylpropanoid biosynthesis pathways [118]. Notably, "miR156" was found to regulate the "SPL9" gene, which, in turn, acted as an activator at transcriptional level of the production of sesquiterpenoid by attaching to the "terpene synthase 21" gene [51]. This discovery revealed that "miR156" modulates the production of both flavonoids and terpenoids by controlling specific genes.

Another investigation focused on the medicinal plant "*Picrorhiza kurroa*," where "miR4995" was discovered. The enzyme "3-deoxy-7-phosphoheptulonate synthase," involved in the picroside production pathway, was identified as the anticipated target of "miR4995." Reducing the target transcript led to increased production and accumulation of picroside [50].

A transcriptome investigation conducted on "*Ferula gummosa*" demonstrated the regulatory functions of miRNAs belonging to the families "miR2919," "miR838," "miR5021," "miR5658," and "miR5251" in controlling terpene production. Additionally, the study revealed the potential involvement of miRNAs in regulating transcription factors such as "SPL7," "SPL11," and "ATHB13," which also play a role in terpene regulation [53].

In "*Ginkgo biloba*," a plant known for its therapeutic properties and containing unique terpenoids called "terpene trilactones," recent research identified miRNAs. Among these, 4 (conserved) and 5 (new) miRNAs of "*G. biloba*" were found to show potential roles for the synthesis of "terpene trilactones".

Furthermore, miRNAs controlling the enzymes involved in sesquiterpene production were discovered and verified in "*Xanthium strumarium*." These miRNAs regulate the enzymes "1-deoxy-D-xylulose 5-phosphate synthase (DXS)," "3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)," "isopentenyl diphosphate (IPP)/dimethylallyl diphosphate (DMAPP) synthase (IDS)," and "isopenteyl diphosphate isomerase (IDI)" [47].

"*Artemisia annua*" stands as the sole natural reservoir of "artemisinin" (ART), a sesquiterpene lactone frequently prescribed to combat malaria. The miRNA families "miR159," "miR172," and "miR166" specifically target the "cytochrome P450 reductase" gene, crucial for artemisinin synthesis [57].

"Alkaloids" serve many functions in plant system. Derived mainly from amino acids, these chemical compounds find application in medicines, stimulants, narcotics, and poisons [119]. In the opium poppy ("*Papaver somniferum*"), "Pso-miR13," "Pso-miR2161," and "Pso-miR408" regulate opium alkaloids, the most widely used alkaloids. According to research, "pso-miR13" regulates the "7-O-methyltransferase" gene involved in morphinan alkaloid production. Similarly, "pso-miR408" targets "reticuline oxidase-like protein," and "pso-miR2161" influences "S-adenosyl-L-methionine, 30-hydroxyN-methylcoclaurine 40-O-methyltransferase 2," both integral to the benzylisoquinoline alkaloids (BIA) biosynthesis pathways [65].

The discovery of miRNA-mediated alkaloid regulation has also seen contributions from "target mimicry" investigations. It was showcased how a specific miRNA controls nicotine production by mimicking the "quinolinate phosphoribosyl transferase 2" gene, known as "nta-miRNA27" [64].

miR156 plays a regulatory role in controlling the "SPL9" gene, impacting the synthesis of "alkaloids." Additionally, "SPL9" governs the synthesis of jasmonates, which, when expressed at higher levels, promote the biosynthesis of "glucosinolates" [63]. As a result, miR156 continues to regulate the major class of secondary metabolites.

In the Thale cress, "miR826" and "miR5090" were found to influence the synthesis of glucosinolates by regulating the function of the shared target "AOP2," gene [61,62].

Research on Turmeric revealed that miRNAs simultaneously regulate the biosynthesis of secondary metabolites, including curcuminoids, flavonoids, alkaloids, and terpenoids.

Among the identified miRNAs, "miR5021" was found to control terpenoid and alkaloid pathways, while "miR2919" was shown to regulate the flavonoid pathway [56]. An analysis of miRNA expression in Potato demonstrated the "miRNA-TOR", an area of plant biology that remains less explored. Inhibiting TOR in Potato led to increased initiation of many miRNAs that impact various natural product pathways [56].

The potential of medicinal plants to yield a vast array of secondary metabolites opens avenues for exploring their applications in medications, pesticides, poisons, dyes, and various other products. However, the regulation of these secondary metabolite biosynthesis pathways in medicinal plants remains poorly understood. Recent investigations have focused on identifying miRNAs involved in biosynthesis, understanding their mechanisms of action, and uncovering the pathways leading to the degradation of secondary metabolites.

One such medicinal plant, known for its anti-inflammatory and hypolipidemic effects, is "*Lonicerae japonicae*," also called honeysuckle. In a recent study, it was identified several new miRNAs that may control the genes involved in the manufacture of flavonoids in the honeysuckle plant [70].

Another medicinal plant (subtropical) found in Asia, *Murraya koenigii*, synthesizes various beneficial chemicals. MiRNA profiling of this plant led to the discovery of 142 conserved and 7 new miRNAs from *"M. koenigii"* that control target genes for producing flavonoid and terpenoid manufacturing pathways [120].

"*Withania somnifera*," a therapeutic plant belonging to the solanaceae family, underwent a transcriptome analysis, unveiling the role of "miR5140," "miR159," "miR477," and "miR530" in enhancing withanolide production. This research could prove crucial in preventing the overproduction of highly important secondary metabolites [58].

A single RNAi pathway for riboregulation of thebiosynthesis of many secondary metabolites

In the realm of plant research, a single RNAi pathway emerges as a potent tool for controlling the synthesis of numerous secondary metabolites. By skillfully riboregulating specific gene circuits involved in biosynthesis pathways, researchers can modify the levels of biosynthesis through the use of designed miRNAs targeting those genes. This breakthrough approach allows for precise fine-tuning of secondary metabolite synthesis in plants, presenting exciting possibilities for applications in "biotechnology," "pharmaceuticals," and "agriculture".

To achieve this, synthetic miRNAs are crafted using the target genes' sequences as a guide and then introduced into plant cells through injection. Inside the plant cells, these synthetic miRNAs become part of the "RISC", a multiprotein entity. The RISC-loaded miRNAs identify and bind to the complementary mRNA sequences of the target genes, leading to mRNA degradation.

"Figure 16" illustrates the role of miRNAs as riboregulators, orchestrating the synthesis of multiple types of secondary metabolites found in plants.

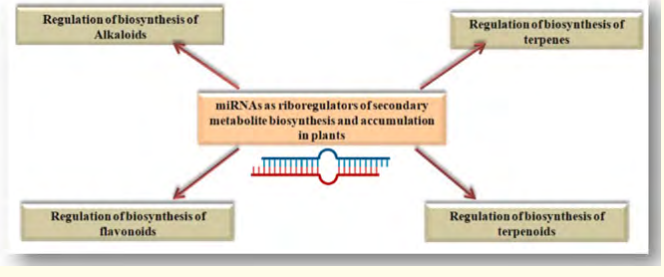


Figure 16: miRNAs as riboregulators of multiple types of secondary metabolites found in plants.

Regulation of the synthesis of natural products by secondary siRNA

Other than their traditional roles as mediators in "post-transcriptional cleavage and translational repression", plant miRNAs showcase an extraordinary ability to activate "siRNA-based secondary regulatory pathways" [106].

Figure 17 illustrates the process wherein miRNAs trigger the biogenesis of secondary siRNAs.

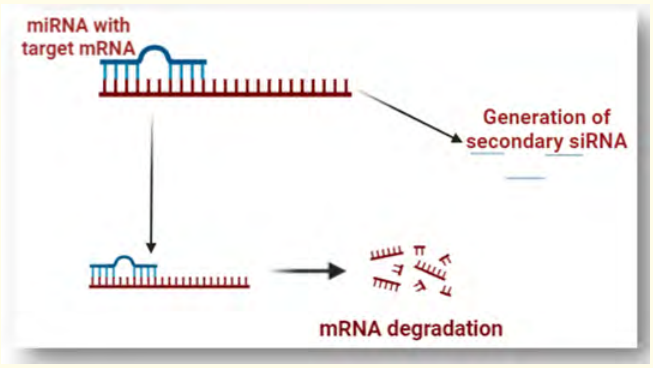


Figure 17 : miRNA triggering biogenesis of secondary siRNAs.

The secondary siRNAs produced by "initiator-miRNAs" have a wide-ranging impact on plant growth, development, stress responses, and specific metabolic activities [121]. These siRNAs play a crucial role in regulating phenylpropanoids, a well-known class of plant phytochemicals with essential functions in various aspects of plant growth. Working in conjunction with "MYB" and "miR828" trigger genes, "TasiRNAs" oversee the manufacturing of phenylpropanoids. The regulation of "anthocyanins," which belong to the flavonoid family and are produced through the phenylpropanoid pathway, involves a cascade of "miR828-AtTAS4-siR81()-MYBs." Interestingly, overexpressing "miR828" in *A. thaliana* leads to reduced expression of several MYB genes, such as "AtMYB82," "AtMYB75/PAP1," "AtMYB90/PAP2," and "AtMYB113," thereby suppressing the enzyme-coding genes essential for anthocyanin biosynthesis and resulting in decreased pigment accumulation [122].

Significant "miR828b-mediated" direct silencing of the MYB gene occurs in "gymnosperms," "monocots," and "dicots". However, only dicots implement this regulation through tasiRNAs produced from miR828-induced TAS4 or MYB transcripts. The emergence of these dual regulatory mechanisms in anthocyanin biosynthesis took place during the monocot-dicot separation [123]. Additionally, a similar regulatory pathway involving the "miRNA-MYB/TAS4-tasiRNA pathway" controls carbohydrate metabolism in some cotton tissues [124].

"Carbohydrates," vital components for secondary metabolites, can form various phytochemicals through glycosidation connections. "Figure 18" visually represents the formation of tasiRNA from miR828-induced TAS4.

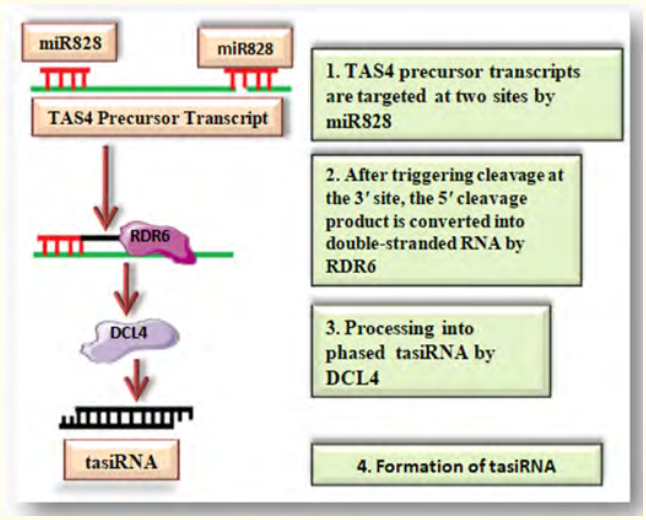


Figure 18: Formation of tasiRNA from TAS4 precursor transcript by miR828 intervention.

An "intriguing" cotton study proposed that "miR828" induces the "GhMYB2D" gene to produce "tasiRNAs," which suppress carbohydrate metabolism and inhibit the development of cotton fibers. This mechanism allows "sugar molecules" to be utilized for other vital plant growth and developmental processes [124].

The transcription factor "R2R3-MYB," responsible for regulating the production of "anthocyanin 1" or "TT19" ("PAP1/MYB75"), plays a crucial role in preventing anthocyanin buildup. In "*A. thaliana*," the "RDR6-SGS3-DCL4-sRNA pathway" involves "TAS4-siRNA81(-)," a "tasiRNA", that finds specific target transcripts like "PAP1," "MYB113" "PAP2," in certain conditions. It impacts both "TT19" and "PAP1" expression. The study in "*A. thaliana*" reveals the potential for a siRNA-based monitoring system capable of controlling central carbon metabolism, which is essential for synthesizing phytochemical precursors [125].

Secondary siRNA production can be initiated not only by miRNAs but also by particular primary siRNAs. For example, secondary siRNAs are activated by primary siRNAs originating from the "CHS" gene, which codes for "chalcone synthase"—an important enzyme needed for many secondary metabolites. In the seed coat, "siRNAs" produced by "CHS7", "CHS8" play a regulatory role in metabolic processes in a tissue-specific manner, while cotyledons and other vegetative tissues lack such siRNAs [66]. Further research

is required to identify new siRNAs for creating improved plant varieties that produce desired secondary metabolites [126].

A single secondary metabolite biosynthesis can be riboregulated by multiple RNAi pathways

Anthocyanin represents a prime example of a secondary metabolite in plants, whose production can be regulated through various RNAi pathways. These pigments are responsible for the red, blue, and purple hues in fruits, flowers, and leaves, play essential roles in plant defense against both biotic and abiotic stressors, pollinator attraction, and protection against UV radiation.

Numerous critical genes involved in anthocyanin production encode enzymes required for various enzymatic stages. By leveraging RNAi technology, the expression of these genes can be modulated, consequently influencing anthocyanin synthesis. Employing a range of RNAi pathways, specific genes responsible for anthocyanin-producing enzymes can be targeted with siRNAs to reduce anthocyanin production, leading to plants with altered coloration.

Moreover, RNAi technology enables the targeting of TFs, such as "WD40", "MYB", "bHLH", repeat proteins that regulate the function of genes involved in anthocyanin production. By silencing these transcription factors, the entire anthocyanin biosynthesis process can be efficiently inhibited, resulting in plants with minimal or no anthocyanin production.

This ability to regulate anthocyanin biosynthesis using diverse RNAi pathways serves as a powerful tool for studying anthocyanin functions in plants and engineering plants with desired pigmentation traits, benefiting various applications such as improving fruit quality or enhancing ornamental value. However, it is crucial to consider potential off-target effects and unintended consequences to ensure the safe and ethical application of this technique in plant biotechnology, as with any RNAi-based strategy.

"Figure 19" illustrates the regulation of anthocyanin biosynthesis, which can be controlled by both secondary siRNAs and miRNAs.

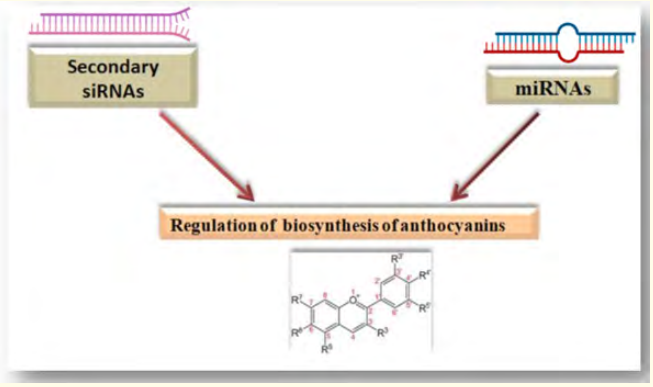


Figure 19: Regulation of biosynthesis of anthocyanins can be controlled by both secondary siRNAs and miRNAs.

Regulation of the biosynthesis of secondary metabolites through lncRNA

The "lncRNAs" have attracted considerable attention in plant research for their diverse controlling effects on vital cell functions and also act as mimic to miRNA target sites [127]. In the context of oolong tea (*Camellia sinensis*), specific lncRNAs and their targets related to many natural product pathways which play a crucial role in determining the beverage's flavor and aroma. Notably, two lncRNAs, "LTCONS_00054003" and "LTCONS_00060939", have a favorable connection with their target genes, 4CL and CHI, which are essential for flavonoid metabolism.

Several RNA molecules "LTCONS_00026271-novel_miR44-LOX", "LTCONS_00026271-novel_miR44", as well as "LTCONS_00026271-novel_miR44-LOX","LTCONS_00020084-miR169d5p_1", "LTCONS_00020084-miR169d5p_1" have been found to exhibit the ability of eTM-based corresponding "miR169d-5p_1-ACX" pairings [49].

In various plant species, lncRNAs have been observed to function as miRNA target mimics, influencing important genes in pathways related to terpene and tartrate production. Several enzymes involved in these pathways, including polygalacturonase and hexokinase in the tartrate pathway, as well as terpene synthase and hydroxymethylglutaryl-CoA reductase in the terpene pathway, are directly targeted by lncRNAs [54].

Similarly, in *Nicotiana* species, the biosynthesis of nicotine, a primary alkaloid, is partially controlled by lncRNAs through a process involving "nta-eTMX27" as a mimic for the "miRNA nta-miRX27". This mechanism reduces the function of nta-miRX27, which would normally target the crucial gene QPT2 responsible for nicotine biosynthesis [128].

"Figure 20" visually represents the interaction between lncRNA (nta-eTMX27) and miRNA (nta-miRX27) in reducing the miRNA's function to enhance nicotine production. The research on lncRNAs and their regulatory roles opens up promising possibilities for various applications in plant biotechnology, such as enhancing desired traits in crops. Nonetheless, it is important to carefully consider potential unintended effects and ensure ethical use in plant research and improvement.

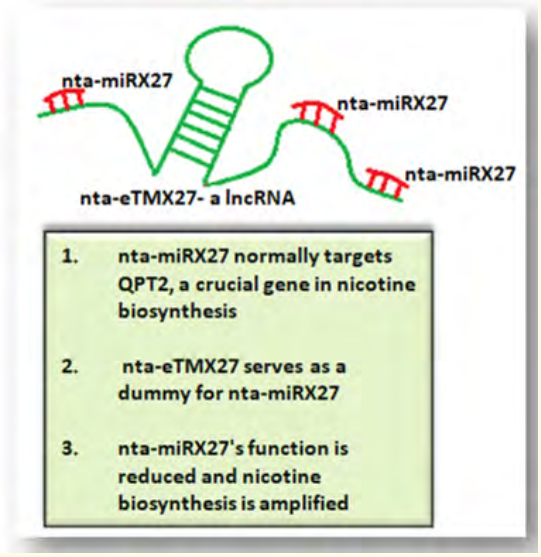


Figure 20: nta-eTMX27 acting as a dummy for nta-miRX27.

In sea buckthorn transcriptome analysis, the fruit is abundant in many valuable metabolites which unveiled the impact of "cis/trans-acting lncRNAs" on elements of various pathways. These lncRNAs were found to play a significant role in controlling enzymes involved in the first steps and end steps of these biosynthetic processes. The lncRNAs "TCONS_00082246", "TCONS_00085219" were shown to interact with "phytoene synthase" and "chalcone synthase" enzymes, respectively, indicating their regulatory effect on the biosynthesis of carotenoids and flavonoids [129].

Studying the role of lncRNAs in modifying crucial phytochemical pathways in sea buckthorn can provide insights into the hidden mechanisms influencing fruit ripening and coloring in other fruit-producing species. Additionally, the role of lncRNAs in response to heavy metal stress, particularly cadmium (Cd) in rice roots, was investigated. The study revealed specific lncRNAs, such as "XLOC_058523, XLOC_104363", and "XLOC_059778", that can modify the gene "OS11G0552000" associated with phenylpropanoids and phenylalanines. These heavy metal-induced lncRNAs and their connection to phytochemical pathways suggest their potential role in activating transporter proteins to facilitate the removal of hazardous concentrations of Cd from the cell [68].

An extensive study that integrates lncRNAs induced by Cd-stress and additional genetic components linked to them can complement regulatory network of elements responsible for stress responses related to heavy metal. This research may be instrumental in mitigating the impact of toxic substances like cadmium on both plants and animals. In response to salt stress in *Pistacia vera*, intriguing evidence was found of "lncRNA_PveLR34269" targeting "Laccase genes". These genes play a role in enhancing "monolignol polymerization" required for the production of "lignin", a distinguishing property of cells acclimated to salinity [59,60].

In depth study of the functional characteristics of "regulatory lncRNAs" and the identification of new lncRNAs associated with synthesis of metabolites hold the potential to precisely harness these lncRNAs for the production of specific phytochemicals in desired quantities in plants. Additionally, exploring the interaction of bioactive secondary metabolites with cancer-related lncRNAs is another aspect of the relationship between lncRNAs and phytochemicals.

Many reports have shown that metabolites such as resveratrol, curcumin, baicalin, berberine genistein, etc. can regulate specific lncRNAs. Additionally, these phytochemicals have been found to impact the expression of TTTY18 (a testis-specific transcript, Y-linked 18), CASC2 (a candidate for cancer susceptibility 2), and CAAT29 (a cancer-associated transcript 29), leading to the production of anticancer effects [130].

Utilizing synthetic ncRNAs to increase plant phytonutrients

The "amiRNAs", "syn-tasiRNAs" are promising technologies offering target specific gene silencing that is more advantageous

as compared to traditional double-stranded RNA (dsRNA)-induced silencing methods. These novel approaches have several benefits, including a lower likelihood of off-target and transitive silencing compared to dsRNA. Additionally, the use of a single promoter for a single transgenic cassette allows the implementation of multiple amiRNAs or syn-tasiRNAs targeting various pathways in complex metabolic engineering [131].

To create "amiRNA/amiRNA* duplexes" that precisely silence target transcripts, "amiRNAs" are generated by modifying the "miRNA and miRNA* sequences" in "miRNA precursors" [132]. On the other hand, syn-tasiRNAs or artificial tasiRNAs (atasiRNAs) are produced by substituting desired sequences for tasiRNA precursors found in the TAS DNA. Once inserted into plants, these modified TAS genes utilize the standard tasiRNA biogenesis pathway to generate syn-tasiRNAs that target specific sequences [133].

Successful downregulation of 2 "monolignoid biosynthetic genes", "ferulate 5-hydroxylase (F5H)", "coumarate 3-hydroxylase (C3H)" in *Corchorus olitorius* (jute) has been achieved using amiRNA vectors. This resulted in lower lignin concentration and improved digestibility without compromising the plant's development or defense abilities [134]. In tobacco, the use of amiRNA called amiFLS to silence the tobacco flavonol synthase (NtFLS) genes led to increased rutin production, enhancing resistance against *Spodoptera litura*, an insect pest. Conversely, overexpressing the ATMYB12 gene in tobacco reduced rutin production, consequently reducing insect resistance mediated by rutin [135]. In *Phaeodactylum tricornutum*, a model marine diatom, successful gene silencing of phytoene synthase (PSY) using amiRNAs resulted in decreased carotenoid production [136].

While these technologies show promising results in targeted gene regulation and metabolic engineering, there is still a need for further research to fully exploit their potential for secondary metabolite production in plants. Nonetheless, amiRNAs and syn-tasiRNAs hold great potential in advancing plant biotechnology and improving crop traits.

A research found that amiRNA-mediated regulation of the autophagy-carotenoid biosynthetic pathway resulted in an increase in the function of various carotenoids. Also the yield of "saturated and monounsaturated fatty acids", which could serve as a promising resource of biodiesel could be stimulated [137]. However, using multiple precursor-miRNAs in a single transgenic construct may pose challenges as it could hinder the processing of amiRNAs during their maturation and potentially impact their downstream targeting, limiting the effectiveness of amiRNA-mediated gene silencing.

In contrast, syn-tasiRNAs offer advantages in targeting multiple genes within the same or distinct gene families, as well as specific locations within a single gene, from a single TAS locus. The introduction of atasiRNA constructs along with associated miRNA triggers further enhances the ability to selectively silence desired target genes [131].

Several successful studies have utilized syn-tasiRNA-mediated multiple virus resistance against various pathogens, including "Tomato spotted wilt virus (TSWV)", "Potato spindle tuber viroid (PSTVd)", "Cucumber mosaic virus (CMV)", "Turnip mosaic virus (TuMV)", and in different plant species such as Nicotiana benthamiana, Arabidopsis thaliana, and Solanum lycopersicum.

To fully utilize syn-tasiRNA-mediated gene silencing in plant metabolic engineering and address the increasing demand for secondary metabolites obtained from plants, further in-depth research in this field is necessary. These emerging technologies hold immense promise for advancing plant biotechnology and contributing to the production of valuable bioactive compounds.

Outlooks for the future

Recent studies have delved into the concept of the miRNA-epigenetic feedback loop in eukaryotes, revealing the powerful role of miRNAs as epigenetic regulators. MiRNAs can control gene expression without altering the gene sequences they target, but they could be influenced modifications of epigenetic nature [138]. Future research on phytochemical production is likely to explore the potential of utilizing this miRNA-epigenetic modulator connection. Additionally, the coexistence of miRNAs and phytochemicals in edible nanoparticles (ENPs) and exosome-like nanoparticles (ENPs) has been discovered, raising the intriguing possibility of miRNA-phytochemical interactions regulating cross-kingdom regulation in humans and other animals [139]. However, in-depth investigation is needed to fully harness ENPs as a powerful dietary supplement containing desired phytochemicals and corresponding regulatory miRNAs.

Unlike tasiRNAs, which can have an impact across several cell layers, miRNAs' silencing activity is spatially restricted [140]. The nature of "tasiRNAs", with some other vital components and "initiator miRNAs", calls for comprehensive examination to uncover their potential in controlling various biosynthetic pathways. Artificial small RNAs, such as synthetic tasiRNAs (syn-tasiRNAs) and artificial miRNAs (amiRNAs), are emerging techniques with tremendous potential for use in crop development [141]. These artificial sRNA-mediated gene silencing approaches offer advantages such as highly efficient duplication methods (cloning), and minimal off-target silencing, avoiding interference with highly similar sequences [142]. However, to enable widespread implementation of treatments based on "artificial sRNA" effective validation using *in vivo* and in vitro procedures are essential for identification of the most suitable "amiRNA/atasiRNA". In vitro screening techniques like epitope-tagged protein-based amiRNA (ETPamir) screenings have been recently developed to enhance the utilization of artificial sRNA-mediated technologies [131].

Another avenue to explore in controlling phytochemical production is examining lncRNAs that interact with genetic components linked to plant metabolic pathways in either a cis or trans manner. Although evidence shows that lncRNAs modulate important genes involved in rate-limiting steps of phytochemical production, their investigation is hindered by low sequence conservation and restricted expression levels [48]. Despite their low expression levels, these regulatory transcripts have diverse functions, including alternative splicing, epigenetic alterations, acting as molecular cloaks, and even scaffolding [127]. Leveraging emerging data on the useful links between "lncRNAs", "sRNAs", and important genes could be instrumental for meeting the demand for enhanced plant varieties with increased phytochemical output through metabolic engineering [127].

Future of RNAi biology could be influenced by new artificial intelligence (AI) and deep learning

Researchers from Stanford University in the US have made a significant breakthrough in RNAi biology by using a computer to predict the three-dimensional (3D) structures of RNA molecules, including non-coding RNA with various physiological functions. Their method utilized a machine learning strategy called "deep learning", stimulated by the "neural networks" in the human brain that process information by identifying patterns in data. The deep learning model they developed is called the Atomic Rotationally Equivariant Scorer (ARES) neural network.

To train ARES, the scientists used only 18 short RNA molecules with known experimental structures, providing the network with structural models denoted solely by their atomic structure and chemical content. The next footstep was to test whether ARES could accurately select the most suitable structural model for RNA sequences it had not encountered before, using a set of more than 1,500 potential 3D structures generated by different computer software. Remarkably, ARES excelled in this task, even without any prior knowledge of properties like nucleotides, steric restraints, or hydrogen bonds crucial for determining RNA structures.

ARES consistently outperformed humans and other existing techniques, and it emerged as the top performer in a competition for RNA-puzzles within the scientific community, outperforming at least nine other methods. Furthermore, the model demonstrated the ability to predict the 3D structures of larger and more complex RNA molecules beyond the ones it was initially trained on.

The achievement of ARES and the "deep learning" approach holds great promise for advancing our understanding of both coding and non-coding RNA molecules, contributing to their better elucidation [143]. This breakthrough has the potential to revolutionize RNAi biology and open up new avenues for research in the field.

Conclusion

Noncoding RNAs have emerged as a captivating area of interest in phytochemical research, as mounting evidence suggests their involvement in regulating the production and functioning of plant secondary metabolites. With advancements in sequencing technologies and computational methods, researchers can now detect various regulatory noncoding RNAs that play a role in phytochemical production, in addition to the well-established structural genes and enzymes. Small non-coding RNAs, in particular, hold significant potential for metabolic engineering aimed at enhancing phytochemical production in plants.

These small regulators serve as crucial silencing agents, thanks to their ability to precisely silence their target genes, target multiple transcripts, and exert their effects in specific spatial and temporal patterns [144]. Despite the already known regulatory

role of "miRNAs" and "secondary siRNAs" on the development of plant and responses to different stimuli, their role and importance in phytochemical biosynthesis have only recently begun to be explored.

The use of "deep sequencing" techniques and new tools of bioinformatics has improved the prophecy and presise confirmation of these "regulatory sRNAs", enabling additional investigation of their object at each stage of the metabolic and other biosynthetic route. Understanding these "sRNA-target" communications may be instrumental in metabolic pathway engineering approaches, facilitating the synthesis of desired natural products in specific quantities and arrangement. As research in this field continues to advance, noncoding RNAs are poised to revolutionize our understanding and manipulation of phytochemical production in plants.

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