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PHYSIOLOGY AND BIOCHEMISTRY

Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L.

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Abstract This paper provides evidence that salicylic acid (SA) can activate artemisinin biosynthesis in *Artemisia annua* L. Exogenous application of SA to *A. annua* leaves was followed by a burst of reactive oxygen species (ROS) and the conversion of dihydroartemisinic acid into artemisinin. In the 24 h after application, SA application led to a gradual increase in the expression of the 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR*) gene and a temporary peak in the expression of the amorpha-4,11-diene synthase (*ADS*) gene. However, the expression of the farnesyl diphosphate synthase (*FDS*) gene and the cytochrome P450 monooxygenase (*CYP71AV1*) gene showed little change. At 96 h after SA (1.0 mM) treatment, the concentration of artemisinin, artemisinic acid and dihydroartemisinic acid were 54, 127 and 72% higher than that of the control, respectively. Taken together, these results suggest that SA induces artemisinin biosynthesis in at least two ways: by increasing the conversion of dihydroartemisinic acid into artemisinin caused by the burst of ROS, and by up-regulating the expression of genes involved in artemisinin biosynthesis.

Keywords *Artemisia annua* L. · Artemisinin · Gene expression · Reactive oxygen species · Salicylic acid

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Abbreviations

ADS	Amorpha-4,11-diene synthase
CYP71AV1	Cytochrome P450 monooxygenase
DW	Dry weight
FDP	Farnesyl diphosphate
FDS	Farnesyl diphosphate synthase
FW	Fresh weight
HMGR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
MEP	Methylerythritol phosphate
MVA	Mevalonate
ROS	Reactive oxygen species
SA	Salicylic acid
SAG	SA-glucoside

Introduction

Malaria is a serious infectious disease in many parts of the world, affecting more than one-third of the global population and killing approximately 2 million people annually (Korenromp et al. 2005). Artemisinin, a sesquiterpene lactone originally extracted from the medicinal plant *Artemisia annua* L., is an effective antimalarial agent, particularly for multi-drug resistant and cerebral malaria. By February 2005, 43 countries had adopted artemisinin-based combination therapies (ACTs) as first- or second-line malaria treatment: most of these countries are in sub-Saharan Africa. However, the low concentrations of artemisinin in *A. annua*, which range from less than 0.1 to 1% of the plant dry weight (DW), make artemisinin relatively expensive and difficult to meet the demand of over 100 million courses of ACTs each year (Mutabingwa 2005). Moreover, the chemical synthesis of artemisinin has

not been shown to be commercially feasible at present. Therefore, it is necessary to increase the concentration of artemisinin in *A. annua* to reduce the cost of artemisinin-based antimalarial drugs.

Artemisinin is biosynthesized through the terpenoid pathway (Fig. 1). The C5 building blocks from both the conventional mevalonate (MVA) pathway and the newly found plastid methylerythritol phosphate (MEP) pathway are involved in the formation of farnesyl diphosphate (FDP) in *A. annua* (Akhila et al. 1987; Towler and Weathers 2007). The cyclization of FDP to generate amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (ADS) has been suggested as the first committed step in artemisinin biosynthesis (Bouwmeester et al. 1999). Recently, a cytochrome P450 monooxygenase cDNA (*CYP71AV1*) has been independently cloned by both the Covello group (Teoh et al. 2006) and the Keasling group (Ro et al. 2006). *CYP71AV1* encodes a multifunctional enzyme capable of oxidizing amorpha-4,11-diene to generate artemisinic acid via artemisinic alcohol and artemisinic aldehyde intermediates. The pathway beyond artemisinic alcohol is somewhat less well established (Covello et al. 2007; Li et al. 2006; Zeng et al. 2008). However, there is biochemical (Bertea et al. 2005) and molecular (Zhang et al. 2008) evidence supporting a route to dihydroartemisinic acid via artemisinic aldehyde and dihydroartemisinic aldehyde.

Salicylic acid (SA) is a phenolic phytohormone with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport (Hayat and Ahmad 2007). It also has a role in plant responses to different abiotic stresses such as UV radiation and ozone exposure (Rao and Davis 1999; Senaratna et al. 2000), different developmental conditions or various biological stimuli such as those that occur during nodulation (Stacey et al. 2006). In recent years, SA has received particular attention because it plays an important signaling role in the activation of various plant defense responses such as the induction of the expression of pathogenesis-related genes (PR genes) and the biosynthesis of special secondary metabolites, which function as phytoalexins in plants (Bulgakov et al. 2002; Kang et al. 2004; Sirvent and Gibson 2002). The induction mechanism of plant defense is generally thought to be related to the elevation of H_2O_2 and other reactive oxygen species (ROS) levels, which can then serve as second messengers in the defense signaling pathway (Lamb and Dixon 1997; Ebel and Mithofer 1998; Qian et al. 2006).

Several SA-binding proteins in the SA-signaling pathway have been identified, including (1) the H_2O_2-scavenging enzymes catalase (SABP) (Chen and Klessig 1991; Chen et al. 1993) and ascorbate peroxidase (Durner and Klessig 1995), (2) methyl salicylate (MeSA) esterase (SABP2), which converts MeSA into SA (Song et al. 2008), (3) chloroplast carbonic anhydrase (SABP3) (Slaymaker et al. 2002), and (4) aconitase (Ruffer et al. 1995). Interestingly, three of these proteins (catalase, ascorbate peroxidase, and carbonic anhydrase) exhibit antioxidant activity. It is possible that increase in H_2O_2 and other ROS levels by SA results from the inhibition of antioxidant enzyme activity.

Since SA might increase antioxidant levels and strongly induce secondary metabolite biosynthesis in some plants, it would be interesting to know its effects on artemisinin biosynthesis. Here, we report evidence that exogenously applied SA can activate artemisinin biosynthesis in *A. annua*. The possible mechanisms are also discussed.

Materials and methods

Plant growth and SA treatment

An *Artemisia annua* plant from the high artemisinin yielding strain 001 (Han et al. 2005) was used in this study. On 1 March 2007, plant cuttings were rooted in a growth chamber and were illuminated using fluorescent lamps (100 μmol photons m^{-2} s^{-1}) for 16 h a day at 23°C. After 20 days, the plantlets were transplanted into individual pots (1 L, PVC) with growth medium (Voltilon Agriculture Technology, Inc., Beijing, China). Water was slowly added to each pot until field moisture capacity was reached. The pots were kept in an experimental greenhouse at Beijing, WV (39°59′23″N and 116°12′36″E, 74 m altitude). The daily photoperiod was 14 h (8:00–22:00) with controlled temperature (25°C/18°C, day/night), and 70% relative humidity all day.

On 1 May 2007 (20 days after transplanting), uniform plants (25–30 cm in height) were selected for the experiments. The pots were organized in a randomized complete block design with three blocks. Pots were spaced at a density of 5 pots per square meter. Each block was surrounded with the same *A. annua* plants to guarantee the coherence of the experimental plants. Eighty plants were combined in each block. On 5 May 2007, SA solution (1.0 mM, adjusted to pH 7.0 with NaOH) was sprayed using an atomizer onto the leaves until it ran off. The control plants were sprayed with distilled water, also adjusted to pH 7.0 with NaOH. Sampling was carried out: at 8:00, 12:00, 16:00 and 24:00 on 5 May, 8:00 on 6 May, 8:00 on 7 May and 8:00 on 8 May for internal SA, ROS, artemisinic acid, dihydroartemisinic acid and artemisinin analysis; at 8:00, 10:00, 12:00, 16:00 and 24:00 on 5 May and 8:00 on 6 May for RNA analysis. The first sample ($t = 0$) was taken before spraying. For internal SA, ROS and RNA analysis, tagging was done to recognize leaves of similar age before spraying. Five leaves under the third visible leaf from the apex of the tagged branch were harvested and mixed, frozen in liquid nitrogen and stored at −80°C immediately after fresh weight

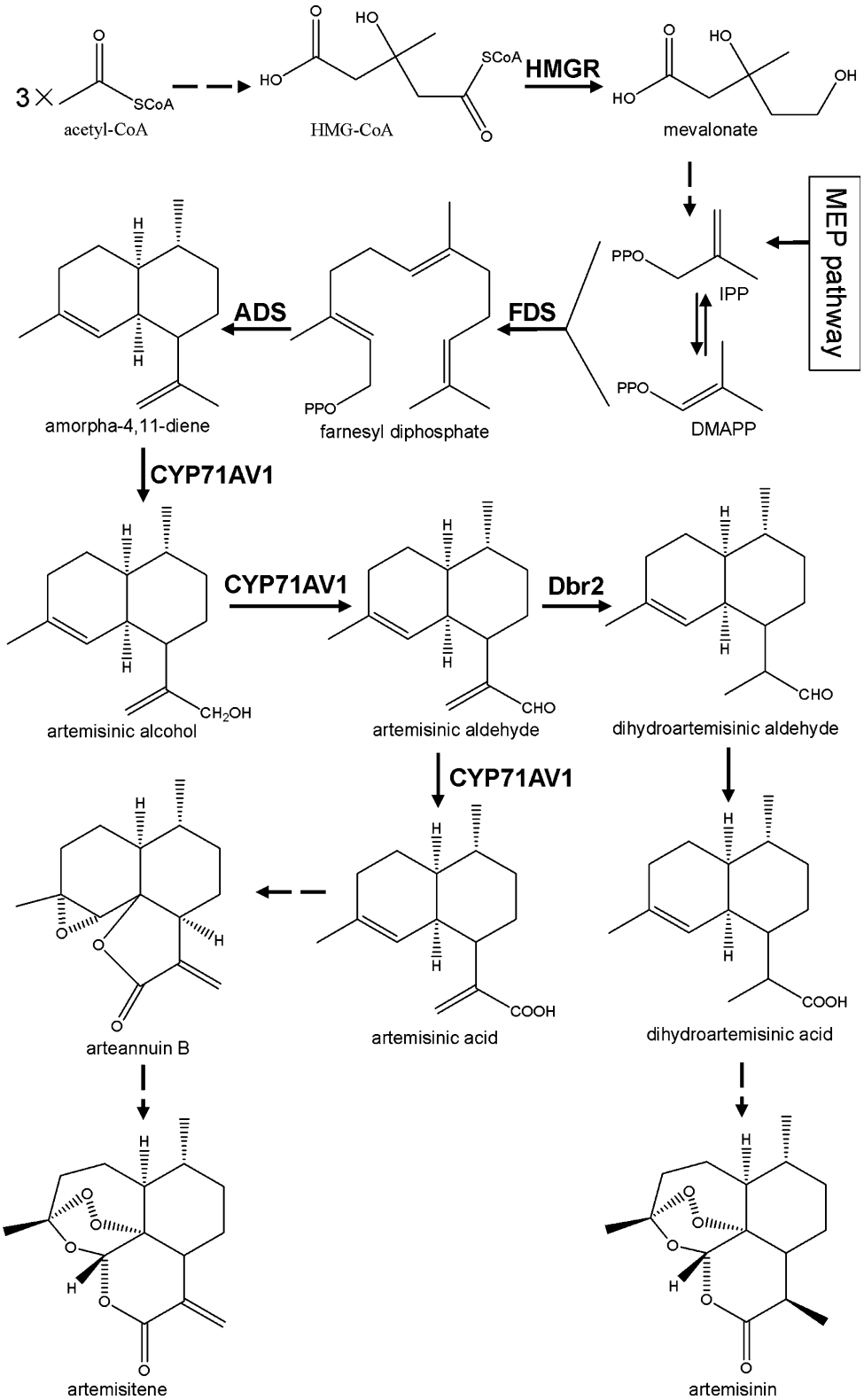

Fig. 1 An integrative artemisinin biosynthetic pathway in *A. annua*, adopted from Lommen et al. (2006) with modifications. *DMAPP* dimethylallyl diphosphate, *HMGR* 3-hydroxy-3-methylglutaryl coenzyme A reductase, *IPP* isopentenyl diphosphate, *FDS* farnesyl diphosphate synthase, *ADS* amophadiene synthase, *CYP71AV1* cytochrome P450 monooxygenase, *Dbr2* artemisinic aldehyde reductase. Broken arrows indicate more than one step

determination. There were about 50, 50 and 5 leaves in each sample for SA, ROS and RNA analysis, respectively. For the quantification of artemisinic acid, dihydroartemisinic acid and artemisinin concentrations, plants were cut 3 cm above the soil level and dried in a forced-air oven at 40–45°C for 24–48 h. One plant was used for one sample. Leaves were separated from the stems and stored at −20°C until high-performance liquid chromatography (HPLC)

analysis (within 2 days). The experiment was repeated in 2008 from 1 March to 10 May, to ensure that the data obtained were reliable.

Measurement of internal SA after exogenous SA treatment

The SA concentration was measured using the method of Meuwly and Metraux (1993) with some modifications. As much as 1 g (fresh weight, FW) of *A. annua* leaves was ground in liquid nitrogen with a mortar and pestle. The tissue was transferred to a centrifugation tube and mixed with 1.5 ml 90% methanol. The mixture was sonicated for 10 min and centrifuged at 12,000*g* for 10 min. The pellet was resuspended in 1.5 ml 100% methanol, vortexed, resonicated and recentrifuged as above. Supernatants from both extractions were combined, added to 20 µl of 2 M HCl and evaporated at room temperature under vacuum. After drying, the residue was resuspended in 500 µl of 5% (w/v) trichloroacetic acid (TCA), and the mixture was centrifuged at 12,000*g* for 10 min. The supernatant was gently partitioned twice with 1.5 ml of a 1:1 (v/v) mixture of ethyl acetate/cyclohexane. The organic phase was dried using an SPD 111 V speedvac concentrator (Thermo Fisher Scientific, USA) and then redissolved in 120 µl of 0.2 M acetate buffer (pH 5.5). The pellets were resuspended with 500 µl mobile phase for the HPLC analyses of free SA. SAG was analyzed by hydrolysis of the TCA phase. To the TCA phase, 500 µl of 8 M HCl was added, incubated at 80°C for 1 h, and partitioned twice with 1.5 ml of a 1:1 (v/v) mixture of ethyl acetate/cyclohexane. The organic phase was dried using a Speedvac concentrator with the addition of 120 µl of 0.2 M acetate buffer (pH 5.5). The pellets were resuspended with 500 µl mobile phase for the HPLC analyses. The mobile phase consisted of 90% 0.2 M sodium acetate, pH 5.5 and 10% methanol with a flow rate of 0.8 ml min^{-1}. SA was quantified fluorimetrically (W474 scanning fluorescence detector, Waters, USA), with excitation at 305 nm and emission at 407 nm.

Determination of artemisinic acid, dihydroartemisinic acid and artemisinin concentrations

Samples of approximately 100 mg of dried leaves were extracted once in 20 ml of petroleum ether in an ultrasonic bath for 30 min. The supernatant was transferred to 50-ml glass beakers and evaporated to dryness in a hood. The residue was redissolved with 5 ml acetonitrile and filtered through a nylon filter (0.45 µm) attached to a 5-ml syringe. The samples were transferred to HPLC flasks and analyzed on the same day. Artemisinin, artemisinic acid and dihydroartemisinic acid were quantified using an Agilent (Agilent Technologies, Inc., Pickerington, OH, USA) HPLC (1100 Series) system according to the method of Ferreira (2007). A PL-ELSD 2100 (Polymer Laboratory, Amherst, MA, USA) with the evaporator at 40°C, nebulizer at 30°C, and a nitrogen flow of 1.2 L min^{-1} was used for artemisinin quantification. Artemisinic acid and dihydroartemisinic acid were detected using a C-18 column coupled to a G1315B diode array detector set at 194 nm. The mobile phase consisted of 60% acetonitrile and 40% water (acidified with acetic acid to pH 3.2) with a flow rate of 0.8 ml min^{-1}. Artemisinin, artemisinic acid and dihydroartemisinic acid were calculated from a 10-point standard curve varying from 0.05 to 0.5 mg ml^{-1} for artemisinin and artemisinic acid and from 0.04 to 0.4 mg ml^{-1} for dihydroartemisinic acid, with an injection volume of 10 µl.

Determination of H_2O_2 and O_2^- concentrations

The concentration of H_2O_2 in the leaves was determined according to the method of Mukherjee and Choudhuri (1983). *A. annua* leaves (0.5 g, FW) were homogenized in a cold mortar with a pestle in pre-cooled acetone (5 ml) and the homogenate was centrifuged at 12,000*g* for 5 min. A volume of 1 ml of the supernatant was mixed with 0.1 ml of 5% $Ti(SO_4)_2$ and 0.2 ml 19% ammonia. After a precipitate was formed, the reaction mixture was centrifuged at 12,000*g* for 5 min. The resulting pellet was dissolved in 3 ml 2 M H_2SO_4 and the absorbance was quantified at 415 nm using a spectrophotometer (UV-1700, SHIMADZU, Japan). The H_2O_2 level was calculated according to a standard curve of H_2O_2 ranging from 0 to 10 µM.

O_2^- production was measured as described by Able et al. (1998) with some modifications. The reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H- tetrazolium-5-carbo-xanilide) in the presence of O_2^- was monitored. *A. annua* leaves (0.5 g, FW) were homogenized with 1 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 12,000*g* for 15 min. The reaction mixture contained 50 mM Tris–HCl buffer (pH 7.5), 100 µl supernatant and 0.5 mM XTT. The reduction of XTT was determined by measuring the absorbance at 470 nm in a spectrophotometer (UV-1700, SHIMADZU, Japan). The quantity of O_2^- was determined using the molar extinction coefficient (ε) 2.16×10^4 M^{-1} cm^{-1}.

RNA gel blot analysis

The cDNAs of *HMGR*, *FDS*, *ADS* and *CYP71AV1* were synthesized using RT-PCR based on the *A. annua* nucleotide sequences published in GenBank (GenBank accession nos. AF142473, AF112881, AF138959 and DQ315671, respectively). The full-length cDNA was used as the hybridization probe. Total RNA was extracted using the RNApure Kits (Bioteke, China) according to the manufacturer's instructions. The concentration and quality of each RNA

sample was examined by measuring A_{260} and A_{280}. The integrity of RNA samples was also assessed by agarose gel electrophoresis. To study the expression of these genes, total RNA (10 μg) was separated on MOPS-formaldehyde (6.2%) agarose gels (1.0%) and transferred to Hybond N^+ nylon membrane (Amersham, Sweden) by capillary transfer. Labeling, hybridization and detection followed the instructions of the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche Applied Science, Germany). Prehybridization was carried out at 42°C for 2 h and hybridization was performed for 16 h at 42°C. Blots were washed with 2× SSC (saline-sodium citrate buffer) and 0.1% SDS (sodium dodecyl sulfate) twice for 5 min at 25°C, followed by 0.5× SSC and 0.1% SDS twice for 15 min at 65°C, and chemiluminescence detection was carried out according to the protocol supplied by Roche. The density of the signal was measured using the NIH Image Program (http://rsb.info.nih. gov/nih-image/).

Statistical analysis

Except RNA gel blot, all parameters are expressed as the mean of three blocks. Significant differences between the control and SA treatment were analyzed using the Student's *t* test.

Results

Changes in SA concentration in *A. annua* leaves after exogenous SA treatment

The free SA concentration was rapidly and significantly ($P < 0.01$) increased to more than 350% of the control, 4 h after treatment (Fig. 2a). At 16 h, the free SA concentration increased to a maximum. Following this abrupt increase, free SA concentration declined to the concentration of the control during the next 80 h. The SAG concentration also increased significantly ($P < 0.01$) after treatment, but declined more slowly than the free SA concentration (Fig. 2b).

Effects of exogenous SA on the concentration of artemisinin and related precursors

The concentration of artemisinin increased slowly during the first 8 h, and increased rapidly between 8 and 96 h after treatment (Fig. 3A). The artemisinin concentration was 21.7 and 75.8% higher than the control at 8 and 96 h, respectively. The concentration of artemisinic acid was unchanged up to 16 h after treatment, but increased thereafter to 4.98 mg g^{-1} DW at 96 h, which was 127% higher than that in the control plant (Fig. 3b). The dihydroartemisinic acid concentration decreased during the first 8 h, then increased gradually from 16 to 96 h (Fig. 3c). Treatment with SA also increased the total yield of artemisinin and the putative precursors, artemisinic acid and dihydroartemisinic acid in *A. annua* leaves by 1.3-, 1.5- and 1.8-fold compared with the controls on days 1, 2 and 4 after treatment, respectively (data not shown).

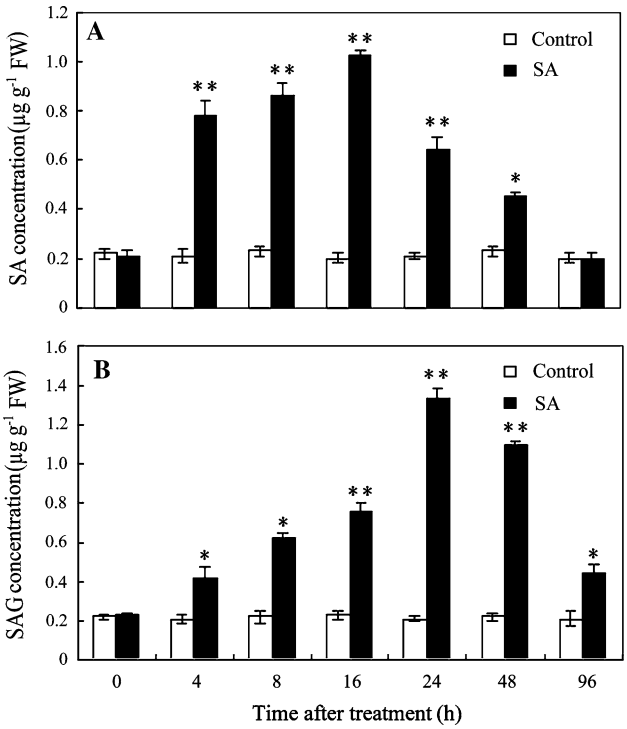

Fig. 2 The concentration of free SA (**a**) and SAG (**b**) in *A. annua* leaves treated with 1.0 mM SA. Average values are given. *Error bars* represent 2× SE ($n = 3$). *Asterisks* indicate significant differences from controls (*$0.01 \leq P < 0.05$; **,$P < 0.01$). *FW* fresh weight, *SA* salicylic acid, *SAG* SA glucoside

Exogenous SA treatment activates artemisinin biosynthesis genes

RNA gel blot analysis was performed to determine the transcription level of putative genes in the artemisinin biosynthetic pathway. The accumulation of *HMGR* transcripts increased gradually, and was 130% higher than the control at 24 h after treatment (Fig. 4a). During the period of sampling, few changes in *FDS* and *CYP71AV1* transcription levels were observed (Fig. 4b, d). Accumulation of the *ADS* transcript increased to a maximum at 8 h after treatment and then gradually decreased (Fig. 4c).

Exogenous SA treatment activates the accumulation of ROS

The H_2O_2 concentration was significantly increased ($P < 0.01$) by more than 2.1-fold compared with the

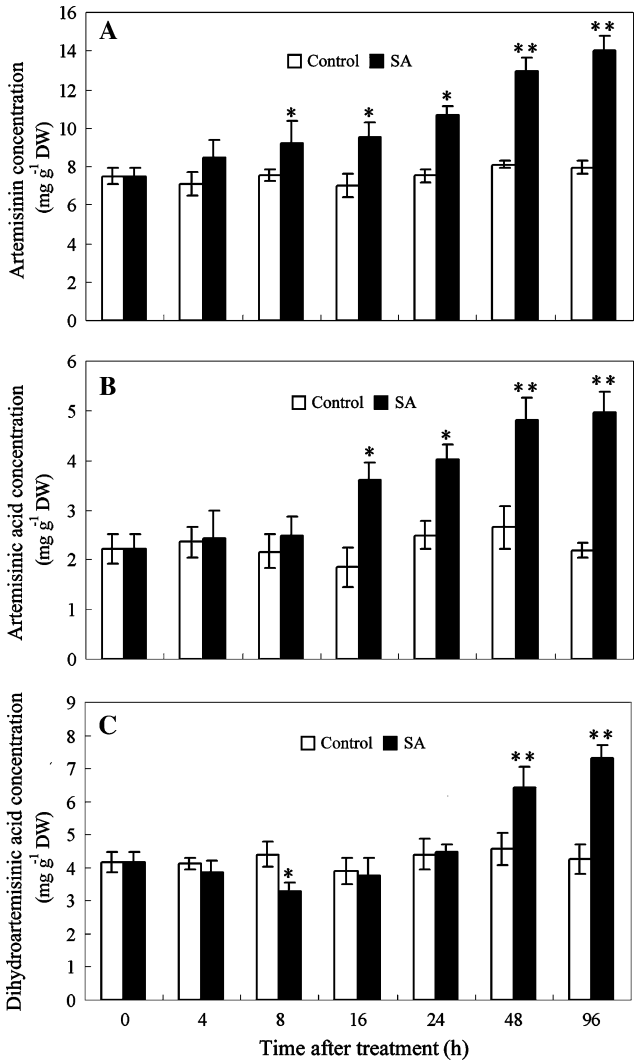

Fig. 3 The effects of 1.0 mM SA on the concentration of artemisinin (**a**), artemisinic acid (**b**) and dihydroartemisinic acid (**c**) in *A. annua* leaves. Average values are given. *Error bars* represent 2× SE ($n = 3$). *Asterisks* indicate significant differences from controls (*$0.01 \leq P < 0.05$; **,$P < 0.01$). *DW* dry weight; *SA* salicylic acid

control level at 4 h after treatment (Fig. 5a). Following this abrupt increase, H_2O_2 declined to the control level during the next 92 h. This elicitation also led to the induction of O_2^- accumulation with a similar trend to that of H_2O_2 (Fig. 5b). The levels of H_2O_2 and O_2^- remained significantly higher than that of the control ($P < 0.05$) at 16 h after treatment.

Discussion

Our results indicated that SA regulates gene transcription in the artemisinin biosynthetic pathway and increases artemisinin concentration in *A. annua*. Half of the genes studied were up-regulated by SA (Fig. 4), which might explain the increased accumulation of artemisinin. The up-regulated gene *HMGR* catalyzes the first step in the biosynthesis of isoprenoids by converting 3-hydroxy-3-methylglutaryl-CoA to mevalonate. It is generally believed that the C5 building blocks of sesquiterpenoids are mainly provided by the MVA pathway, although there are some reports that the C5 units from the MEP pathway are also involved in the biosynthesis of sesquiterpenoids (Dudareva et al. 2005; Laule et al. 2003; Schuhr et al. 2003). Our results suggest that up-regulation of *HMGR* can supply more building blocks for artemisinin biosynthesis. This finding is similar to that of Stermer and Bostock (1987), who also reported the importance of *HMGR* in the regulation of sesquiterpenoid phytoalexin accumulation in potato.

ADS catalyzes the first branch step in the separation of artemisinin from other sesquiterpenoids in the artemisinin biosynthetic pathway. The transcriptional level of *ADS* was very low in control plant tissues (Fig. 4c). This is consistent with the characteristics of terpene synthases, which are known to occur in very low intracellular concentrations (Croteau et al. 1985). The increase in *ADS* transcripts following SA treatment may account for the increase in artemisinin concentration. Therefore, this might be an important regulatory step in the biosynthesis of artemisinin. Evidence that the regulation of terpenoid metabolism is located at the first dedicated step (terpene cyclase) of the pathway has been shown in several other species, including linalool synthase in *Clarkia breweri* (Pichersky et al. 1994) and limonene synthase in *Carum carvi* (Bouwmeester et al. 1998).

It is interesting to note that *FDS* and *CYP71AV1* transcriptional levels showed little change after SA treatment. One possible reason for this might be that the catalytic efficiency of these enzymes is high and it is not necessary to increase the amount of these enzymes to enhance the conversion of substrates to products.

In the present study, the concentrations of H_2O_2 and O_2^- instantaneously increased after SA treatment and then gradually declined (Fig. 5). The increase in ROS was followed by an early (8 h after treatment), temporary decrease in dihydroartemisinic acid concentration and an increase in artemisinin concentration (Fig. 3). Wallaart et al. (1999) hypothesized that dihydroartemisinic acid may act as a scavenger of singlet oxygen, which is often released from plant cells when they are exposed to oxidative stress, to generate dihydroartemisinic acid hydroperoxide and then yield artemisinin as a final product. Given that dihydroartemisinic acid spontaneously converts to artemisinin by reacting with ambient oxygen, it is reasonable to assume that exogenous SA treatment led to the conversion of dihydroartemisinic acid into artemisinin,

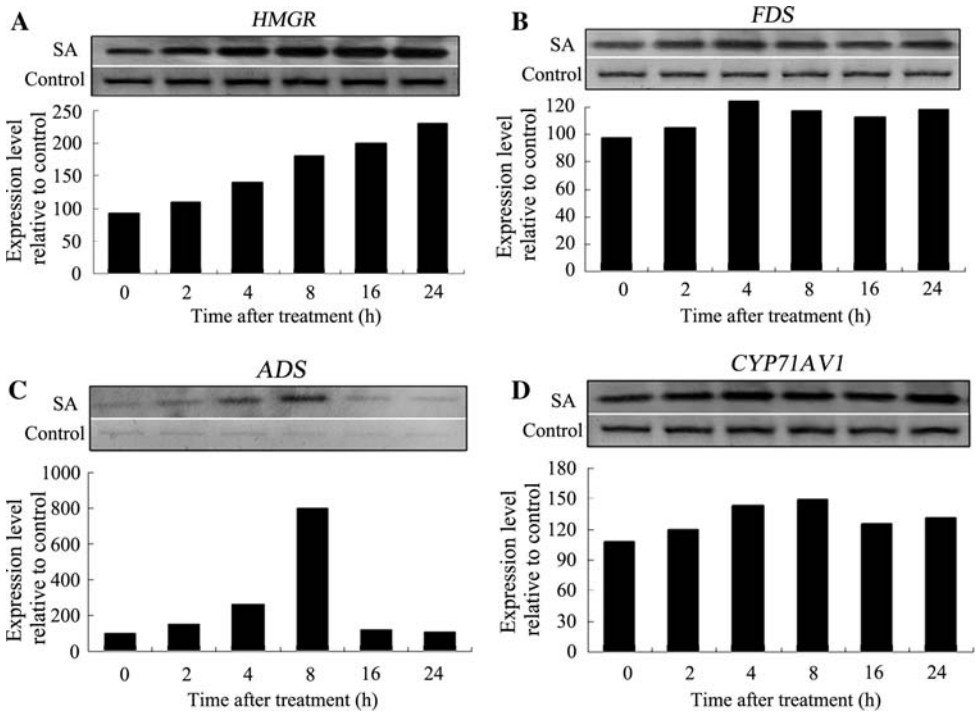

Fig. 4 RNA gel blot analysis of the genes *HMGR* (**a**), *FDS* (**b**), *ADS* (**c**) and *CYP71AV1* (**d**) after 1.0 mM SA treatment. The experimental values were plotted relative to the corresponding control value that was set at 100. *ADS* amophadiene synthase; *CYP71AV1* cytochrome P450 monooxygenase, *FDS* farnesyl diphosphate synthase, *HMGR* 3-hydroxy-3-methylglutaryl coenzyme A reductase, *SA* salicylic acid

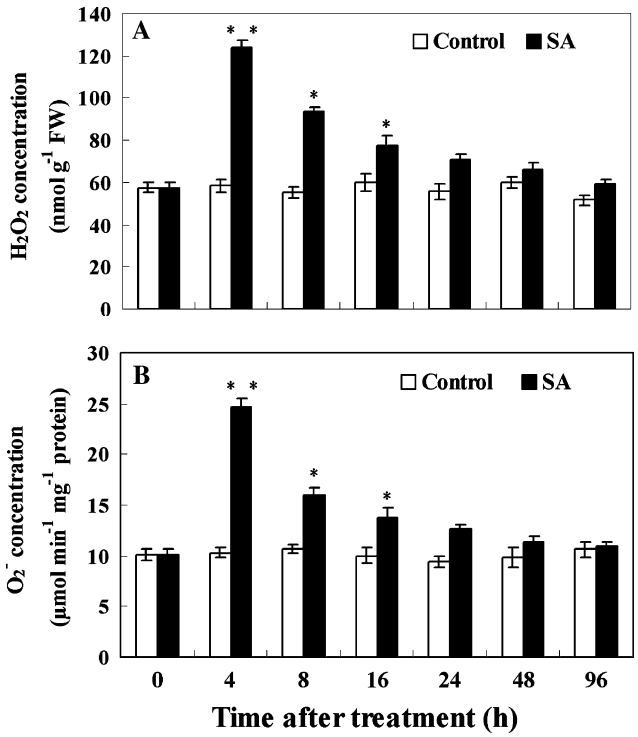

Fig. 5 The effects of 1.0 mM SA on H_2O_2 (**a**) and O_2^- (**b**) concentration in *A annua* leaves. Average values are given. *Error bars* represent 2× SE ($n = 3$). *Asterisks* indicate significant differences from controls (*$0.01 \leq P < 0.05$; **,$P < 0.01$). *FW* fresh weight, *SA* salicylic acid

which was mediated by generating ROS. This conjecture correlates with the observation that night frost (Wallaart et al. 2000) and K deficiency (Ferreira 2007) triggered the conversion of dihydroartemisinic acid to artemisinin, during which relatively high levels of ROS were formed.

Previous studies have indicated that H_2O_2 and SA can induce each other and thus form a feed-forward loop (Bi et al. 1995; Rao et al. 1997). We also found a continuous increase in SA from 4 to 16 h after treatment (Fig. 2). This SA may have come from *A. annua* itself and is thought to be associated with the H_2O_2 burst (Fig. 5a). SAG is the major metabolite of exogenous and endogenous SA (Enyedi et al. 1992; Malamy et al. 1992). The enzyme, SA-glucosyltransferase, which catalyzes SA conjugation is strongly and specifically induced by SA in *Arabidopsis thaliana* (Song 2006). In our study, maximum SAG concentration was noted 8 h after the maximum of SA concentration was achieved, and the SAG concentration declined slower than the free SA concentration (Fig. 2), which possibly indicates the removal of free SA by conjugation.

From these findings, we conclude that SA increased artemisinin concentration in *A. annua* in two ways: by converting dihydroartemisinic acid to artemisinin using the burst of ROS, and by positive regulation of the genes involved in artemisinin biosynthesis. In addition, these results can be applied to increase field artemisinin production in the short term.

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