Review

RNA interference (RNAi) technology: a promising tool for medicinal plant research

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RNA interference (RNAi) is a process of dsRNA-mediated gene silencing. This dsRNA-triggered sequence-specific RNA degradation pathway has been termed post-transcriptional gene silencing (PTGS) in plants. RNA interference technology has already had a major impact on the study and manipulation of plant secondary metabolites. To date RNAi has mainly been used as a readily available, rapid, reverse genetic tool to create medicinal plants with novel chemical phenotypes, and to determine the phenotypes of genes responsible for the synthesis of many pharmaceutically important secondary metabolites. This dsRNA-mediated gene silencing system has facilitated the improvement of specific medicinal plants for their greater exploitation to produce commercially valuable, plant-derived drugs, flavoring agents, etc.

Key words: Medicinal plants, RNA interference, post-transcriptional gene silencing, small interfering RNA.

INTRODUCTION

Medicinal plants are one of the most important sources of life saving drugs for majority of the world's population. Plant secondary metabolites are economically important as drugs, fragrances, pigments, food additives and pesticides. The biotechnological tools are important for selecting, multiplying, improving and analyzing medicinal plants (Khan et al., 2009). It is estimated that 70 - 80% of the people worldwide rely mainly on traditional, largely herbal, medicines to meet their primary healthcare needs. The global demand for herbal medicine is not only large, but also growing. Over the years, several technologies have been adopted for enhancing bioactive molecules in medicinal plants (Khan et al., 2009). Biotechnological tools are important for the multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformation (Liew and Yang, 2008; Abdin and Kamaludin, 2006). It could also be harnessed for the production of secondary metabolites using plants as bioreactors. Recent advances in the combined molecular biology and enzymology through post-transcriptional gene silencing (PTGS) of medicinal plants suggest that this system is a viable source of important secondary metabolites. This review describes the basics of PTGS, the current understanding of siRNA (small interfering RNA, sometimes known as short interfering RNA or

silencing RNA) of PTGS in medicinal plants, and potential roles of PTGS towards improving the medicinal plants and their products. In the present write-up, few examples have been highlighted to indicate the powerful approach of gene knockdown as a means for enhancing secondary metabolite production from medicinal plants.

What is RNAi?

Interruption or suppression of the expression of a gene at transcriptional or translational levels is called gene silencing. This type of RNA interference (RNAi) is a process of dsRNA-mediated gene silencing in which only the mRNA cognate to dsRNA is specifically degraded. RNAi mediated gene silencing is referred as cosuppression or post-transcriptional gene silencing in plants; quelling in fungi and gene silencing in animals (Price and Gatehouse, 2008; Nakayashiki and Nguyen, 2008). The term "RNA interference (RNAi)" was initially coined by Fire et al. (1998).

DISCOVERY OF RNAi

A decade has passed since the initial discovery of RNA

interference (RNAi) in the nematode *Caenorhabditis elegans* (Fire et al., 1998), and it is now clear that double-stranded RNA (dsRNA) mediated gene silencing is a conserved mechanism in many eukaryotes including plants, animals and fungi (Grishok et al., 2000; Hannon, 2002; Geley and Muller, 2004). RNAi-related phenomena had been demonstrated in plants before the discovery of RNAi in worms. One of these phenomena is co-suppression, that is, gene silencing mediated by a sense transgene. In cosuppression, expression of the transgene itself is suppressed together with that of endogenous homologous genes. Co-suppression was subsequently shown to involve either transcriptional gene silencing (TGS) or post-transcriptional gene silencing (PTGS) (Kusaba, 2004). Another example of an RNAi-related phenomenon is coat protein mediated protection (CPMP). Virus resistance is conferred by a sense coat protein transgene. Initially, protection was thought to be induced by the coat protein, but later it was shown that untranslatable coat protein transgenes could also confer virus resistance. Because CPMP was found to act post-transcriptionally, it was thought that CPMP and PTGS shared similar mechanisms (Waterhouse et al., 2001; Kusaba, 2004). The gene-silencing concept was first adopted by Mahmoud and Croteau (2001) to reduce the level of the undesirable menthofuran medicinal plant, *Mentha*x *piperita* through antisense suppression of the *mfs* gene, coding for the cytochrome P450 (+) menthofuran synthase.

COMPONENTS OF RNAi PROCESS

Dicer

Dicer is a ribonuclease in RNase III family enzyme whose function is the processing of dsRNA to short double-stranded RNA fragments called siRNA (Bernstein et al., 2001) of uniformed size. Dicer cleaves dsRNA at 21 - 25 bp distance and produce siRNA with 2-nt 3' overhangs and 5' phosphorylated ends. Dicer contains helicase domain, dual RNase III motifs and a region homologous to the protein of RDE1 or QDE2 or ARGONAUTE family (Bernstein et al., 2001). Dicer works on the first step of RNAi pathway as a catalyst starting production of RNA-induced silencing complex (RISC). Argonaute, a catalytic component of dicer, has the capability to degrade mRNA complementary to that of the siRNA guide strand (Jaronczyk et al., 2005).

RNA induced silencing complex (RISC)

RISC or RNA-induced silencing complex is a siRNA directed endonuclease contains proteins and siRNA. It targets and destroys mRNAs in the cell complementary to the siRNA strand. When RISC finds the mRNA comple-

mentary to siRNA, it activates RNAse enzyme resulting in cleavage of targeted RNA. About 20 - 23 bp siRNA are able to associate with RISC. The RISC associated with siRNA targets mRNA, and degrades them, resulting in decreased levels of translation that is gene knockdown (Hammond et al., 2000; Hammond et al., 2001). RISC acts as catalyst to cleave single phosphodiester bond of mRNA (Schwarz et al., 2004).

RNA dependent RNA polymerase

Aberrant single-stranded (ss) RNA transcribed into dsRNA by RNA-dependent RNA polymerase (RdRP).

Primary siRNA and secondary siRNA

Several aspects of PTGS cannot be readily explained by a simple model, which postulates that the catalysis of dsRNA produces a population of primary siRNAs that act as the sole determinants of RISC specificity. For example, it is difficult to explain the massive RNA degradation response triggered in *C. elegans* by microinjection of tiny amounts of short (400 - 500 bp) dsRNA without the existence of some mechanism of dsRNA amplification (Fire *et al.*, 1998). Further, several studies have shown that PTGS can target RNA sequences outside the original dsRNA inducer molecule. Sijen et al. (2001) reported that a transcriptional fusion of the endogenous gene *unc-22* and green fluorescent protein (*unc-22*:GFP) could be silenced in *C. elegans* by microinjection of GFP dsRNA. However, the endogenous *unc-22* gene (which possesses no homology to GFP) was also silenced, suggesting that target sequences for silencing were somehow expanded through some interaction with the *unc-22*::GFP transcript. This phenomenon was termed transitive silencing. Subsequent RNase protection experiments showed accumulation of siRNAs homologous to *unc-22*, with a higher abundance of *unc-22* sequences, which lie down closer to the *unc-22*/GFP junction in the fusion transcript (Sijen et al., 2001; Escobar and Dandekar, 2009). Mutant analyses demonstrated that the RNA-dependent RNA polymerase rrf-1 was required for production of these "secondary" siRNAs, which could not have arisen directly from digestion of the introduced dsRNA molecule. In addition, rrf-1 mutants displayed a large decrease in total siRNA accumulation and were incapable of RNAi in somatic tissues (Escobar and Dandekar, 2009).

Based upon this data, a model was proposed in which a relatively small population of primary siRNAs is derived from direct digestion of the introduced dsRNA molecules (Escobar and Dandekar, 2009). These primary siRNAs can pair with homologous mRNA and directly or indirectly prime extension of an antisense RNA strand in the 5' - 3' direction by rrf-1. This catalytic activity would produce

more dsRNA, ultimately generating a large population of secondary siRNAs, some of which would extend beyond the boundaries of the original dsRNA trigger. This amplification effect would appear to be required for PTGS, at least in the somatic tissues of *C. elegans.* However, the fact that transitive silencing has been described in *Nicotiana benthamiana* suggests that a similar mechanism operates in plants, though it may play a less vital role in plants constitutively producing large amounts of dsRNA from integrated transgenes. It is also possible that systemic silencing in plants is caused by a related amplification effect in which a mobile signal molecule produced at the local PTGS initiation site primes *de novo* dsRNA synthesis from homologous mRNA templates in distant tissues (Escobar and Dandekar, 2009).

BASIC MECHANISM OF RNAi

The molecular mechanism of RNAi and RNAi-related phenomena in plants is presented in Figure 1. The basic mechanism of RNAi is a multi-step process. PTGS involves the generation of dsRNA by RdRP. It is thought that, in PTGS, 'aberrant single-stranded (ss) RNA' transcribed from a transgene of transgenic plant with an intron-hairpin construction, that triggers the generation of dsRNA by RNA-dependent RNA polymerase (RdRP), and consequently the RNAi pathway is activated (Waterhouse et al., 2001). Micro RNA (miRNA) is an endogenous siRNA-like RNA known to be involved in the developmental regulation of gene expression in animals (Hannon, 2002) and plants (Aukerman and Sakai, 2003; Palatnik et al., 2003). Its precursor (pre-miRNA) is a small hpRNA with 'bulges' in its stem region. All dsRNA, hpRNA and pre-miRNA are processed by dicer into 21 - 25 nt RNA duplexes. When the dsRNA enter into the cell, it is targeted by the enzyme Dicer. Dicer when activated by ATP cuts the dsRNA in to smaller segments of 21 - 25 siRNAs. These siRNAs are incorporated into a nuclease complex labeled as the RNA-induced silencing complex (RISC). The incorporated siRNAs are then unwound, the antisense strand remaining in RISC, resulting in complex activation. The activated RISC cleaves mRNA that is complementary to the siRNA or blocks the translation (Kusaba, 2004).

APPLICATIONS OF RNAi TECHNOLOGY IN MEDICINAL PLANT RESEARCH

To make a novel compound in a plant, precursors might be formed by the introduction of appropriate heterologous genes. To reduce or eliminate levels of undesirable compounds, flux in the pathway leading to such compounds can be reduced or redirected to competing pathways. Particular steps in the pathway leading to such undesirable compounds can be blocked by antisense, cosuppression or RNA interference (RNAi) methods. Similar results might be achieved by suppressing genes that upregulate the pathway or by increasing catabolism (Gomez-Galera et al., 2007). Successful RNAi mediated gene-silencing efforts in medicinal plants are listed in Table 1; some of the recent observations are also discussed in detail in the current review.

Using antisense gene for blocking activity of an enzyme is widely accepted, but in case of enzymes encoded by multigenes, it sometimes fails to block the activity. RNAi technology provided an alternative to block the activity of such enzymes that are not only encoded each by a multigene family but are also expressed across a number of tissues and developmental stages, as was the case with codeinone reductase involved in the biosynthesis of morphinan alkaloid presented in Table 1 (Larkin et al., 2007). Allen et al. (2004) was able to knockdown the activity of codeinone reductase through DNA-directed RNAi in transgenic opium poppy, which resulted in accumulation of precursor (s)-retculine (these are seven enzymatic steps before the substrate codeinone) at the expense of morphine, codeine, oripavine and thebaine. Similarly, Fujii et al. (2007) used RNAi technology to block the activity of berberine bridge enzyme (BBE) in the California poppy, *E. californica* resulting in the accumulation of (S)-reticuline, an important intermediate of metabolic pathways of isoquinoline alkaloid biosynthesis; previous attempts involving antisense RNA-mediated suppression of this enzyme in the California poppy failed to achieve any substantial accumulation of reticuline (Park and Facchini, 2002, 2003). Thus, in recent years, RNAi technology has become an important tool for accelerating the breeding of medicinal plants, where a conventional mutation breeding approach failed (Allen et al., 2004).

Han et al. (2006) applied RNA interference (RNAi) technology to silence the gene for dammarenediol synthase (DDS) in transgenic *Panax ginseng,* successfully they silenced the DDS expression which leads to a reduction of ginsenoside production to 84.5% in roots (Figure 2) using DDS-RNAi produced using the RNAi destination vector pK7GWIWG2(I) by *Agrobacterium tumefaciens*-mediated genetic transformation. From this observation they concluded that expression of DDS played a vital role in the biosynthesis of ginsenosides in *P. ginseng*. It was highly important that there is no obvious morphological change in DDS-RNAi transgenic plants compared with the wild type (Han et al., 2006). Recently Allen and coworkers (Allen et al., 2008) demonstrated that both over-expression and suppression of the gene encoding the morphinan pathway enzyme salutaridinol 7-*O*-acetyltransferase (SalAT) in opium poppy affects the alkaloid products that accumulate (Allen et al., 2008).

Very recently Kempe et al. (2009) has transformed *Papaver somniferum* with an RNAi construct designed to reduce transcript levels of the gene encoding the mor-
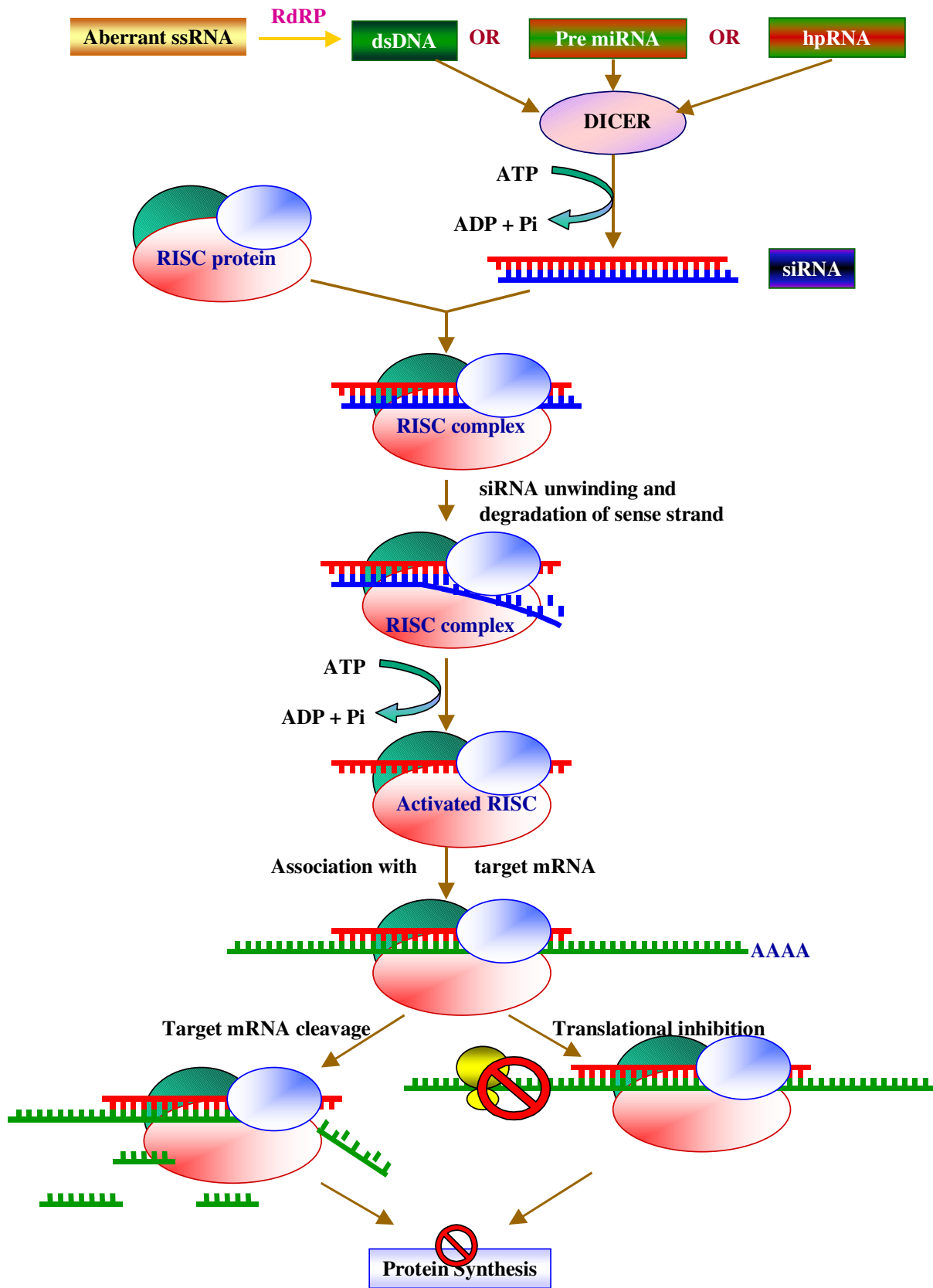
Figure 1. The molecular mechanism of post-transcriptional gene silencing.

Table 1. Examples of RNAi mediated gene-silencing efforts in medicinal plants.

S. No.	Enzyme (gene)	Host species	Product	References
1	cytochrome P450 (+) menthofuran synthase	*Mentha x piperita*	Menthofuran	Mahmoud and Croteau 2001
2	berberine bridge enzyme (BBE)*	*Eschscholzia californica*	Benzophenanthridine alkaloids	Park and Facchini, 2002
3	BBE*	*Eschscholzia californica*	(S)-reticuline	Park and Facchini 2003
4	Putrescine N-methyltransferase (PMT)	*Nicotiana tabacum*	Pyridine and tropane alkaloids	Chintapakorn and Hamill 2003
5	Codeinone reductase (COR)*	*Papaver somniferum*	Codeine and morphine	Allen et al., 2004
6	Limonene-3-hydroxylase gene	*Mentha x piperita*	Limonene	Mahmoud et al 2004
7	Berberine bridge enzyme (BBE) and the N methylcoclaurine 3'-hydroxylase (CYP80B1).	*Papaver somniferum*	Morphine, codeine, sanguinarine,	Frick et al. 2004
8	De-etiolated1 DET1	*Solanum lycopersicum*	Carotenoid and flavonoid contents of fruits	Davuluri et al., 2005
9	Benzoic acid/salicylic acid carboxyl Methyltransferase *PhBSMT1*	*Petunia x hybrida*	Methylbenzoate	Underwood et al., 2005
10	R2R3 MYB-type transcription factor ODORANT1	*Petunia hybrida*	fragrance	Verdonk, et al., 2005
11	Glutatione STransferase 1 and 2, ubiquitin conjugating enzyme, putative cullin, anthocyanidin synthase, putative flowering-time gene, GPT and the glutathione conjugate transporter.	*Torenia fournieri*	Anthocyanin	Nagira et al., 2006
12	Dammarenediol synthase gene	*Panax ginseng*	Ginsenoside	Han et al., 2006
13	Phenylacetaldehyde synthase (PAAS)	*Petunia hybrida*	Complete suppression of phenylacetaldehyde and 2-phenylethanol emission	Kaminaga et al., 2006
14	Benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase (BPBT)	*Petunia hybrida*	Benzylaldehyde	Orlova et al., 2006
15	Cinnamoyl-CoA Reductase	*Solanum lycopersicum*	Phenolics	Van der Rest et al., 2006
16	Codeinone reductase	*Papaver somniferum*	Morphinan alkaloid	Larkin et al., 2007
17	BBE	*Eschscholzia californica*	(S)-reticuline	Fujii et al., 2007
18	Norcoclaurine 6-O-methyltransferase	*Coptis japonica*	Benzylisoquinoline alkaloid	Inui et al., 2007
19	Coniferyl alcohol acyltransferase (*Ph*CFAT)	*Petunia x hybrida*	Coniferyl aldehyde and homovanillic acid	Dexter et al., 2007
20	Cinnamyl alcohol dehydrogenase	*Linum usitatissimum*	Lignin reduction	Wrobel-Kwiatkowska et al., 2007
21	Endogenous dihydroflavonol 4-reductase (DFR) and overexpression of both iris DFR and viola flavonoid 3'5'- hydroxylase	*Rosa hybrida*	Delphinidin	Katsumoto et al., 2007
22	VEGF-R2 protein	*Commiphora mukul*	z-Guggulsterone	Xiao and Singh, 2008
23	Salutaridinol 7-O-acetyltransferase	*Papaver somniferum*	Morphinan alkaloids	Allen et al., 2008
25	Salutaridinol 7-O-acetyltransferase (SalAT).	*Papaver somniferum*	Morphine	Kempe et al., 2009
26	Tryptophan Decarboxylase	*Catharanthus roseus* plant culture	Tryptamine	Runguphan et al., 2009

*Either partial or incomplete silence of the respective gene.

Squalene

Squalene epoxidase

2,3-oxidosqualene

Dammarenediol synthase

β amyrin synthase

Dammarenediol II

β amyrin

Dammarene-type triterpene (ginsenoside Rb1, Rb2, Rc,- Rd, Re, Rf, Rg1)
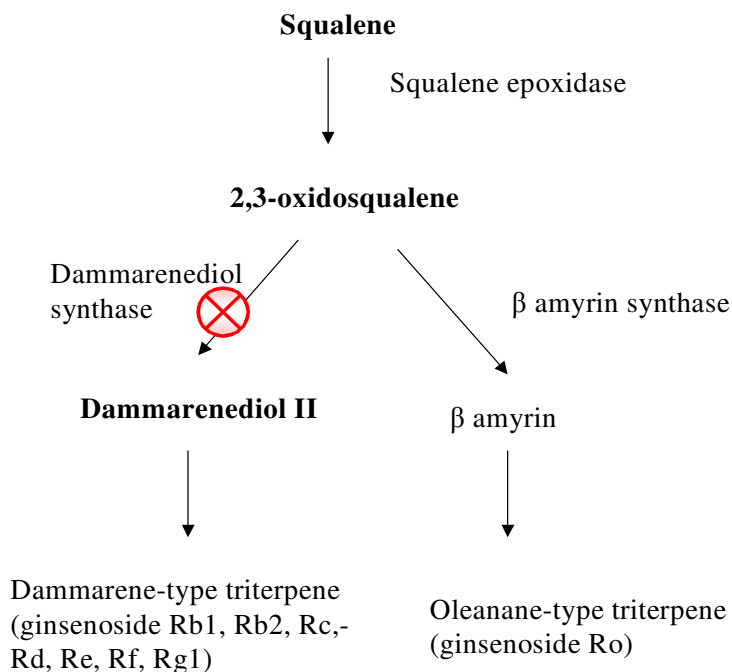
Oleanane-type triterpene (ginsenoside Ro)

Figure 2. Biosynthetic pathways of ginsenosides from squalene in *P. ginseng*. (Modified from Han et al., 2006). Han and coworkers successfully silenced a gene for dammarenediol synthase.

phine biosynthetic enzyme, salutaridinol 7-O-acetyltransferase (SalAT). RNA interference of salAT led to accumulation of the intermediate compounds, salutarydine and salutaridinol, in a ratio ranging from 2:1 - 56:1 (Kempe et al., 2009).

Production of unnatural alkaloids

Natural products have long served as both a source and inspiration for pharmaceuticals. Modifying the structure of a natural product often improves the biological activity of the compound. Metabolic engineering strategies to ferment "unnatural" products have been enormously successful in microbial organisms. However, despite the importance of plant derived natural products, metabolic engineering strategies to yield unnatural products from complex, lengthy plant pathways have not been widely explored. Runguphan et al. (2009) reported the RNA mediated suppression of tryptamine biosynthesis in *Catharanthus roseus* during hairy root culture eliminates all production of monoterpene indole alkaloids, a class of natural products derived from two starting substrates, tryptamine and secologanin. To exploit this chemically silent background, they introduced an unnatural trypta-mine analog to the production media and demonstrated that the silenced *C. roseus* culture could produce a variety of novel products derived from this unnatural starting substrate. The novel alkaloids produced by

Runguphan et al. (2009) were not contaminated by the presence of the natural alkaloids normally present in *C. roseus*.

Conclusion

RNAi is a potentially powerful tool for a wide variety of gene silencing applications. Since its initial discovery in 1998 by Fire et al. RNAi has taken the scientific community by storm. Despite many rapid advances, RNAi is still in its infancy in medicinal plant research. As described, the RNAi and gene disruption methods differ in principle, and therefore, have their own strengths and limitations. The drawbacks of RNAi, such as incomplete repression and possible unintended targets, are often described for pharmacological studies. In this regard, RNAi seems to be somewhere between gene disruption and pharmacological approaches. RNAi might prove to be useful for the studies towards production of important biomedical products by medicinal plants, which in turn can provide novel and rapid applications. Compounds like Ginsenoside, morphinan alkaloid and (S)-reticuline may be produced from the RNAi incorporated medicinal plants in near future. To make RNAi a better tool to increase the production of compound in a medicinal plant, the next challenges are to understand the extent of complete effects in medicinal plant cells and to develop an inducible RNAi system with a combination of a strictly

controlled promoter and a convenient inducer applicable to a wide range of medicinal plant. Finally, the generation of additional RNAi protocols for genome-wide screening might assist in the rapid identification of genes involved in novel compound production. Thus, though the promise of RNAi is yet to be fulfilled, its potential is begun to be realized.

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