

An ecological implication of glandular trichome-sequestered artemisinin: as a sink of biotic/abiotic stress-triggered singlet oxygen

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Abstract Artemisinin is accumulated in wormwood (*Artemisia annua*) with uncertain ecological implications. Here, we suggest that artemisinin is generated in response to biotic/abiotic stress, during which dihydroartemisinic acid, a direct artemisinin precursor, quenches singlet oxygen ($^1\text{O}_2$), one kind of reactive oxygen species. Evidence supporting artemisinin as a sink of $^1\text{O}_2$ emerges from that volatile isoprenoids protect plants from biotic/abiotic stress; biotic/abiotic stress induces artemisinin biosynthesis; and stress signaling pathways are involved in the biosynthesis of volatile isoprenoids among plants as well as the biosynthesis of artemisinin in *A. annua*. In this review, we address the ecological implication of glandular trichome-sequestered artemisinin as a sink of biotic/abiotic stress-triggered $^1\text{O}_2$, and also summarize the cumulating data on the transcriptomic and metabolic profiling of stress-enhanced artemisinin production upon eliciting $^1\text{O}_2$ omission from chloroplasts and initiating retrograde $^1\text{O}_2$ signaling from chloroplasts to nuclei.

Keywords Artemisinin; singlet oxygen; oxidative stress; signal transduction

Abbreviations

ADS	Amorphadiene synthase
ALDH1	Aldehyde dehydrogenase 1
CYP71AV1	Cytochrome P450 monooxygenase
DBR2	Double bond reductase
JA	Jasmonic acid
MeJ	Methyl jasmonate
FPS	Farnesyl pyrophosphate synthase
HMGR	3-hydroxy-3-methyl glutaryl coenzyme A reductase
RED1	Dihydroartemisinic aldehyde reductase 1
ROS	Reactive oxygen species
SA	Salicylic acid

Introduction

Artemisinin, a sesquiterpene lactone with a unique endoperoxide bridge, naturally occurs in the medicinal plant species wormwood (*Artemisia annua*). As an effective antimalarial agent, artemisinin has been urgently needed for artemisinin-based combination therapies (ACTs), which are designed for combating the endemic multi-drug resistant malarial parasite (*Plasmodium falciparum*) (Mutabingwa 2005). However, artemisinin exists in trace amounts in *A. annua*, and *A. annua* rich in artemisinin is distributed in the narrow-distributed area, so naturally available artemisinin remains insufficient for minimizing the annual rates of morbidity (380 million) and mortality (4.6 million) due to the malarial infection (Mathers et al. 2007). Fortunately, Sanofi has recently established a pilot plant for the large-scale semi-synthesis of artemisinin from the engineered yeast-derived artemisinin precursor artemisinic acid (Paddon et al. 2013). During 2013, 35-ton artemisinin has been manufactured, and 60-ton artemisinin is expected in 2014 (Corsello and Garg 2014).

Although all those of encouraging progresses hold a promise to allow the tailor-made artemisinin available (Zeng et al. 2008a; Barbacka and Baer-Dubowska 2011), it is anticipated that the genetically modified *A. annua* varieties with the high-content artemisinin should be the most economic resources of artemisinin. During last two decades, a lot of initiatives to engineer

artemisinin biosynthesis genes towards enhanced artemisinin production has been lunched (Farhi et al. 2013). The transgenic endeavors in *A. annua* include 'pulling carbon flux' to artemisinin via the introduction of a single or multiple artemisinin biosynthesis genes (Alam and Abdin 2011; Aquil et al. 2009; Banyai et al. 2010; Elfahmi and Chahyadi 2014; Nafis et al. 2011; Shen et al. 2012; Yuan et al. 2014) or 'shutting carbon flux' from steroids or other isoprenoids to artemisinin through anti-sense/RNA interference (Chen et al. 2011; Feng et al. 2009; Yang et al. 2008; Zhang et al. 2009). In the former situation, artemisinin biosynthesis is enhanced due to the increase of the copy numbers of artemisinin biosynthesis genes; in the latter one, other isoprenoid biogenesis is suppressed due to the knockdown of one or more genes (Lange and Ahkami 2013).

Some fast-growing model plants such as tobacco (*Nicotiana tabacum*/*N. benthamiana*) have been also engineered to produce artemisinin precursors and even artemisinin *per se*. In a pioneering experiment, *N. tabacum* was transformed by amorphaadiene synthase gene (*ADS*) cloned from *A. annua*, leading to the accumulation of amorpha-1,4-diene, a committed precursor of artemisinin, in trace amounts (Wallaart et al. 2001). After years, an innovative strategy has been used to increase the substantial accumulation of amorpha-1,4-diene in *N. tabacum*, in which *ADS* gene from *A. annua* and farnesyl pyrophosphate synthase gene (*FPS*) from chicken were designed to be expressed in nucleus and targeted to plastid, resulting in 5,000-fold increases of amorpha-4,11-diene (Wu et al. 2006). Later, three newly cloned artemisinin biosynthesis genes including cytochrome P450 monooxygenase gene (*CYP71AV1*), artemisinic aldehyde D11(13) double bond reductase gene (*DBR2*), and aldehyde dehydrogenase 1 gene (*ALDH1*), were introduced into transgenic *N. tabacum* plant cells that express *ADS* and accumulate amorpha-4,11-diene. As results, three kinds of artemisinin precursors including amorpha-1,4-diene, artemisinic alcohol, and dihydroartemisinic alcohol were produced, but no artemisinin was detectable (Zhang et al. 2011). Recently, as many as 12 kinds of artemisinin biosynthesis genes were introduced into the plastid genome of *N. tabacum*, resulting in the accumulation of artemisinic acid, but still no artemisinin was found (Saxena et al. 2014).

Alternatively, van Herpen et al. (2010) used a multi-gene vector that carries *ADS* from *A. annua*, *FPS* from *Arabidopsis*, and a truncated sequence of 3-hydroxy-3-methyl glutaryl coenzyme A reductase gene (*HMGR*) from *Arabidopsis* to transform *N. benthamiana*, consequently leading to 7-fold increases of amorpha-1,4-diene. When the multi-gene transfer was employed with *HMGR*, *FPS*, *ADS*, and *CYP71AV1* in *N. benthamiana*, the modified artemisinic acid-12- β -diglucoside was measurable. It is believed that the glycosylation of artemisinic acid prohibits its further conversion to artemisinin. The most exciting news is that an entire artemisinin biosynthesis pathway has been re-established in *N. tabacum* by introducing a truncated *HMGR* from yeast, and *ADS*, *CYP71AV1*, *DBR2*, and cytochrome P450 reductase (*CPR*) from *A. annua*, from which artemisinin was successfully produced albeit only in a trace amount of 0.0007% in the dry weight (Farhi et al. 2011).

In regard to the questions why artemisinin content in transgenic *N. tabacum* plants is extremely low and why engineering of *A. annua* for increasing artemisinin yield has limited success, one answer is that a bottleneck of the non-enzymatic step from a direct artemisinin precursor to artemisinin is impossible to overcome by gene transfer (Zeng and Bao 2011). In this sense, genetic modifications to the enzymatic pathway and artificial interventions at the non-enzymatic step would be equally important for enhanced artemisinin production. However, the former strategy has been extensively explored, while the latter strategy has not been critically considered until recently. This is because the details of such a light-dependent non-enzymatic reaction remains unclear.

Since it was suggested that singlet oxygen ($^1\text{O}_2$), one kind of reactive oxygen species (ROS), might be responsible for the catalysis of conversion from dihydroartemisinic acid to artemisinin (Wallaart et al. 1999), whether $^1\text{O}_2$ is certainly engaged in the auto-oxidative process is debating, and direct evidence supporting the involvement of $^1\text{O}_2$ in artemisinin biosynthesis remains lacking (Brown 2010). Nevertheless, the multiple enzymatic routes to artemisinic acid and dihydroartemisinic acid have been validated, as illustrated in Figure 1 (Ting et al. 2013). Overall, intermediates were identified and assigned sequentially, enzymes were purified and structurally/functionally characterized, and encoding genes were cloned and homologously/heterologously expressed. Additionally, it remains deductive that artemisinic acid is converted to artemisinin via arteannuin B, and the putative $^1\text{O}_2$ -catalyzed conversion from arteannuin B to artemisinin also needs further elucidations.

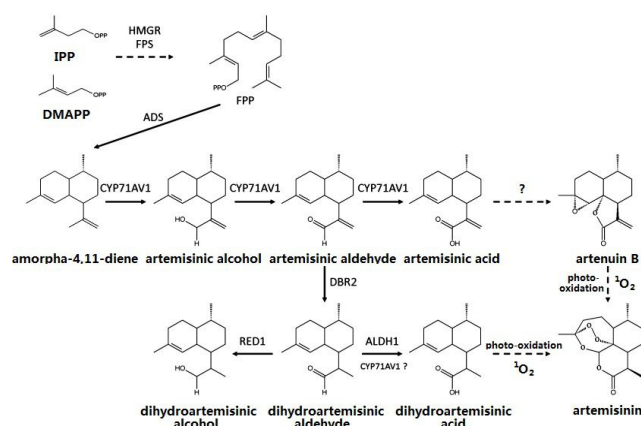


Figure 1 The currently recognized artemisinin biosynthesis pathway in *A. annua*. ADS, amorphadiene synthase; ALDH1, aldehyde dehydrogenase 1; CYP71AV1, amorphadiene oxidase; DBR2, artemisinic aldehyde double-bond reductase; DMAPP, dimethylallyl diphosphate; FPP, farnesyl diphosphate; FPS, farnesyl diphosphate synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; IPP, isopentenyl diphosphate; RED1, dihydroartemisinic aldehyde reductase 1. Broken arrows indicate reactions more than one step (Ting et al. 2013 with modifications).

Given that $^1\text{O}_2$ is involved in the conversion of artemisinin from dihydroartemisinic acid or other artemisinin precursors, it would be reasonable to suppose that artemisinin is accumulated with certain evolutionary benefits, such as scavenging reactive oxygen species including $^1\text{O}_2$, for survival, growth and propagation. However, artemisinin is by no means a unique $^1\text{O}_2$ quencher, some isoprenoids including carotene and tocopherol are known to quench $^1\text{O}_2$. It might be possible that artemisinin has been conserved by a random selection together with other isoprenoids. Therefore, we propose here that an ecological implication of the presence of artemisinin in *A. annua* is as a sink of $^1\text{O}_2$ generated from biotic/abiotic stress, thereby lending support to the hypothesized mechanism of $^1\text{O}_2$ -involved conversion from dihydroartemisinic acid to artemisinin (Wallaart et al. 1999). In the present review, we will correlate the induction of biotic/abiotic stress with the acceleration of artemisinin biosynthesis from the data of transcriptomic and metabolic profiling. Additionally, we will also depict the molecular episodes of stress-driven $^1\text{O}_2$ generation within chloroplasts and retrograde $^1\text{O}_2$ signaling from chloroplasts to nuclei. We wish that current achievements should shed light into an understanding of stress-guided improvements by genetic engineering and environmental simulation.

Why does *A. annua* accumulate artemisinin?

Although artemisinin exists in *A. annua* with or without ecological implications is unknown, plant volatile isoprenoids have been suggested to play protective roles against damage from abiotic stress-triggered ROS (Vickers et al. 2009). Indeed, the emission of volatile isoprenoids frequently increments under abiotic stress conditions at least including intense light, extreme heat, and drought stress (Brilli et al. 2007; Loreto and Sharkey 1990; Loreto et al. 1998; Loreto et al. 2006; Sharkey and Loreto 1993; Sharkey and Yeh 2001; Tingy et al. 1981). Strikingly, even the stored carbon resource for growth and development can be urgently mobilized to produce the volatile isoprenoids for fighting abiotic stress (Brilli et al. 2007; Loreto and Sharkey 1990; Monson and Fall 1989). On the other hand, isoprenoids are also accumulated when plants are exposed to the pathogen-originated biotic stress. For example, it has been reported that fungal elicitors stimulate the production of phytoalexin, a diterpene derivative, in tobacco suspension cells (Vogeli et al. 1998). Similarly, fungal elicitors also induce the generation of saponin, a triterpene derivative, in cultured cells of *Panax ginseng* (Xu et al. 2005).

Considering that artemisinin is a kind of sesquiterpene belonging to a huge family of isoprenoids, or terpenoids, it is reasonable to envisage that artemisinin may also exert a similar role in oxidative protection. Ironically, however, artemisinin has been demonstrated to function as a carbon-centered free radical to alkylate and inactivate heme-containing enzymes (Zeng and Zhang 2011; Zeng et al. 2011a). Logically, we do believe that artemisinin should be also cytotoxic to *A. annua* and other plants, which has been actually confirmed in the *in vitro* cultured plant cells (Chen et al. 1987; Duke et al. 1987). Fortunately, artemisinin is safely sequestered in the essential oil-rich glandular trichomes without any phytotoxicity (see below). To *A. annua* itself, artemisinin

seems to serve as a phytoalexin that confers a resistance to pathogenic microorganisms (Stoessel et al. 1976) and attraction to protective insects (Kappers et al. 2005). More importantly, while artemisinin precursors such as artemisinic acid and dihydroartemisinic acid serve as the direct ROS quenchers, artemisinin *per se* is only a side-product after ROS scavenging. Once a correlation of artemisinin biosynthesis with biotic/abiotic stress induction has been established, we would be confident to draw a conclusion that artemisinin is accumulated as an essential consequence of artemisinin precursors as a sink or a pool for trapping ROS from oxidative stress.

In a broad definition, the term 'oxidative stress' indicates all kinds of extreme environmental conditions that trigger ROS generation. The exogenous stress signals are classified as those from biotic stress such as pathogen infection, pest ingression, and mechanical wounding, etc., and those from abiotic stress such as cold, heat, irradiation, anoxia, drought, saline, and alkaline, etc (Xiong et al. 2002). Although our knowledge regarding the relationship between oxidative stress and artemisinin biosynthesis remains fragmentary, the oxidative stress-induced overproduction of plant secondary metabolites has been documented in *Oryza sativa* (Nojiri et al. 1996; Tamogami et al. 1997), *Catharanthus roseus* (Menke et al. 1999), *Arabidopsis thaliana* (Brader et al. 2001), and *Cupressus lusitanica* (Zhao and Sakai, 2001).

Abiotic stress-induced artemisinin biosynthesis

The first hint implying artemisinin is likely induced by environmental stress signals emerges when the artemisinin biosynthesis responsible transcript *ADS* mRNA was amplified only from those samples of *A. annua* shoots treated by water deficit, drought and high light, but not from shoots without stress pre-treatments (Wallaart et al. 1999). In similar, *ADS* mRNA and *CYP71AV1* mRNA were also amplified only from chilling-exposed *A. annua*, but not from unexposed plants (Yin et al. 2008). It was later confirmed that the copy numbers of *ADS* mRNA and *CYP71AV1* mRNA in chilled *A. annua* have 11-fold and 7-fold increases, respectively (Zeng et al. 2008c). When the promoter of *ADS* (*pADS*) was fused with the coding sequence of β -glucuronidase gene (*GUS*), and *pADS-GUS* was transformed to *N. tabacum*, 1.5-fold and 2-fold increases of GUS activity were determined upon exposure to ultraviolet and chilling (Feng et al. 2009). Similarly, it was also detected that *pCYP71AV1-GUS* allows an increase of GUS activity up to 1.4-2.7 folds in *N. tabacum* under chilling, dehydration, and ultraviolet irradiation (Yang et al. 2011). Han et al. (2013a; 2013b; 2014) observed that a series of chimeric cassettes with the fused sesquiterpene promoters are expressed in *A. annua* in a similar stress-inducible mode.

In view of artemisinin production, post-harvest drying of *A. annua* under sun, shade or dark with ambient temperature increases artemisinin yield (Laughlin 2002). Lead acetate and NaCl promote artemisinin accumulation (Quareshi et al. 2005). Chilling of transgenic *A. annua* plants leads to 3.7-fold increases of artemisinin content, while the ground dry powder of chilled transgenic *A. annua* plants surprisingly shows 5.2-fold increases of artemisinin content after 15-month storage (Feng 2009). *A. annua* plants can normally grow with low-level arsenic and NaCl, during which artemisinin biosynthesis genes including *HMGR*, *ADS*, and *CYP71AV1* exhibit upregulation (Paul and Shakya 2013).

Biotic stress-induced artemisinin biosynthesis

Supplement of *A. annua* hairy roots with cell extracts of the fungus *Aspergillus oryzae* increases artemisinin content up to 550 mg/L (Liu et al. 1997). After addition of fungal chitosan to *A. annua* hairy root cells for six days, artemisinin content increases for 6-fold to 1.8 μ g/mg of dry weight (Putalun et al. 2007). Application of two strains of fungi, *Glomus macrocarpum* and *Glomus fasciculatum*, to field-grown *A. annua* also elevates artemisinin yield (Kapoor et al. 2007). Colonization of *A. annua* by *Rhizophagus intraradices* that forms arbuscular mycorrhiza upregulates the expression of artemisinin biosynthesis genes and also increases artemisinin content (Mandal et al. 2014).

Because $^1\text{O}_2$ ubiquitously bursts among the plant kingdom, some people may argue why only *A. annua* accumulates artemisinin but others do not. We thought that dihydroartemisinic acid/artemisinic acid is chosen as a quencher of $^1\text{O}_2$ in *A. annua* may be an evolutionary consequence of random mutation and beneficial selection. As discussed below, $^1\text{O}_2$ originated within chloroplasts can be scavenged by β -carotene and α -tocopherol, but overdosed $^1\text{O}_2$ emitting to the cytosol must be depleted by other isoprenoids (Vickers et al. 2009).

What are transducers conveying stress signals?

According to the 'single biochemical mechanism for multiple physiological stressor's model (Vickers et al. 2009), abiotic stress signals such as intense light, extreme temperature, drought, and

ozone can cause oxidative stress via triggering ROS including $^1\text{O}_2$, superoxide (O_2^-), hydroxy radical (OH^\cdot), and hydrogen peroxide (H_2O_2). On the other hand, there are also reactive nitrogen species (RNS) including nitric oxide (NO) and peroxyxynitrite (ONOO^\cdot). Both ROS and RNS can directly initiate signal transduction and can also indirectly act through interactions with salicylic acid (SA), jasmonic acid (JA), gibberellic acid (GA_3), and other phytohormones. SA, JA and its derivative methyl jasmonate (MeJ) are important signaling messengers in plants, especially in defense reactions against oxidative stress.

SA, JA and MeJ

SA, JA, and MeJ are ubiquitous signal molecules in plants and known as the ‘second messengers’, which can transduce most of the extreme environmental stimuli to initiate the cellular response and protection from biotic/abiotic stress. Application of JA/MeJ stimulates the production of volatile isoprenoids in many plant species (Filella et al. 2006; Martin et al. 2003). Some *Artemisia* species have 2- to 8-fold higher JA when grown outside, and wounding also increases MeJ levels (Afitlhile et al. 2005). MeJ emitted by *A. tridentate* induces approximated tomato plants to express a protease inhibitor gene and exhibit a protective role from pest insect feeding (Farmer et al. 1990). The foliar application of MeJ enhances the photosynthetic efficiency in both stressed and non-stressed *A. annua* plants, but stressed plants have reduced rates of lipid peroxidation, elevated levels of antioxidant enzymes, and increased content of artemisinin (Aftab et al. 2010). Lu et al. (2014) found that overexpression of allene oxide cyclase, a key enzyme for JA biogenesis, accelerates artemisinin biosynthesis in *A. annua*.

The *pADS-GUS* cassette in transgenic *N. tabacum* plants is highly responsive to SA and MeJ, and SA/MJ-treated *A. annua* exhibits a correlation of *ADS* upregulation with $^1\text{O}_2$ emission, suggesting SA/MJ inducing artemisinin overproduction by triggering $^1\text{O}_2$ burst (Guo et al. 2011). Mycorrhization of *A. annua* by *Rhizophagus intraradices* induces allene oxidase synthase gene, which upregulates the expression of artemisinin biosynthesis genes and increase the accumulation of artemisinin. In contrast, the JA biogenesis inhibitor ibuprofen represses the accumulation of artemisinin in both non-mycorrhizal and mycorrhizal plant shoots (Mandal et al. 2014). Based on the finding that artemisinin biosynthesis is JA inducible, Yu et al. (2012) identified two *A. annua* JA-responsive transcription factors, AaERF1 and AaERF2, capable of binding to *ADS* and *CYP71AV1* promoters. Overexpression of either transcription factor in *A. annua* leads to 60% increases of artemisinin yield up to 0.8% of dry weight.

***GA*₃ and other phytohormones**

In cultural *A. annua* cells, supplementation with 10 mg/L GA_3 increases artemisinin content as 6-fold as the control (Baldi et al. 2008). GA_3 -treated *A. annua* plants have also significant increases of artemisinin content (Fulzele et al. 1995; Paniego and Giulietti 1996). Zhang et al. (2005) found that exogenous GA_3 diverts the carbon flux to artemisinin by feedback inhibition of GA_3 biosynthesis. Addition of cytokinin to *A. annua* tissue cultures increases artemisinin yield (Whipkey et al. 1992). Similarly, *A. annua* hairy roots supplemented with the cytokinin analogue 2-isoprenyl adenine also substantially increases artemisinin content (Weathers et al. 2005). After introducing isopentenyl transferase gene (*ipt*) into *A. annua*, cytokinin levels elevate for 2- to 3-fold and artemisinin content increase for 30-70% in transgenic *A. annua* plants (Geng et al. 2001). Additionally, overexpression of the abscisic acid receptor gene *AaPYL9* enhances sensitivity to abscisic acid (ABA) and improves artemisinin yield in *A. annua* (Zhang et al. 2013).

It has been indicated that the transcription factor *A. annua* basic helix-loop-helix 1 (AabHLH1) can bind to E-box elements within both *ADS* and *CYP71AV1*. Upon induction by ABA and chitosan, expression of the fused *AabHLH1*-green fluorescent protein gene (*GFP*) in yeast shows transactivation activity, and co-transformation of *AabHLH1* with *pCYP71AV1-GUS* into *A. annua* exhibits a significant activation of *GUS*. The transient expression of *AabHLH1* in *A. annua* increases *ADS* and *CYP71AV1* mRNA levels, suggesting AabHLH1 positively regulating artemisinin biosynthesis (Ji et al. 2014).

NO

NO was found to enhance the production of taxol in *Taxus* (Wang et al. 2004) and catharanthine in *Catharanthus* (Xu et al. 2005), but not artemisinin in *A. annua* (Zheng et al. 2007). However, NO can potentiate fungal elicitor-induced overproduction of ginseng saponin (Hu et al. 2003), taxol (Xu et al. 2004), hypericin (Xu et al. 2005), puerarin (Xu et al. 2006), and artemisinin (Zheng et al. 2007). Upon application of an oligosaccharide elicitor for 4 days, artemisinin elevates from 7 mg/g of dry weight to 13 mg/g of dry weight in 20 day-old *A. annua* hairy roots, and a combined

treatment by an oligosaccharide elicitor with the NO donor compound sodium nitroprusside leads to the increases of artemisinin content up to 12-22 mg/g of dry weight (Zheng et al. 2007).

Calcium ion

Chilling-induced expression of *ADS* and *CYP71AV1* in *A. annua* is significantly suppressed after supplemented with the calcium ion (Ca^{2+}) channel inhibitor LaCl_3 or the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA). Following depletion of LaCl_3 or EGTA, chilling-induced expression of *CYP71AV1* is recovered immediately, whereas that of *ADS* is recovered very slowly. Moreover, calmodulin gene (*CaM*) was observed to elevate for 2.5 folds upon chilling exposure (Zeng et al. 2008c). Lin et al. (2004) previously demonstrated that the elevation of CaM titers is accompanied by the increase of antioxidant enzyme activity in *Populus tomentosa* during cold-acclimation. It was also found that an oligosaccharide elicitor derived from *Colletotrichum* sp. triggers the signal transduction involving rapid Ca^{2+} accumulation, plasma membrane NAD(P)H oxidase activation, and ROS release (Wang et al. 2001; 2002).

Why are there different chemotypes with low- and high-level artemisinin?

Artemisinin and precursors may differentially exist in certain levels in *A. annua*, for example, there are cultivars with high artemisinin/low artemisinic acid levels and cultivars with low artemisinin /high artemisinic acid levels, suggesting the natural distribution of various chemotypes (Wallaart et al. 2000). It was reported that the metabolic responses to MeJ elicitation is different between two chemotypes of *A. annua*, in which the type I plants accumulate dihydroartemisinic acid and artemisinin after exogenous MeJ application, whereas the type II plants have decreased artemisinic acid and artemisinin levels upon MeJ elicitation (Wu et al. 2011).

An explanation on the existence of *A. annua* chemotypes has been given in a recent study, from which the length of CYP71AV1 was associated with the alteration of enzyme activity. CYP71AV1 in the low artemisinin-chemotype has extra amino acid residues in its N-terminal, whereas CYP71AV1 in the high-artemisinin chemotype has not such extension. Upon transient expression in *N. benthamiana*, high artemisinin-chemotype CYP71AV1 exhibits higher enzyme activity than low artemisinin-chemotype CYP71AV1 (Ting et al. 2013).

When is artemisinin synthesized?

To investigate the relevance of artemisinin accumulation with the developmental stages, in particular, blooming, Wang et al. (2004; 2007) transformed *A. annua* by flowering-promoting factor 1 gene (*FPF1*) and flowering gene (*CO*) from *Arabidopsis thaliana*. Consequently, artemisinin content was not found to increase significantly in transgenic plants although they do bloom early, suggesting that blooming is unlikely a prerequisite for artemisinin accumulation. From the previous follow-up determination of artemisinin content, it was known that artemisinin content is highest before flowering (Morales et al. 1993) or under flowering (Gupta et al. 2002; Laughlin 1995). However, later investigations surprisingly discovered that a highest artemisinin level was analyzed in dry leaves that experience post-harvest maturation (Lommen et al. 2006), and even in dead leaves subjected to programmed cell death (Lommen et al. 2007).

A discrepancy of the determination results of artemisinin content may be deciphered by that the earlier investigations might have not determined artemisinin content in dry leaves and dead leaves. Intriguingly, it was found that artemisinin biosynthesis genes are upregulated and artemisinin production is also enhanced in senescent yellow and brown leaves (Yang et al. 2009). Another possibility could also come from a difference of artemisinin content in developing leaves. Because it was observed that the maximum artemisinin production occurs by the fully flowering stage within floral tissues, but it can alter in the leafy bracts and non-bolt leaves when the development of plants shifts from budding to fully flowering (Arsenault et al. 2010).

Where is artemisinin synthesized and sequestered?

Leaves are main organs accumulating artemisinin

Although flowers are the organs with the highest artemisinin content, their numbers and sizes are obviously less than leaves. From artemisinin determination throughout the vegetative stage of *A. annua*, it is known that top leaves exhibit generally higher artemisinin content, while bottom leaves display lower artemisinin content (Liersch et al. 1986; Ferreira et al. 1995). An immunoquantitative assay of organ-specific distribution of artemisinin biosynthesis enzymes showed that the abundance of CYP71AV1 is the highest in leaves, moderate in stems and the lowest in roots (Zeng et al. 2009). A study to reveal the ontogenic variation of artemisinin content in mature field grown *A. annua* indicated that artemisinin content is always optimal in the leaves at upper levels of secondary branches, but it is very low in the stem, seed, and seed husk, while no

artemisinin was detected in the root (Nair et al. 2013).

Glandular trichomes are dominant sites storing artemisinin

It was demonstrated that *CYP71AV1* is expressed in *A. annua* at a maximal level in glandular trichomes, a moderate level in leaves, and a minimal level in roots, in accordance with the distribution of artemisinin itself. Glandular trichomes give rise to the highest artemisinin content, leaves have a relatively lower content of artemisinin, and only trace amounts of artemisinin presents in roots (Teoh et al. 2006). Recent studies have provided a clue implying that some transcription factors may guide the glandular trichome-specific expression of artemisinin biosynthesis genes. It was detected that the transcription factor *AaWRKY1* mRNA is highly abundant in the cDNA library prepared from the glandular trichomes of *A. annua*, and *AaWRKY1* can interact with the *cis*-acting elements within the *ADS* promoter. When *AaWRKY1* was transferred into *A. annua*, artemisinin biosynthesis was found to be greatly enhanced (Ma et al. 2009; Han et al. 2014). It was also known that the changes of glandular trichomes are closely correlated with artemisinin levels, and the densities of glandular trichomes are dependent on the developmental stages and organ positions (Arsenault et al. 2010).

The transcriptome profiling of glandular trichomes from *A. annua* showed that artemisinin biosynthesis genes are significantly upregulated in both the apical and sub-apical cells of glandular trichomes in *A. annua* (Soetaert et al. 2013). Interestingly, it was observed that water deficit stress induces a decrease in the density and size of glandular trichomes, thereby negatively modulating artemisinin and its precursors (Yadav 2014).

The cytosol, cooperated with chloroplasts, is responsible for artemisinin biosynthesis

In plants, there are differential organelle-specific isoprenoid biosynthesis pathways, which mainly include the cytosolic mevalonate (MVA) pathway and the chloroplast methylerythritol 4-phosphate (MEP) pathway (Zeng et al. 2008b). The MVA pathway is responsible for the biosynthesis of sesquiterpenes (artemisinin in *A. annua*), triterpenes (such as sterols) and polyterpenes, whereas the MEP pathway is responsible for the biosynthesis of monoterpenes, diterpenes, and tetraterpenes (including carotenoids, chlorophylls and plastoquinone).

Although both pathways are located in different spaces, they can crosstalk between the cytosol and chloroplasts through interchanges of the common intermediate IPP. Besides, IPP is also interchanged between the cytosol and mitochondria. Lovastatin (LV) is an inhibitor of HMGR in the cytosolic MVA pathway, fosmidomycin (FM) is an inhibitor of DXR in the chloroplast MEP pathway, and fluridon (FD) is a carotene biosynthesis inhibitor. Those inhibitors have been used to elucidate the interchange and crosstalk among the subcellular carbon flux. Figure 2 illustrates the distribution and interaction among three types of isoprenoid biogenesis pathways (Zeng et al. 2008b).

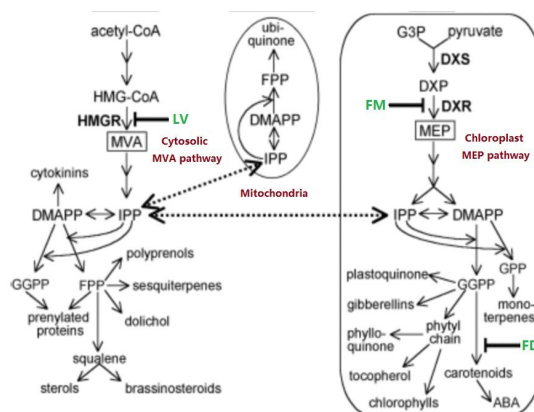


Figure 2 Plant isoprenoid biosynthesis pathways located in distinct organelles. ABA: abscisic acid; DMAPP: dimethylallyl diphosphate; DXP: deoxyxylulose 5-phosphate; DXR: DXP reductoisomerase; DXS: DXP synthase; FD: fluridon; FPP: farnesyl pyrophosphate; FM: fosmidomycin; GGPP: geranylgeranyl pyrophosphate; HMG-CoA: 3-hydroxy-3-lmethylglutaryl coenzyme A; HMGR: HMG-CoA reductase; G3P: glyceraldehyde 3-phosphate; GPP: geranyl pyrophosphate; IPP: isopentenyl pyrophosphate; LV: lovastatin; MEP: methylerythritol 4-phosphate; MVA: mevalonic acid (Zeng et al. 2008b with modifications).

It is clear from Figure 2 that the interchange of subcellular isoprenoid biosynthesis pathways can be realized via the shuttle of IPP, suggesting a dynamic feature of one pathway affecting others.

For example, inhibition of the cytosolic MVA pathway not only transiently decreases sterol levels, but also transiently increases carotenoid and chlorophyll levels (Laule et al. 2003).

Which is the direct artemisinin precursor?

Wallaart et al. (2000) found that the increase of artemisinin is concomitant with the decrease of dihydroartemisinic acid in *A. annua*. Meanwhile, they observed that *A. annua* with increased artemisinin content exhibit high-level dihydroartemisinic acid, but low-level artemisinic acid. Based on these results, they proposed that dihydroartemisinic acid rather than artemisinic acid is the immediate precursor of artemisinin, and also suggested that conversion from dihydroartemisinic acid to artemisinin may represent one of the rate-limiting steps during artemisinin biosynthesis.

Brown and Sy (2004) fed *A. annua* with the isotope-labeled dihydroartemisinic acid and detected 16 kinds of 12-carboxyamorphane and cadinane sesquiterpenes including a small proportion of labeled artemisinin. Furthermore, they also confirmed that the committed product of dihydroartemisinic acid is an allylic hydroperoxide originated from non-enzymatic catalysis. These observations led them to draw a conclusion that the metabolic fate of dihydroartemisinic acid is *in vivo* autoxidation to artemisinin via a hydroperoxide intermediate. Later then, Brown and Sy (2007) fed the isotope-labeled artemisinic acid to *A. annua* and isolated 7 labeled sesquiterpene metabolites, including arteannuin B, annulide, isoannulide, *epi*-deoxyarteannuin B, deoxyarteannuin B, *seco*-cadinane and artemisinic acid methyl ester, but no artemisinin, further validating that artemisinic acid can not be converted to artemisinin.

Brown (2010) has summarized a deductive mechanism for the conversion of dihydroartemisinic acid to artemisinin, which undergoes a spontaneous autoxidation process and involves the following 4 steps: photo-sensitization of the double bond in dihydroartemisinic acid involving $^1\text{O}_2$; Hock cleavage of the tertiary allylic hydroperoxide; oxygenation of the enol product; and cyclization of the vicinal hydroperoxyl-aldehyde to artemisinin (Figure 3).

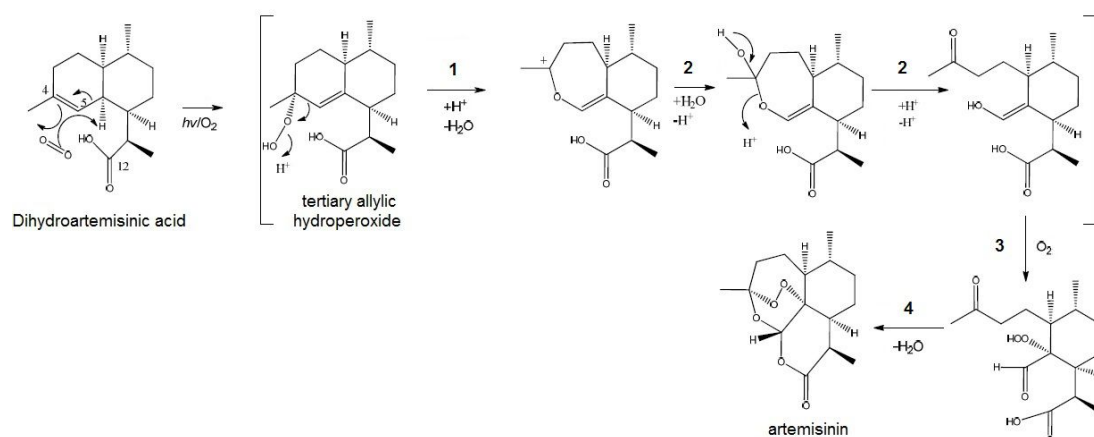


Figure 3 A 4-step mechanism for the $^1\text{O}_2$ -involved spontaneous autoxidation of dihydroartemisinic acid to artemisinin in *A. annua* (Brown 2010 with modifications).

What is the non-enzymatic catalyst?

Wallaart et al. (1999) proposed that dihydroartemisinic acid is likely at first converted to dihydroartemisinic acid hydroperoxide via a light-involved and $^1\text{O}_2$ -catalyzed reaction, and then the resultant dihydroartemisinic acid hydroperoxide is auto-oxidized to artemisinin in air. Such deduced reaction mechanism has been subsequently validated by Sy and Brown (2002), who demonstrated that dihydroartemisinic acid can be slowly auto-oxidized into artemisinin via two steps, in which light is needed in the first step, and the second step is conducted in the dark.

It is well-known that $^1\text{O}_2$ frequently generates in chloroplast thylakoid membranes as plants are exposed to extensive light (Krieger-Liszkay 2005). $^1\text{O}_2$ is harmful to thylakoids due to photo-inactivation, but it can be detoxified by carotenoids and tocopherols within chloroplasts (Krieger-Liszkay and Trebst 2006; Telfer 2005). Although artemisinin production was reported to be influenced by natural stress (Chen and Zhang 1987; Martinez and Staba 1988; Ferreira et al. 1995), no direct evidence indicating the involvement of $^1\text{O}_2$ in artemisinin biosynthesis has been provided for many years. Feng et al. (2008) monitored for the first time the enhanced emission of $^1\text{O}_2$ from chilling-treated transgenic *A. annua* plants using a simple spectrophotometric method.

The sustained release of $^1\text{O}_2$ is recognized from a gradual decline of absorbance at 440 nm (A_{440}) due to the bleaching of the selective $^1\text{O}_2$ acceptor N, N-dimethyl-p-nitrosoaniline.

To inspect the dynamics of $^1\text{O}_2$ emission from stressed samples, a time-course assay of A_{440} was performed in chilling-treated plants. As shown in Figure 4a, the sample from a chilled plant was observed to have declined A_{440} values from 0.3 to below 0.15 during the assay duration of 2.5 h, whereas the sample from an untreated plant was detected to remain a slightly changed A_{440} values above 0.2 within 2.5 h. Interestingly, a natural progression to senescence also triggers $^1\text{O}_2$ burst from *A. annua* leaves, in which yellow and brown leaves release more $^1\text{O}_2$ than green leaves (Figure 4b) (Yang et al. 2009). These results imply that chilling or senescence elicit potent $^1\text{O}_2$ emission following the inhibition of homeotic photosynthetic functionality, whereas untreated samples with normal photosynthetic activity release a stable baseline level of $^1\text{O}_2$.

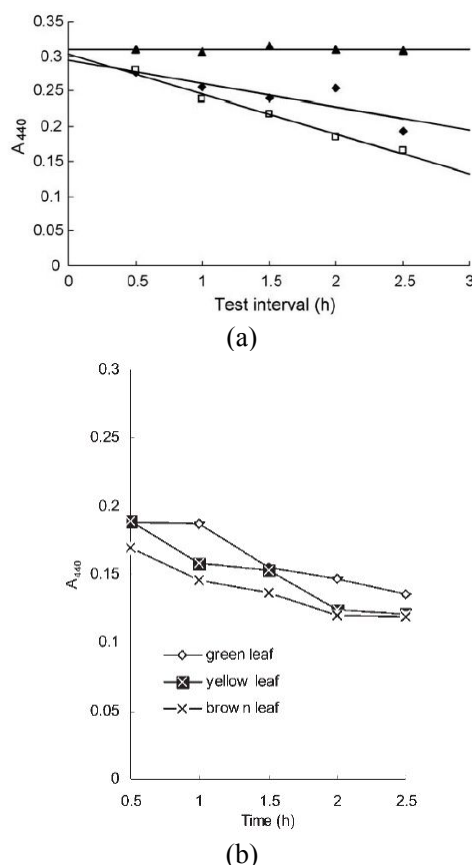


Figure 4 The time-dependent change modes of $^1\text{O}_2$ burst from leaves of treated and untreated *A. annua* plants. (a) A time-course measurement of A_{440} that correlates reversely with the amount of $^1\text{O}_2$ emitted by the chilling-treated transgenic plant sample, T47, and untreated T47. (▲) is the blank sample; (◆) is the untreated T47 sample; and (□) is the chilled T47 sample (Feng et al. 2008). (b) The time course of $^1\text{O}_2$ emission from green, yellow and brown leaves of *A. annua* plants (Yang et al. 2009).

Whether are artemisinin biosynthesis genes induced by $^1\text{O}_2$?

Direct measurement of $^1\text{O}_2$ burst from chilling and senescent leaves has provided strong support to an involvement of $^1\text{O}_2$ in the non-enzymatic conversion to artemisinin, but whether artemisinin biosynthesis genes are also inducible under the stress circumstance that triggers $^1\text{O}_2$ emission remains unclear. In the context of senescence and chilling, it was observed that $^1\text{O}_2$ burst from chilled and senescent leaves can really induces artemisinin biosynthesis genes, reiterating that $^1\text{O}_2$ is a common signal that modulate artemisinin accumulation either in senescent *A. annua* leaves or after *A. annua* leaves acclimatized to chilling (Feng et al. 2008; Yang et al. 2009; Zeng et al. 2008b).

Furthermore a correlation of the upregulation of artemisinin biosynthesis gene with the emission of $^1\text{O}_2$ in SA/MeJ-treated *A. annua* plants was also established. However, it was found that external spraying *A. annua* leaves with Rose Bengal, a photo-sensitizing $^1\text{O}_2$ generator, or direct exposure of *A. annua* to $^1\text{O}_2$ originated from the reaction of H_2O_2 with NaOCl, fails to

increase artemisinin content. The alteration of metabolite profiling in treated *A. annua* also indicated that Rose Bengal represses artemisinin accumulation, implying endogenous $^1\text{O}_2$ rather than exogenous $^1\text{O}_2$ being effective in artemisinin conversion *in vivo* (Guo et al. 2010).

How to transduce $^1\text{O}_2$ from chloroplasts to nuclei?

The conditional *fluorescent (flu)* mutant of *A. thaliana* can elicit $^1\text{O}_2$ from plastids during a transition from dark to light, during which period nuclear genes induced by $^1\text{O}_2$ are distinct from those induced by O_2^- or H_2O_2 (Laloi et al. 2007). In *A. thaliana*, two kinds of nucleus-encoded and chloroplast-targeted proteins, Executer 1 (EX1) and Executer 2 (EX2), were identified to transduce $^1\text{O}_2$ from chloroplast to nuclei, i.e., so-called ‘retrograde $^1\text{O}_2$ signaling’ (Lee et al. 2007; Kim et al. 2008). Upon enhanced $^1\text{O}_2$ burst, programmed cell death is triggered, leading to the bleaching of seedlings and the growth retardation of mature plants in the *flu* mutant of *A. thaliana* (Kim et al. 2012).

Alternatively, inhibition of isoprenoid biosynthesis by LV/FM also elicits $^1\text{O}_2$ emission (see Figure 2). It was observed that enhanced nuclear gene expression is closely correlated with repressed carotenoid production and potent $^1\text{O}_2$ burst when carotenoids are dramatically decreased by FM-inhibited plastidial terpenoid biosynthesis in *A. thaliana* (Laule et al. 2003). Although LV/FM unlikely alters the expression levels of genes involved in sterol, chlorophyll and carotene biosynthesis, it was noted that some antioxidant genes at least including glutathione peroxidase gene (*GPX*) and glutathione S-transferase gene (*GST*) in *A. thaliana* are significantly affected by LV/FM. Interestingly, those antioxidant genes are synchronously upregulated with EX1 gene (*EX1*) after treatment of *A. thaliana* by LV/FM.

Furthermore, FM/LV exposure was also observed to accompany with increase of reduced glutathione (GSH) and decline of H_2O_2 . Importantly, the photo-sensitizing $^1\text{O}_2$ generator RB fails to co-upregulate *GPX*, *GST* and *EX1* as well as to overproduce GSH, suggesting that only endogenous $^1\text{O}_2$ other than exogenous $^1\text{O}_2$ is engaged in the EX1-mediated retrograde activation of nuclear genes in *A. thaliana*. A striking elevation of the nucleus-encoded artemisinin biosynthesis relevant *DBR2* mRNA was detected following incubation of *A. annua* plants with FM. While FM decreases the chloroplast $^1\text{O}_2$ scavengers β -carotene and α -tocopherol, FD only inhibits the biosynthesis of β -carotene but not α -tocopherol (Table 1).

Table 1 The amounts of β -carotene and α -tocopherol in the leaves of *A. annua* incubated for different durations with 200 μM FM or 100 μM FD (Zeng et al. 2011a).

Treatments	β -carotene ($\mu\text{g}/\text{mg}$ fresh weight)	α -tocopherol ($\mu\text{g}/\text{mg}$ fresh weight)
Control	4.725 \pm 0.540	0.1113 \pm 0.0085
FM 3 h	3.692 \pm 0.038**	0.0840 \pm 0.0096**
FM 12 h	1.498 \pm 0.165**	0.0603 \pm 0.0015**
FM 48 h	1.378 \pm 0.080**	0.0693 \pm 0.0032**
FD 3 h	2.986 \pm 0.133**	0.1042 \pm 0.0077
FD 12 h	1.103 \pm 0.035**	0.1370 \pm 0.0171
FD 48 h	1.150 \pm 0.095**	0.0967 \pm 0.0096

Means \pm SD, $n = 9$. The double asterisks (**) represent values very significantly different from the control at $P < 0.01$.

From the comparison of isoprenoid content, it is obviously that FM represents a potent $^1\text{O}_2$ -generator because it strongly inhibits the biosynthesis of both β -carotene and α -tocopherol. In contrast, FD only decreases β -carotene but not α -tocopherol, so it serves as a weaker $^1\text{O}_2$ generator. This conclusion is strongly supported by the finding that $^1\text{O}_2$ -activated *DBR2* gene is highly up-regulated by FM, but almost unaffected by FD (Zeng et al. 2011a). In *A. thaliana*, ROS-dependent signaling was not detectable in seedlings grown with the carotenoid biosynthesis inhibitor norflurizon (NF), whereas enhanced ROS production was noted in seedlings first exposed to NF and then grown under light, during which the induction of $^1\text{O}_2$ -mediated and EX-dependent retrograde signaling was determined (Kim and Apel 2013).

Recently, an alternative approach to provoke $^1\text{O}_2$ emission has been described, by which the *Alternaria alternata* toxin tenuazonic acid blocks the Q_B -binding site along the photosynthetic electron transport chain within chloroplasts. The dysfunction of electron transport enhances the generation of excited triplet-state chlorophylls, promotes $^1\text{O}_2$ burst, activates EX1- and

EX2-dependent signaling, and finally triggers programmed cell death (Chen et al. 2014).

Conclusion

The currently available data have indicated that artemisinin is the end product of dihydroartemisinic acid quenching $^1\text{O}_2$, suggesting artemisinin ecologically exists as a sink of stress-triggered $^1\text{O}_2$ in the cytosol of *A. annua*. It is demonstrated that $^1\text{O}_2$ -catalyzed non-enzymatic reaction represents a rate-limit step for artemisinin biosynthesis, but $^1\text{O}_2$ as a catalyst must be originated from the inside of cells. It is also evident that endogenous $^1\text{O}_2$ not only promotes the auto-oxidative conversion to artemisinin, but also up-regulates artemisinin biosynthesis genes via an EX1-mediated and $^1\text{O}_2$ -sensing retrograde signaling cascade.

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Conflict of interest The authors declare that they have no conflict of interest.

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