Salicylic acid and methyl jasmonate but not Rose Bengal enhance artemisinin production through invoking burst of endogenous singlet oxygen

Xiao-Xia Guo 1, Xue-Qin Yang 1, Rui-Yi Yang, Qing-Ping Zeng*

Laboratory of Biotechnology, Tropical Medicine Institute, Guangzhou University of Chinese Medicine, Guangzhou 510405, China

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A B S T R A C T

As a cis element responsive to oxidative stresses, the promoter of amorphadiene synthase gene (pADS) from Artemisia annua was also found to be inducible by salicylic acid (SA) and methyl jasmonate (MJ), which had been evident by phenotyping pADS-driven β-glucuronidase reporter gene (GUS) in transgenic Nicotiana tabacum incubated with SA/MJ. Furthermore, SA/MJ-treated A. annua exhibited a correlation of the up-regulation of ADS gene with the emission of singlet oxygen (1O2), suggesting that SA/MJ induced artemisinin overproduction through invoking 1O2 burst. Supplement with Rose Bengal (RB), a photosensitizing 1O2 generator, or direct exposure to a 1O2-generating pool, however, failed to facilitate artemisinin accumulation in subjected A. annua. Alteration of metabolite profiles and fluctuation of H2O2 level suggested that RB treatment might have led to a diminished endogenous 1O2 release from A. annua, thereby reiterating an important role of the endogenous 1O2 rather than the exogenous 1O2 in enhanced artemisinin production.

1. Introduction

As a kind of plant secondary metabolites, artemisinin accumulates in Artemisia annua in a trace amount, which has led to the valuable antimalarial agent in short supply [1]. Since a putative enzymatic pathway for artemisinin biosynthesis in planta was previously suggested [2–4], artemisinin biosynthetic genes encoding amorphadiene synthase (ADS), cytochrome P450 monoxygenase (CYP71AV1), cytochrome P450 reductase (CPR) and double-bond reductase 2 (DBR2) have been cloned from A. annua [5–8]. Also, the development-dependent and environment-inducible features for artemisinin biosynthesis have also been addressed [9–11]. For example, it was observed that senescent and even dead leaves of A. annua incredibly produced highest artemisinin [12], which was supported by our report describing that senescent leaves of A. annua are one of the most active organs for overexpression of artemisinin biosynthetic genes [13]. Moreover, we have currently revealed a positive correlation of overexpressed artemisinin biosynthetic genes with enhanced artemisinin production in response to low temperature [14], from which a Ca2+-involved signal transduction pathway responsible for the chilling stress-induced up-regulation of artemisinin biosynthetic genes has been elucidated [15]. Recently, we have also detected a sustainable release of singlet oxygen (1O2), a kind of reactive oxygen species (ROS), in A. annua that exposed to cold and experienced senescence [16]. These results highly implied that artemisinin production can be enhanced by oxidative stresses, especially 1O2 burst, which likely plays a dual role through activating artemisinin biosynthetic genes and promoting non-enzymatic conversion of artemisinin precursors to artemisinin.

Jasmonic acid (JA), methyl jasmonate (MJ) and salicylic acid (SA) are naturally occurring phytohormones and known as ‘secondary messengers’, which are capable of transducing normal developmental signals or adverse environmental stimuli to plant cells for initiating protective responses against oxidative stresses [17]. MJ was known to involve fungal elicitor-induced phytoalexin production in cultured rice suspension cells [20]. MJ induced overproduction of paclitaxel and silymarin in the suspension cultures of Taxus [21] and Silybum marianum [22], respectively. On the other hand, SA has deep implications in response to abiotic stresses (such as ultraviolet light radiation and ozone contamination) and biotic stresses (pathogen infection) [23–25].
An SA-dependent signalling pathway was suggested for fungal elicitor-induced puerarin biosynthesis in *Pueraria thomsonii* Benth [26]. It has been reported that 20–50 mg/L (140–360 μM) SA or 5–10 mg/L (22.3–44.6 μM) MJ significantly increased artemisinin content in suspension cells of *A. annua*, in which the highest yield was 6-fold as untreated control cells [27]. A most recently published report has described that 1 mM SA could activate artemisinin biosynthesis in field-grown *A. annua* plants [28].

Nevertheless, it is unclear how MJ and SA affect artemisinin biosynthesis and whether they behave in an identical or distinct pattern. Here, we report that SA/MJ-mediated \(^{1}\)O\(_{2}\) burst is a mechanism underlying up-regulation of artemisinin biosynthetic genes and overproduction of artemisinin. Also, we draw a conclusion that endogenously generated \(^{1}\)O\(_{2}\) other than exogenously supplied \(^{1}\)O\(_{2}\) enhanced artemisinin production in *A. annua*.

2. Materials and methods

2.1. Plant materials

Transgenic tobacco (*Nicotiana tabacum*) plants were achieved by *Agrobacterium*-mediated transformation of a cultivated varieties (*K326*) as described [16]. For transformation, a plant expression vector with a chimeric promoter of *b* and *s* genes and overproduction of artemisinin. Also, we draw a conclusion that endogenously generated \(^{1}\)O\(_{2}\) other than exogenously supplied \(^{1}\)O\(_{2}\) enhanced artemisinin production in *A. annua*.

2.2. Treatments of cultured plants

For preparing stock solutions, SA (Sigma) and MJ (Sigma) were dissolved in ethanol; RB (Sino Reagent, China) was dissolved in double distilled water. To treat *A. annua* by a different concentration of SA, MJ or RB, 30-day-old cultured young plants without flowers were immersed with their roots into each of above solutions and incubated for certain durations in a luminescent growth chamber. For in situ generating \(^{1}\)O\(_{2}\), a funnel containing 50 ml of NaOCl (10% chloride) was placed above a bottle containing 50 ml of 30% H\(_{2}\)O\(_{2}\). As an aliquot of NaOCl falls into H\(_{2}\)O\(_{2}\) drop by drop, \(^{1}\)O\(_{2}\) generates and approaches to a connected flask growing a cultured plant. Although whole plants were included for treatments, only leaves were collected for further analysis.

2.3. Colorimetric analysis of GUS activity

100 mg of leaves were collected from cultured transgenic tobacco plants treated by SA/MJ. After leaves were lyophilized by liquid nitrogen and ground into fine powder, an extraction buffer containing 50 mM Na\(_{2}\)PO\(_{4}\), 10 mM EDTA, 0.1% Triton X-100, 0.1% Sarcosyl and 10 mM β-mercaptoethanol, pH 7.0 were added by threefold volumes into a mortar with leaves and gently ground with a pestle until a homogenate was obtained. After the homogenate was centrifuged at 4000 rpm for 10 min, the suspension was recovered for determination of protein concentrations by the Bradford method [29]. For assessing GUS activity, 120 μl of suspension was used to mix with a reaction buffer containing 50 mM Na\(_{2}\)PO\(_{4}\), 10 mM EDTA, 0.1% Triton X-100, 10 mM β-mercaptoethanol, and 1 mM p-nitrophenyl-β-D-glucurate, pH 7.0 in a 3 ml of total volume. Following the mixture was incubated at 37°C for 5 h, the reaction was stopped by adding 1.2 ml of 1 M 2-chloro-2-methyl-1,3-propanediol and the colored solution was applied for measurement of the absorbance at 415 nm. The amount of p-nitrophenol was checked from a standard curve plotted by a series of concentrations of p-nitrophenol versus corresponding \(A_{415}\) values. One unit of GUS activity was defined as the enzyme amount that hydrolyze p-nitrophenyl-β-D-glucurate to generate 1 nM p-nitrophenol per min, and the specific GUS activity was represented by enzyme units per mg of total proteins.

2.4. Real-time fluorescent quantitative amplification

100 mg of leaves were collected from *A. annua* treated by 145 μM SA/100 μM MJ for 24 h and immediately frozen in liquid nitrogen for quantitative analysis by polymerase chain reaction (PCR). Total RNAs were isolated by a commercially available Plant RNA Kit (Promega). Reverse transcription and fluorescent quantitative PCR were performed with purchased RevertAid H Minus

<table>
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<tr>
<th>Tested gene names</th>
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First Strand cDNA Synthesis Kit (Fermentas) and SYBR Green Real Time PCR Master Mix (TOYOBO) as described [30]. 18S rRNA was chosen as an internal reference. Standard curves of 18S rRNA and target cDNAs were plotted with corresponding recombinant plasmids being constructed in this laboratory. The primers that were designed for amplification of target genes from A. annua were listed in Table 1. The relative mRNA level was defined as specific mRNA copy numbers/18S rRNA copy numbers.

2.5. Time-course detection of \(^{1}O_2\) burst

\(^{1}O_2\) burst from cut leaves was monitored by a spectrophotometric method [16], in which N,N-dimethyl-p-nitrosoaniline (Sigma) was used as a selective acceptor of \(^{1}O_2\) and bleaching of N,N-dimethyl-p-nitrosoaniline was measured. For practical assay, 124 mg of leaves were collected, sheared into strips with 1 mm width, and put into 5 ml of the assay mixture containing 45 mM phosphate buffered saline (PBS) (NaH\(_2\)PO\(_4\), Na\(_2\)HPO\(_4\) and NaCl, pH 7.1), 10 mM histidine (Sigma) and 50 \(\mu\)M N,N-dimethyl-p-nitrosoaniline (Sigma). Following incubation of cut leaves at 30 °C for 30 min under extensive light (>3000 lx), 200 \(\mu\)l of the reaction mixture was mixed with 1.8 ml of 45 mM PBS for determination of the absorbance at 440 nm (\(A_{440}\)) with an interval of 30 min during a test session of 1–3 h. The relative strength of \(^{1}O_2\) burst (%) was represented by \((A_{440}\text{ of blank} - A_{440}\text{ of sample})/A_{440}\text{ of blank} \times 100\).

2.6. Determination of artemisinin content

Artemisinin was determined by high-performance liquid chromatography (HPLC) as described [30]. Briefly, leaves were toasted for 24 h in a 50 °C oven and ground into fine powder. It was extracted in acetone by sonication, 30 \(\mu\)l of the extract sample was injected into a sampling outlet of the instrument (Dionex-100). Quantitative analysis was carried out by a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 \(\mu\)m particle size) and detection was conducted at 216 nm of the wave length (\(A_{216}\)). The acetonitrile–0.1% ice acetic acid (40:60, v/v) was used as a mobile phase with 1 ml/min of the flow rate, 30 °C of the column temperature, 50 °C of the drift tube temperature, and 2 ml/min of the air flow rate. To prepare the positive control, 8.9 mg of artemisinin (Sigma, 98%) were dissolved in 10 ml of methanol. For plotting a standard curve, prepare the positive control, 8.9 mg of artemisinin (Sigma, 98%) was used as a selective acceptor of \(^{1}O_2\) and bleaching of N,N-dimethyl-p-nitrosoaniline (Sigma). Following incubation of cut leaves at 30 °C for 30 min under extensive light (>3000 lx), 200 \(\mu\)l of the reaction mixture was mixed with 1.8 ml of 45 mM PBS for determination of the absorbance at 440 nm (\(A_{440}\)) with an interval of 30 min during a test session of 1–3 h. The relative strength of \(^{1}O_2\) burst (%) was represented by \((A_{440}\text{ of blank} - A_{440}\text{ of sample})/A_{440}\text{ of blank} \times 100\).

2.7. Measurement of \(H_2O_2\) level

Fresh leaves from SA/MJ- or RB-treated A. annua plants were collected after incubation for a certain duration. For preparing samples, 40 mg of pooled leaves were mixed with 160 \(\mu\)l of PBS and ground until a homogenate was obtained. After the homogenate was centrifuged at 13,000 rpm for 10 min, the suspension was recovered for measurement of \(H_2O_2\) level by a commercial reagent kit purchased from Jiancheng Biotech China. On a multi-well plate, 30 \(\mu\)l of the extract sample was injected into a sampling outlet of the instrument (Dionex-100). Quantitative analysis was carried out by a Phenomenex Luna C18 column (250 mm × 4.6 mm, 5 \(\mu\)m particle size) and detection was conducted at 216 nm of the wave length (\(A_{216}\)). The acetonitrile–0.1% ice acetic acid (40:60, v/v) was used as a mobile phase with 1 ml/min of the flow rate, 30 °C of the column temperature, 50 °C of the drift tube temperature, and 2 ml/min of the air flow rate. To prepare the positive control, 8.9 mg of artemisinin (Sigma, 98%) were dissolved in 10 ml of methanol. For plotting a standard curve, prepare the positive control, 8.9 mg of artemisinin (Sigma, 98%) was used as a selective acceptor of \(^{1}O_2\) and bleaching of N,N-dimethyl-p-nitrosoaniline (Sigma). Following incubation of cut leaves at 30 °C for 30 min under extensive light (>3000 lx), 200 \(\mu\)l of the reaction mixture was mixed with 1.8 ml of 45 mM PBS for determination of the absorbance at 440 nm (\(A_{440}\)) with an interval of 30 min during a test session of 1–3 h. The relative strength of \(^{1}O_2\) burst (%) was represented by \((A_{440}\text{ of blank} - A_{440}\text{ of sample})/A_{440}\text{ of blank} \times 100\).

3. Results

3.1. SA/MJ-induced \(pADS-GUS\) gene in transgenic N. tabacum and artemisinin overproduction in A. annua

Using our previously established phenotyping platform for a chimeric \(pADS-GUS\) reporter gene [16], we evaluated SA/MJ-induced GUS staining in cultured transgenic tobacco plants. After 3-day-incubation with 50 \(\mu\)M SA/250 \(\mu\)M MJ, the specific GUS activity was calculated from a colorimetric assay result. Consequently, both treatments activated GUS gene driven by \(pADS\), in which 250 \(\mu\)M MJ induced a higher specific GUS activity than 50 \(\mu\)M SA, as illustrated in Fig. 1 (left three columns).

Although 5-fold differences of working concentrations of SA from MJ only resulted in 2-fold differences of specific GUS activities, a positive GUS staining of SA/MJ-treated \(pADS-GUS\)-expressing tobacco plants confirmed \(pADS\) being SA/MJ inducible. From this clue, it could be anticipated that \(ADS\) gene in A. annua is also inducible by SA/MJ, which ultimately leads to enhanced artemisinin production.

After incubation with 50 \(\mu\)M SA/250 \(\mu\)M MJ, as expected, A. annua plants gave rise to a higher yield of artemisinin (Fig. 1, right three columns), which was consistent with above consequence that \(pADS-GUS\) gene was up-regulated by SA/MJ. Artemisinin content was estimated as 2-fold in SA-treatment and 1.5-fold in MJ-treatment as the control. This result strongly supported the conclusion that \(ADS\) gene is SA/MJ-inducible although an optimal work concentration of SA/MJ needs to be established.

3.2. SA/MJ-invoked time-dependent \(^{1}O_2\) burst from A. annua leaves

To reveal a plausible relevance of the induction of SA/MJ to generation of \(^{1}O_2\), the relative strength of \(^{1}O_2\) burst was monitored in A. annua incubated with a different concentration of SA/MJ. As illustrated in Fig. 2A, after incubation for 24 h, both 500 \(\mu\)M SA and 50 \(\mu\)M MJ stimulated A. annua releasing a high level of \(^{1}O_2\). However, much accelerated \(^{1}O_2\) burst was detected following incubation of A. annua with 100 \(\mu\)M MJ/145 \(\mu\)M SA for 24 h (Fig. 2B). In contrast, treatment of A. annua by 145 \(\mu\)M SA/100 \(\mu\)M for 7d elicited no \(^{1}O_2\) emission (Fig. 2C). These results indicated that the efficiency of SA/MJ on the induction of \(^{1}O_2\) generation is time- and concentration-dependent.

It can be concluded from Fig. 2 that incubation with 145 \(\mu\)M SA/100 \(\mu\)M MJ for 24 h is likely a better regimen among all attempted
ones for invoking stronger $^{1}$O$_2$ release from cut leaves of subjected A. annua.

3.3. SA/MJ-mediated up-regulation of artemisinin biosynthesis genes

To confirm the assumed SA/MJ-inducible feature of artemisinin biosynthesis, the abundance of three artemisinin biosynthetic mRNAs were quantified in A. annua treated by 145 $\mu$M SA/100 $\mu$M MJ. Consequently, SA-induced ADS mRNA was sevenfold as the control, while MJ-induced ADS mRNA was 4-fold as the control (Fig. 3). This result validated that ADS gene is highly inducible by SA/MJ in A. annua, which was supported by above results from GUS phenotyping and artemisinin quantification as well. For CYP71AV1 gene, however, comparisons among SA/MJ-treated A. annua indicated no significant difference of the corresponding transcript. Synchronously, CPR gene was also no response to SA/MJ (Fig. 3).

The fact that only ADS gene rather than CYP71AV1 and CPR genes was up-regulated by SA/MJ postulated that ADS gene might control a critical step on artemisinin biosynthetic pathway.

3.4. Synchronous elevation of terpene relevant mRNAs upon exposure to SA/MJ

For other genes relevant to artemisinin biosynthesis directly or indirectly, HMGR gene responsible for the cytosolic terpene biosynthesis was also induced by 145 $\mu$M SA/100 $\mu$M MJ, in which SA-induced HMGR mRNA was 5.3-fold as that in the control, while MJ-induced HMGR mRNA was 3.6-fold as that in the control. Besides, two other cytosolic terpene pathway genes, FDS and SQS, seemed not being responsive to treatments (Fig. 4).

As to DXS gene for the plastidial terpene pathway, SA did not activated it effectively, whereas MJ only slightly activated it for 1.5-fold as the control. Another terpene pathway gene, DXR, demonstrated no variation following treatments (Fig. 4).

Among above compared genes relevant to artemisinin biosynthesis, only HMGR gene was obviously induced by SA/MJ, while DXS gene was uniquely induced by MJ.

3.5. Decrease of artemisinin content in $^{1}$O$_2$-treated A. annua

To assess an assumption that $^{1}$O$_2$ might be a candidate inducer for artemisinin accumulation, we chose two different ways to generate $^{1}$O$_2$: one utilizing a photosensitive dye RB to liberate $^{1}$O$_2$ under light; another depending on a reaction between H$_2$O$_2$ and NaOCl to release $^{1}$O$_2$. Unfortunately, both treatments failed to facilitate artemisinin biosynthesis as anticipated, otherwise they dramatically decreased artemisinin content (Fig. 5) although RB-treated plants kept a normal phenotype with a slightly stained red color but without demonstrating any signs of wilt and whitening. Plants that were treated by 290 $\mu$M RB for 6 h or 22 h only accumulated artemisinin for 3.7 mg/g or 2.2 mg/g, as 84% or 50% as
the control (4.5 mg/g), and plants that were exposed to $^{1}$O$_{2}$ for 6 h even gave rise to much less artemisinin amount (1.5 mg/g), as 33% as the control.

These data implied that, unlike the endogenous $^{1}$O$_{2}$, exogenously generated $^{1}$O$_{2}$ did not increase artemisinin yield although no distinguishable phenotypic alterations such as wilt and chlorosis were observed. One possible reason for this outcome was likely that the exogenously supplied $^{1}$O$_{2}$ might react non-specifically with the metabolites other than artemisinin precursors to synthesize versatile by-products and thereby to have decreased artemisinin accumulation simultaneously.

### 3.6. Metabolite profiling of A. annua following RB exposure

To uncover the manner by which RB decreased artemisinin content dramatically, the metabolite profiling of an acetone extraction of artemisinin from 290 $\mu$M RB-treated A. annua was analyzed by HPLC. Table 2 listed the analytical data regarding the retention time (RT) and corresponding height to each main peak.

![Fig. 5. Artemisinin content in RB-treated or $^{1}$O$_{2}$-exposed A. annua plants.](image)

The peak at RT = 12 min that represents artemisinin in each treatment was shown in Fig. 6.

From Table 2, it was worthy of noting that either RB incubation or $^{1}$O$_{2}$ exposure led to lost of two main peak at RT = 5.43 min and RT = 5.85 min that was seen from the control, and $^{1}$O$_{2}$ exposure further lost another main peak at RT = 4.72 min. In contrary, both treatments allowed appearance of additional peaks, in which 290 $\mu$M RB incubation for 6 h produced four novel peaks, while a same titer of RB incubation for 22 h and $^{1}$O$_{2}$ exposure for 6 h derived one extra peak. Although each peak was not yet identified, an altered metabolite profiling reflected that the exogenous $^{1}$O$_{2}$ did not benefit to active artemisinin biosynthesis.

### 3.7. Attenuation of $^{1}$O$_{2}$ burst from RB-incubated A. annua

It was known that RB exogenously generates $^{1}$O$_{2}$ by light excitation, but no data had been documented regarding whether RB also invokes the burst of endogenous $^{1}$O$_{2}$. To obtain such information, the relative strength of $^{1}$O$_{2}$ burst was monitored in A. annua during incubation with 290 $\mu$M RB for 4.5 h and 6 h. As shown in Fig. 7, RB in the present concentration significantly diminished $^{1}$O$_{2}$ burst in a time-dependent manner, i.e., the longer of the incubation duration, the weaker of $^{1}$O$_{2}$ burst.

Above results appeared to exclude the possibility of $^{1}$O$_{2}$ emission from cells incubated with RB. It was unclear, however, why presence of the external $^{1}$O$_{2}$ inhibited generation of the internal $^{1}$O$_{2}$.

### 3.8. Comparison of H$_{2}$O$_{2}$ level in SA/MJ- and RB-treated A. annua

To investigate the correlation of exogenous $^{1}$O$_{2}$ exposure to endogenous ROS generation, the cellular level of H$_{2}$O$_{2}$ was determined in SA/MJ- or RB-incubated A. annua. After treatment by 50 $\mu$M SA/250 $\mu$M MJ for 6 h, A. annua only generated a limited amount of H$_{2}$O$_{2}$. In contrast, incubation of A. annua with 290 $\mu$M RB caused a much higher level of H$_{2}$O$_{2}$, during which the induction of H$_{2}$O$_{2}$ was enhanced in first 2–3 h incubations, while it was attenuated and recovered to a base-line level following incubation for 4 h (Fig. 8).

A declined H$_{2}$O$_{2}$ level following an elevated one in A. annua incubating with 290 $\mu$M RB for 4 h might have induced an anti-oxidant enzyme such as peroxidase or activated the activity of
catalase, both of which could scavenge the cellular ROS and lead to a declined H$_2$O$_2$ level.

4. Discussion

Phenotyping of chimeric pADS-GUS gene was previously conducted in transgenic Arabidopsis thaliana plants, by which an organ-specific expression of pADS-driven GUS gene in anthers and trichomes has been verified [31]. By harnessing the similar phenotyping platform established in N. tabacum, we disclosed the cold and ultraviolet radiation-inducible features of pADS [16]. In the present assay, we further attributed a SA/MJ-inducible property to pADS by determination of SA/MJ-induced GUS activity in transgenic tobacco plants.

In such context, we predicted that SA/MJ may also induce the up-regulation of ADS and other artemisinin biosynthetic genes in A. annua. From quantification of artemisinin amount following induction by SA/MJ, it is manifested that artemisinin production enhanced by SA/MJ was most probably via the up-regulation of relevant artemisinin biosynthetic genes. To validate the results available from reporter phenotyping and artemisinin quantification, we investigated SA/MJ-induced expression of eight A. annua genes relevant to artemisinin biogenesis directly or indirectly. As consequences, both upstream HMGR gene and downstream ADS gene directly responsible for artemisinin biosynthesis were up-regulated by 145 μM SA/100 μM MJ following incubation for 24 h. Some clues supporting a positive correlation of overexpressed ADS and HMGR genes with enhanced artemisinin production occurs frequently. For example, ADS represents a key enzyme for artemisinin biosynthesis in A. annua [5]; and introduction of HMGR gene is responsible for artemisinin overproduction in transgenic A. annua plants [32]. It has been described recently that A. annua plants spraying 1 mM SA on leaves allowed accumulation of ADS mRNA to a maximal level and that of HMGR mRNA to 130% higher than the control [28]. These latter result available from RNA blot is coincident to our analytical data on artemisinin biosynthetic mRNAs determined by quantitative PCR.

Considering MJ/SA capable of up-regulating artemisinin biosynthetic genes, we assumed that both inducers share a common mediator to respond external stimuli. What is the bona fide mediator for SA/MJ induction? In the present study, we found that incubation of A. annua with SA/MJ allowed potent 1O$_2$ burst, suggesting that 1O$_2$ may serve as such a candidate mediator for SA/ MJ signalling. As indicated previously by our group, 1O$_2$ burst during senescence [13] or under chilling stress [16] rendered the up-regulation of artemisinin biosynthetic genes and overproduction of artemisinin as well. It has also been observed that a fungal elicitor-induced generation of 1O$_2$ conferred highly effective saponin accumulation in cell cultures of Panax ginseng [33]. Hence, we draw a conclusion here that 1O$_2$ might be involved in SA/MJ-mediated signalling for artemisinin biogenesis in A. annua. In our knowledge, this is the first report describing an involvement of 1O$_2$ in SA/MJ-enhanced artemisinin biosynthesis.

Given that SA and MJ behave in almost a similar fashion of action [34], a question must be raised in regard to why both SA and MJ were employed by A. annua for one purpose. In this aspect, a complicated ROS signalling pathway for secondary metabolite production was described in Pueraria thomsonii Benth cells [26], in which fungal elicitor-induced puerarin biosynthesis was mainly mediated by an SA-dependent signalling pathway, but JA might be used as a substitute of SA for signalling when SA biosynthesis was impaired. Interestingly, an investigation in the genus Arctemisia showed that JA accumulated eightfold in field-grown plants as in greenhouse-cultivated plants, which can be divided into a high level group (30 nmol/g DW), a moderate level group (10−20 nmol/ g DW) and a low level group (10 nmol/g DW) [35]. At present, however, it is still not sure for us whether JA/MJ or SA being a major mediator for signalling in A. annua.

It has been known that 1O$_2$ generates in a photosensitized process by energy transfer from dye molecules such as RB, methylene blue or porphyrins, and 1O$_2$ is also available from a chemical reaction of H$_2$O$_2$ with NaOCl [36]. To provide 1O$_2$ exogenously, A. annua plants were either incubated with RB under light or linked to a chemical reaction pool that generates 1O$_2$ continuously. Unlike abovementioned inducers of 1O$_2$, however, neither RB-incubation nor 1O$_2$-exposure increased artemisinin content. In contrast, these treatments not only caused a gradual drop of artemisinin content in a time-dependent pattern, but also altered the metabolite profiles of artemisinin extracts. In RB-treated plants, some characteristic peaks of ingredients existing in untreated plants disappeared, while extra peaks representing novel by-products appeared. Such unexpected outcome might be originated from the non-specific reaction of intermediates with 1O$_2$ and/or the induction of endogenous antioxidant enzymes, which can effectively quench 1O$_2$ and exert an adverse effect on artemisinin production as well.

It was found that overexpression of either glutathione peroxidase gene or glutathione S-transferase gene in Chlamydomonas reinhardtii was sufficient enhancing resistance to 1O$_2$ from excitation of RB by light radiation [37]. Interestingly, we found in the present investigation that incubation of A. annua with RB for 2-3 h led to an elevated H$_2$O$_2$ level, whereas only a slightly elevated H$_2$O$_2$ level was measured in A. annua treated by 50 μM SA/250 μM MJ for 6 h. A previous discovery regarding that ascorbate reacts with 1O$_2$ to produce H$_2$O$_2$ [38] seemed to provide an interpretation to our findings as that the reaction of light-excited 1O$_2$ with cellular

### Table 2

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<td>–</td>
<td>–</td>
<td>12.137</td>
<td>1.551</td>
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</tbody>
</table>

Note: A dash represents a disappeared or declined peak (height <1 mV), The peak with RT =12 min indicates artemisinin. CK: an untreated control plant; RR6h: a plant incubated with RB for 6 h; RR22h: a plant incubated with RB for 22 h; 1O$_2$6h: a plant exposed to 1O$_2$ for 6 h.
ascorbate might represent a mechanism behind which RB transiently elicited H₂O₂ release. Alternatively, an elevated H₂O₂ level implied that ⁷O₂-mediated signaling will be suppressed because H₂O₂ serves as an antagonist to ⁷O₂ through a distinct pathway for signal transduction [39]. This conclusion was strongly supported by our results that RB stimulated potent H₂O₂ release and thereby antagonized ⁷O₂ signalling, while SA/MJ elicited endogenous ⁷O₂ generation due to without interference by H₂O₂.

It was also likely that an elevated H₂O₂ level might have led to a gradual induction of antioxidant enzymes like glutathione peroxidase, which would in turn reduce the level of H₂O₂ as this enzyme serves as a scavenger and a consumer of H₂O₂. This deduction was supported by our result that continuous RB-incubation for 4 h caused a declined or a base-level of H₂O₂.

Besides, there were no significant changes in β-carotene, lutein, or xanthophyll cycle pigments during 2 h pre-treatment or 1 h challenge by RB. Similarly, changes in the content or composition of vitamin E (tocopherols) were not observed in RB-treated plants [40]. These results have convinced us that RB did not elicit the

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**Fig. 6.** The chromatogram of artemisinin extracts in RB-treated or ⁷O₂-exposed *A. annua* plants. RT = 12 min is a specific peak of artemisinin. Artemisin is the standard; 1–4 are samples representing ⁷O₂ exposure for 6 h, RB incubation for 6 h, RB incubation for 22 h, and the untreated control, respectively.

**Fig. 7.** Time-course monitoring of the relative strength of ⁷O₂ burst invoked by incubation with 290 μM RB for 4.5 h and 6 h. CK represents the control.

**Fig. 8.** The cellular level of H₂O₂ in 50 μM SA/250 μM MJ-treated *A. annua* for 6 h (left three columns) or 290 μM RB-treated *A. annua* for 2, 3 and 4 h (right four columns). CK represents the control.
endogenous \( \text{O}_2 \) burst from chloroplasts since plastidic \( \text{O}_2 \) scavengers like carotenoids and tocopherols can even sense the slight fluctuation of \( \text{O}_2 \) in this photosynthetic organelle. In this regard, we found that some herbicides inhibiting the biosynthesis of carotenoids and tocopherols led to the extremely elevation of endogenous \( \text{O}_2 \) to be published elsewhere.

Most recently, we have suggested plastid-originating \( \text{O}_2 \), so-called an endogenous \( \text{O}_2 \), as a candidate retrograde transducer in \( A. \text{annua} \) for participating plastid signalling and activating nuclear genes relevant to terpene biosynthesis [13]. As indicated by others [37], plastid oxidative stress signals are essential not only for coordinating photosynthetic genes in both the nucleus and in chloroplasts but also for mediating plant stress responses. Fascinatingly, artemisinin was proposed as a peroxidized product of dihydroartemisinic acid by scavenging \( \text{O}_2 \) derived from oxidative stresses [5]. It seems that the endogenous \( \text{O}_2 \) being invoked from chloroplasts by SA/MJ as well as other oxidative stresses can only activate nuclear-encoded artemisinin biosynthetic genes [6], while \( \text{O}_2 \) generated from dihydroartemisinic acid to artemisinin.

In conclusion, SA/MJ-enhanced artemisinin production can be attributed to SA/MJ-induced up-regulation of artemisinin biosynthetic genes and SA/MJ-elicited potent endogenous \( \text{O}_2 \) burst. RB can not facilitate artemisinin accumulation due to a failure in invoking endogenous \( \text{O}_2 \).

References