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Singlet oxygen as a signaling transducer for modulating artemisinin biosynthetic genes in *Artemisia annua*

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Abstract

Although crosstalk between cytosolic and plastidic terpenoid pathways has been validated in many plant species, we report here for the first time a striking elevation of the nucleus-encoded artemisinin biosynthesis relevant *DBR2* mRNA following the incubation of plants with fosmidomycin (FM). FM decreased singlet oxygen ($^1O_2$) scavengers such as β-carotene and α-tocopherol and subsequently invoked $^1O_2$ burst. The treatment of plants with fluridone (FD) neither decreased α-tocopherol content nor triggered $^1O_2$ emission. In conclusion, FM can up-regulate $^1O_2$-sensitive nuclear genes responsible for artemisinin biogenesis by mitigating the accumulation of plastidic scavenging terpenoids, thereby eliciting $^1O_2$ generation and initiating $^1O_2$ retrograde signaling.

Additional key words: fosmidomycin, fluridone, gene expression, reactive oxygen species, upregulation.

Introduction

Terpenoids, also known as terpenes or isoprenes, are a huge cluster of natural products, which serve as pollinator attractants, phytoalexins, gibberellins, brassinosteroids, cytokinins, abscisic acid, carotenoids and chlorophylls (Wu *et al*. 2006). With the subcellular compartmentation, terpenoids are separately synthesized in the cytosol, plastids and mitochondria (Rodriguez-Concepción *et al*. 2004). It has been evident that sesquiterpenoids (C_{15}), triterpenoids (C_{30}) and polyterpenoids ($>C_{40}$) are derived from the cytosolic mevalonic acid (MVA) pathway beginning with acetyl-coenzyme A and proceeding *via* MVA, while monoterpenoids (C_{10}), diterpenoids (C_{20}) and tetraterpenoids (C_{40}) are stemmed from the plastidic non-MVA pathway involving deoxyxylulose-5-phosphate and methyl-4-erythritol phosphate as upstream precursors (Chappell 2002).

Artemisinin, a sesquiterpene lactone endoperoxide, uniquely exists in *Artemisia annua* and is an effective antimalarial agent. Artemisinin biogenesis *in planta* has been suggested to comprise both enzymatic and non-enzymatic reactions (Bertea *et al*. 2005, 2006). Amorpha-4,11-diene synthase (ADS) initially catalyzes the cyclization of farnesyl pyrophosphate (FPP) to generate amorpha-4,11-diene, which is subsequently converted to artemisinic alcohol, aldehyde and acid derivatives sequentially through three consecutive redox reactions by cytochrome P_{450} monooxygenase (CYP71AV1) in cooperation with cytochrome P_{450} reductase (CPR). Following catalysis by $\Delta^{11(13)}$-double-bond reductase 2 (DBR2), artemisinic aldehyde is reduced to dihydroartemisinic aldehyde, which is then oxidized to dihydroartemisinic acid (DHAA) by a trichome-specific aldehyde dehydrogenase, ALDH1 (Covello 2008). Finally, DHAA transiently forms DHAA hydroperoxide non-enzymatically in the presence of singlet oxygen ($^1O_2$), and DHAA hydroperoxide is spontaneously oxidized to artemisinin in the air (Sy and Brown 2002, Covello *et al*. 2007). Due to involving $^1O_2$, *A. annua* becomes the best model plant for

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Abbreviations: ADS - amorpha-4,11-dienesynthase; CPR - cytochrome P_{450} reductase; CYP71AV1 - cytochrome P_{450} monooxygenase; DBR2 - $\Delta^{11(13)}$-double-bond reductase 2; DHAA - dihydroartemisinic acid; DXP - 1-deoxy-*O*-xylulose-5-phosphate; DXR - DXP reductoisomerase; DXS - DXP synthase; FD - fluridone; FM - fosmidomycin; FPP - farnesyl pyrophosphate; MVA - mevalonic acid.

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elucidating the mechanism of endogenous $^1O_2$ signaling and retrograde gene activation in plants.

Since 1995, artemisinin biosynthetic genes including *ADS* (Wallaart *et al*. 1999), *CYP71AV1* (Teoh *et al*. 2006), *CPR* (Ro *et al*. 2006), and *DBR2* (Zhang *et al*. 2008) have been cloned from *A. annua* and functionally expressed in microorganisms, as reviewed by Zeng *et al*. (2008a). Evidence on the regulatory mechanism behind artemisinin biosynthesis has been also documented in recent years (Covello *et al*. 2007, Covello 2008, Zeng *et al*. 2008b). For example, it is known that the promoter of *ADS* gene is tissue-specific and development-modulated (Kim *et al*. 2008), and that *CYP71AV1* gene is differentially expressed with a high level in glandular trichomes, with a moderate level in flower buds, and with a low level in leaves and roots (Teoh *et al*. 2006). We have reported that *ADS* and *CYP71AV1* genes are inducible by low temperature and UV radiation, which is mediated by Ca^{2+}-involving signaling (Yin *et al*. 2008). Most recently, we have further demonstrated that senescence leads to potent $^1O_2$ burst from leaves of *A. annua*, which up-regulates *ADS*, *CYP71AV1* and *DBR2* genes and enhances artemisinin production (Yang *et al*. 2010). As indicated by others, the primary function of $^1O_2$ in plants is to activate a stress-related signaling cascade that encompasses numerous signaling pathways known to respond to pathogen attack, wounding, irradiance and drought stress (Xu *et al*. 2005, Laloi *et al*. 2006).

Until recently, however, it is still unknown the exact source of $^1O_2$ and how $^1O_2$ induces artemisinin biosynthetic genes in *A. annua*. Considering facts that fosmidomycin (FM) specifically inhibits plastidic terpenoid biosynthesis and impedes carotenoid biosynthesis (Laule *et al*. 2003), carotenoids are $^1O_2$ scavengers (Hirayama *et al*. 1994), and $^1O_2$ enhances artemisinin production (Zeng *et al*. 2008c), we suggest a hypothesis of the $^1O_2$-driven activation of nuclear genes to account for how FM enables temporal and spatial modulation on artemisinin biosynthesis. We assume that FM may lead to decrease of $^1O_2$ quenchers, such as carotenoids and tocopherols, emission of excess $^1O_2$, initiation of $^1O_2$-mediated retrograde signaling, and up-regulation of $^1O_2$-hypersensitive nuclear genes.

Here, we present evidence that supports our proposition of $^1O_2$ as a candidate transducer involving in a retrograde signaling pathway from plastids to the nucleus and up-regulating artemisinin biosynthetic genes. Meanwhile, we also try to demonstrate the relationship between the decrease of carotenoids and/or tocopherols and a quantitative profile of $^1O_2$-activated artemisinin biosynthetic genes. These findings should be beneficial to advance our understanding of the mechanistic pattern regarding the homeostatic regulation of artemisinin biosynthesis in *A. annua*.

Materials and methods

Artemisia annua L. cv. Huayang 2, was originally cultivated in Youyang of Sichun, China, and transplanted to Fengshun of Guangdong, China. Seeds of *A. annua* were surface-sterilized in 70 % ethanol for 30 s and in 3 % sodium hypochlorite for 30 min, and then rinsed with sterile double distilled water for 4 - 5 times. For germination, disinfected seeds were sown on filter paper (*Whatman*) saturated with Murashige and Skoog (MS) liquid medium in Petri dishes. All seedlings were cultured on the phytohormones-free 1/2 MS medium supplemented with MS vitamins and solidified by 0.8 % agar at 25 °C , irradiance of 250 μmol m^{-2} s^{-1} (PAR) and a 16-h photoperiod.

Seedlings (30 d-old) were transferred to 1/2 MS liquid medium containing filter sterilized inhibitors 200 μM fosmidomycin (FM; *Invitrogen*, Carlsbad, USA) or 100 μM fluridone (FD; *Aladdin*, Shanghai, China) and kept for 3 - 48 h or 7 d under the same conditions. After the treatment, seedlings were collected and immediately frozen in liquid nitrogen for further analysis.

RNA isolation and mRNA quantification were performed as described (Yang *et al*. 2008, 2010). The relative mRNA level = (specific mRNA copy numbers)/(18S rRNA copy numbers). Preparation of recombinant antigens in *Escherichia coli*, induction of polyclonal antibodies against recombinant antigens in rabbits, plotting of standard curves, and immune-quantification were conducted as described (Zeng *et al*. 2009).

Leaf samples (100 mg) were ground to powder in liquid nitrogen. For extraction of β-carotene, the frozen powder was mixed with 2 - 3 cm^3 of the solution chloroform + methanol (2:1, v/v) and vortexed for 2 min. The extract was filtered through a layer of the filter paper, subsequently covered by 1 g of sodium sulfate and rinsed repeatedly by the extracting solution until sodium sulfate was whitening again. The filtrate was collected and adjusted to 25 cm^3 of a total volume with the extracting solution for measurement of the absorbance at 450 nm. The concentration of β-carotene was determined from the standard curve of β-carotene (National Institute for the Control of Pharmaceutical and Biological Products in China). For extraction of α-tocopherol, the frozen powder was mixed with 1 cm^3 of *n*-hexane and vortexed for 2 min. After extraction for 2 h in 60 °C bath and centrifuged, the supernatant was collected for quantification of α-tocopherol content by a commercial vitamin E assay kit (*Nanjing Jiancheng*, Nanjing, China).

Measurement of $^1O_2$ emitted from leaves of *A. annua* was carried out by a modified spectrophotometric method

(Feng *et al*. 2009), in which N,N-dimethyl-*p*-nitroso-aniline (*Sigma*, St. Louis, USA) was used as a selective acceptor of $^1O_2$ and bleaching of N,N-dimethyl-p-nitrosoaniline was spectrophotometrically monitored.

All data were represented as the means ± SD and the statistical analysis of the independent sample tests was performed with the *SPSS 11.5* for *Windows* software package (*Statistica*, San Jose, CA, USA).

Results

Artemisinin biosynthesis relevant *DBR2* mRNA was substantially elevated in *A. annua* by incubation with 200 μM FM, but the elevated level of *DBR2* mRNA declines dramatically after maximum at 3 h and even drops to a baseline level after 12 h-incubation (Table 1). As compared with the control (0 h treatment), the elevation of *DBR2* mRNA in maximum was 250-fold (in plants 1, 2 and 3) or 20-fold (in plants 4, 5 and 6), demonstrating a huge variation among different plant samples. After incubation of *A. annua* with 200 μM FM, artemisinin biosynthesis responsible *ADS*, *CYP71AV1* and *CPR* mRNAs also elevated. Maximum *ADS* and *CYP71AV1* mRNAs occurred after 3 h, whereas a high level of *CPR* mRNA emerged after 12 h (Table 2). These results indicate that FM eventually led to the decline of artemisinin biosynthetic mRNA although transiently upregulated the corresponding genes.

Table 1. The relative content of mRNA of *DBR2* gene expressed as *DBR2* mRNA/18S rRNA ($\times 10^{-5}$) in leaves of different *A. annua* plants incubated with 200 μM FM for 3, 12 and 48 h.

Time [h]	Plant 1	Plant 2	Plant 3	Plant 4	Plant 5	Plant 6
0	0.26	0.34	0.32	0.74	0.73	0.78
3	245.83	194.66	313.38	17.72	22.15	18.58
12	2.88	2.79	2.72	3.09	2.18	2.13
48	0.19	1.68	1.89	0.46	0.60	0.20

Table 2. The relative contents of mRNA of artemisinin biosynthetic genes expressed as *DBR2* mRNA/18S rRNA in leaves of *A. annua* incubated with 200 μM FM or 100 μM FD. Means ± SD, n = 6 - 9, *,**- values significantly different from the control at $P < 0.05$ and $P < 0.01$, respectively.

Treatments	*ADS* ($\times 10^{-8}$)	*CYP71AV1* ($\times 10^{-8}$)	*CPR* ($\times 10^{-5}$)	*DBR2* ($\times 10^{-5}$)
Control	0.19 ± 0.01	0.09 ± 0.03	0.58 ± 0.19	5.58 ± 2.50
FM 3 h	0.70 ± 0.13**	0.27 ± 0.03**	0.31 ± 0.11**	20.91 ± 6.98
FM 12 h	0.09 ± 0.02	0.02 ± 0.002**	1.09 ± 0.10**	135.39 ± 132.4**
FM 48 h	0.50 ± 0.18**	0.11 ± 0.03	0.47 ± 0.13	2.63 ± 0.39
Control	7.22 ± 1.57	1.73 ± 0.23	4.96 ± 3.77	12.80 ± 10.50
FD 3 h	12.30 ± 3.27	1.97 ± 0.26	5.66 ± 2.07	10.80 ± 1.08
FD 6 h	31.60 ± 42.50*	2.70 ± 2.02	5.09 ± 2.82	16.80 ± 7.36
FD 6 h+ 42 h FD-free	17.80 ± 20.54	2.63 ± 2.27	10.80 ± 0.87	17.10 ± 4.15

Table 3. The relative strength of $^1O_2$ burst from leaves of *A. annua* incubated with 200 μM FM or 100 μM FD for 0.5 - 2.5 h. The relative strength of $^1O_2$ burst [%] = [(A_{440} of blank - A_{440} of sample) / A_{440} of blank] × 100.

Treatments	0.5 h	1 h	1.5 h	2 h	2.5 h
Control, uncut leaves	8.72	13.05	17.37	23.61	27.46
Control, cut leaves	7.18	18.29	24.92	34.12	38.03
FM 7 d, uncut leaves	20.82	35.66	46.33	57.22	64.87
FM 7 d, cut leaves	29.85	49.95	62.98	73.40	81.45
FD 7 d, cut leaves	19.91	19.25	44.50	57.14	64.73

After incubation of *A. annua* with 100 μM FD for 6 h, *ADS* mRNA elevates for more than 4 folds, whereas other artemisinin biosynthetic mRNAs including *CYP71AV1*, *CPR* and *DBR2* mRNAs keep homeostasis during an entire period of incubation (Table 2). This result demonstrates that the extent of FD affecting artemisinin biosynthesis is different from FM.

The $^1O_2$ burst from *A. annua* seedlings that were incubated with FM or FD was monitored in a time-course manner (Table 3). $^1O_2$ burst occurs from leaves of plants that were treated by 200 μM FM for 7 d, in which cut leaves release more $^1O_2$ than uncut leaves of FM-treated or untreated plants, suggesting that $^1O_2$ may emit from the internal space of cells. However, no sign indicating enhanced $^1O_2$ release appears in 100 μM FD-treated plants. Comparison of the relative strength of $^1O_2$ burst shows that 200 μM FM leads to a highest $^1O_2$ burst (80 % at 2.5 h of assay), but 100 μM FD hardly release $^1O_2$. These results suggest that FM affects terpenoid

metabolism probably *via* impacting on *DBR2* gene, in which FM extremely up-regulates *DBR2* gene due to potent $^1O_2$ burst. In contrary, FD hardly induces *DBR2* gene because of only stimulating slight $^1O_2$ liberation.

Table 4. Immunoquantification of ADS, CYP71AV1 and CPR enzymes [μg mg^{-1}(protein)] in *A. annua* incubated with 100 μM FD. Means ± SD, n = 3, *, ** - values significantly different from the control at P<0.05 and P<0.01, respectively.

Organ	Treatments	ADS	CYP71AV1	CPR
Whole plant	control	78.50±10.98	72.64±7.48	45.10±5.96
	FD 0.5 h	109.14±11.94*	97.26±10.57*	50.63±4.84
	FD 3 h	79.81±14.55	62.50±6.04*	39.52±4.26
	FD 6 h	74.51±25.55*	47.28±5.64*	27.63±2.11
	FD 6 h+42 h FD-free	60.31±3.06*	42.10±10.29*	30.27±2.21
Root	control	40.71±1.19	71.28±2.07	22.05±7.02
	FD 7 d	37.79±2.37	52.06±1.95*	15.81±1.14
Stem	control	32.04±2.89	85.25±4.20	17.39±2.09
	FD 7 d	50.82±0.65*	88.18±3.21	23.21±1.05
Leaf	control	35.11±2.49	62.41±2.98	17.13±0.86
	FD 7 d	61.84±2.43*	94.63±2.08*	16.01±1.15

Similarly as its corresponding mRNA, ADS activity rapidly elevated (2.5-folds) due to exposure to FD for a short time (0.5 h), but gradually declined thereafter in different treatment schemes (3 h FD, 6 h FD, 6 h FD + 42 h FD-free). Also, CYP71AV1 slightly increased (1.3-folds) shortly after treatment by FD and rapidly declined until recovery to an original level. At the same time, CPR demonstrated only a minor variation (Table 4). However, after exposure to FD for 7 d, ADS again increased in stems and leaves, CYP71AV1 recovered in stems but increased in leaves, and CPR increased in stems (Table 4).

Table 5. The amounts of β-carotene and α-tocopherol in leaves of *A. annua* incubated with 200 μM FM or 100 μM FD. Means ± SD, n = 9, ** - values very significantly different from the control at P<0.01.

Treatments	β-Carotene [μg mg^{-1}(f.m.)]	α-Tocopherol [μg mg^{-1}(f.m.)]
Control	4.725±0.540	0.1113±0.0085
FM 3 h	3.692±0.038**	0.0840±0.0096**
FM 12 h	1.498±0.165**	0.0603±0.0015**
FM 48 h	1.378±0.080**	0.0693±0.0032**
FD 3 h	2.986±0.133**	0.1042±0.0077
FD 12 h	1.103±0.035**	0.1370±0.0171
FD 48 h	1.150±0.095**	0.0967±0.0096

Much lower amounts of β-carotene and α-tocopherol were measured in *A. annua* treated by 200 μM FM for 3, 12 and 48 h. Only β-carotene but not α-tocopherol decreased in 100 μM FD-treated plants. From comparison of the content of terpenoids, it was obviously that FM represented a potent $^1O_2$-generator because it strongly inhibited the biosynthesis of both β-carotene and α-tocopherol. In contrast, FD only decreased β-carotene but not α-tocopherol, so it served as a weaker $^1O_2$ generator. Additionally, the finding that *DBR2* gene was highly up-regulated by FM, but not affected by FD suggested a possibility of *DBR2* gene being induced by a higher threshold level of $^1O_2$.

Discussion

Although reduced artemisinin production in *A. annua* after exposure to FM was noticed (Towler and Weathers 2007), no suppressed transcriptomic and proteomic profiles regarding artemisinin biosynthesis have been described in *A. annua*. In the present investigation, we observed for the first time that *DBR2* gene was extremely up-regulated by FM although the elevation of *DBR2* mRNA as well as other MVA mRNAs were transient, not longer than 3 h, and gradually declined to a level lower than that in the control. On the other hand, we found that FM constantly down-regulated non-MVA genes during the entire period of incubation. This result seems to be supported by a previous conclusion that chlorophyll and carotenoid contents in *A. thaliana* are substantially lower after addition of FM than in controls (Laule *et al*. 2003). Most recently, we recognized the relevance of chilling-evoked $^1O_2$ burst to artemisinin overproduction (Feng *et al*. 2009), and addressed an implication of the inducible endogenous $^1O_2$ in enhancing artemisinin production (Guo *et al*. 2010).

Interestingly, the analytical data available from the present experiments can be fairly interpreted by our proposed hypothesis for $^1O_2$-driven retrograde gene activation. According to the hypothesis, any inhibitors capable of decreasing amounts of $^1O_2$-scavengers within chloroplasts can activate $^1O_2$-sensitive nuclear genes. Given that FM leads to a dramatic decrease of β-carotene and α-tocopherol contents, it can be concluded that FM is able to up-regulate the $^1O_2$-sensitive *DBR2* gene. As a major class of $^1O_2$ scavengers residing in chloroplasts, carotenoids are responsible for quenching the excited $^1O_2$ in the chlorophyll antenna (Hirayama *et al*. 1994), while tocopherols accumulate in chloroplasts to protect membranes from $^1O_2$-mediated lipid peroxidation (Maeda *et al*. 2005). It seems that *DBR2* gene may simply sense the chloroplast-originating $^1O_2$ signal and be fine-tuned by a threshold level of $^1O_2$ burst within chloroplasts. In such context, it can be concluded that FM induced

abundant *DBR2* mRNA due to invoking stronger $^1O_2$ burst.

A photo-bleaching herbicide FD was chosen to validate this tentative conclusion as it only decreases β-carotene (Bartels and Watson 1978). Due to the presence of α-tocopherol, it is predicted that FD-treated plants may keep a slightly elevated $^1O_2$ level or remain an unchanged $^1O_2$ level, thereby not inducing *DBR* gene. Indeed, FD-treated plants exhibit no induction of *DBR2* gene, which is in contrast to the abundant *DBR2* mRNA induced by the FM treatment. Importantly, the $^1O_2$ burst is much higher after FM treatment than after FD treatment, which also seems to suggest that the induction of *DBR2* gene is likely controlled by a threshold level of $^1O_2$. However, other $^1O_2$ scavengers including pyridoxine, ascorbic acid, plastoquinones and wide arrays of flavonoids (Benderliev *et al*. 2003, Triantaphylides and Havaux 2009) may add complexicity to account for the relevance of $^1O_2$.

Although no elevated *DBR* mRNA was detected in FD-treated plants, *ADS* mRNA was observed to elevate up to 10 folds following treatment by FD. Accordingly, ADS activity was higher after exposure to FD although it gradually declined in a time-dependent manner. After 7 d incubation with FD, however, ADS activity increased again to the level above the control. This result implies that *ADS* gene may be activated by a mechanism different from *DBR2* gene, which is consistent with our recent findings that cold, heat shock and UV radiation activate *ADS* gene but not *DBR2* gene (Yang *et al*. 2010). Therefore, it can be concluded that potent $^1O_2$ burst may bear importance in the activation of nuclear genes including those for artemisinin biosynthesis in *A. annua*. In regard to how plastid-generating $^1O_2$ is transmitted to nuclei, Laloi *et al*. (2007) and Lee *et al*. (2007) have indicated that $^1O_2$ signaling can be transduced by EXECUTER1 (EXE-1) and EXE-2, through which the transcription of $^1O_2$-responsive nuclear genes is initiated.

In conclusion, we demonstrated that $^1O_2$ is likely a modulator of the nucleus-encoded $^1O_2$-sensitive *DBR2* as well as other artemisinin biosynthetic genes of *A. annua*, in response to the insufficient content of β-carotene and α-tocopherol. We also conclude that $^1O_2$-driven activation of *DBR2* gene is likely fine-tuned by a threshold level of $^1O_2$, which can be invoked potently by FM but not by FD. Our future endeavors will focus on the finding of a $^1O_2$-inducible EXE-like candidate gene from *A. annua* for expediting the organization and operation of a detailed $^1O_2$-involving signaling pathway.

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