Foliar application of chitosan activates artemisinin biosynthesis in Artemisia annua L.

Caiyan Lei, Dongming Ma, Gaobin Pu, Xiaofang Qiu, Zhigao Du, Hong Wang, Guofeng Li, Hechun Ye, Benye Liu

Abstract

There has been much interest in artemisinin owing to its excellent activity against malaria, an infectious disease threatening the tropical world. However, the low artemisinin content (0.01–0.8%, DW) in Artemisia annua, which is the only commercial source of artemisinin, makes artemisinin expensive to produce and not yet available on a global scale. Here we show that foliar application of 100 mg l−1 chitosan improved artemisinin biosynthesis in A. annua. The content of dihydroartemisinic acid and artemisinin in chitosan-treated leaves increased by 72% and 53% compared with control values, respectively. Chitosan induced the expression of ADS and DBR2, which could explain the increase in level of artemisinic metabolites. After chitosan treatment, the amounts of hydrogen peroxide (H2O2) and superoxide anion (O2−) in leaves of A. annua were 1.4 and 3.0 times higher than those of the control, respectively. Accumulation of reactive oxygen species (ROS) probably accelerated the conversion of dihydroartemisinic acid to artemisinin. Foliar application of 100 mg l−1 chitosan had no harmful effect on A. annua growth. The simple method described here could be an effective method to improve artemisinin production in A. annua field cultivation.

1. Introduction

Malaria is a major health problem in many developing countries, particularly in Africa and southeast Asia (Snow et al., 2005). Each year, there are 500 million new cases of malaria, which result in 1–2 million deaths, mainly in children under 5 years old (Lindahl et al., 2006). Artemisinin is an endoperoxide sesquiterpene lactone isolated from the traditional Chinese herb Artemisia annua by Chinese scientists more than 30 years ago in the efforts for finding new antimalarial drugs. Several derivatives of artemisinin have shown excellent activity against susceptible and multi-drug-resistant strains of Plasmodium falciparum, the causative agent of malaria (Woerdenbag et al., 1994). An increasing number of countries have adopted artemisinin–based combination therapies (ACTs) as the first- or second-line malaria treatment. Consequently, approximately 400–600 million therapeutic doses of ACT are likely to be required per year, while fewer than 100 million doses per year are presently available. In addition to antimalarial activity, artemisinin has been reported to have activities against a wide variety of cancers such as human leukemia, breast cancer, colon cancer and small-cell lung carcinomas (Efferth, 2006).

A. annua is the only commercial source of artemisinin at the moment; however, lower content (0.01–0.8%, DW) of artemisinin in leaves and flowers of A. annua has seriously limited its commercialization and triggered numerous efforts for improving artemisinin production (Van Agtmael et al., 1999). Chemical synthesis of artemisinin is still commercially unfeasible due to the low yields (Yadav et al., 2003). Therefore, increasing the artemisinin content in A. annua is a potential approach to reduce the cost of artemisinin and increase the supply of ACTs.

It is generally believed that artemisinin, as a sesquiterpene, is synthesized in the cytosolic compartment of A. annua glandular secretory trichome cells and that it originates from IPP/DMAPP.
Fig. 1. Possible routes of artemisinin biosynthesis (Towler and Weathers, 2007; Zhang et al., 2008).

derived from mevalonate, as indicated by the results of feeding experiments with [2-14C]mevalonolactone (Huang et al., 1990). However, recent results have indicated that both C5 biosynthesis pathways contribute to artemisinin biosynthesis in A. annua (Towler and Weathers, 2007; Schramek et al., 2010). The first committed step in artemisinin biosynthesis is the cyclization of farnesyl diphosphate to amorpha-4,11-diene catalyzed by amorpha-4,11-diene synthase (ADS) (Bouwmeester et al., 1999). This step is followed by oxidation at C12 to form artemisinic alcohol. A multifunctional cytochrome P450 monooxygenase (CYP71AV1) has been shown to hydroxylate amorpha-4,11-diene at C12 and oxidize the resulting alcohol to generate artemisinic acid via artemisinic aldehyde intermediate (Ro et al., 2006; Teoh et al., 2006). Recent studies have shown that artemisinic aldehyde reductase (DBR2) can reduce the C11–C13 double bond of artemisinic aldehyde to give dihydroartemisinic aldehyde (Zhang et al., 2008). Furthermore, ALDH1, a gene encoding aldehyde dehydrogenase was recently isolated from A. annua and shown to catalyze the oxidation of dihydroartemisinic aldehyde to give dihydroartemisinic acid, a late precursor of artemisinin (Teoh et al., 2009) (Fig. 1).

Chitosan (β-1,4-linked glucosamine) is a deacetylated derivative of chitin, the second most abundant natural polymer on earth that is found in the composition of cell walls of many fungi (Bartnicki-Garcia, 1968; Khan et al., 2002). This compound has two biological functions. First, it has antifungal properties at defined concentration; it inhibits the mycelial growth of a number of pathogenic fungi such as Pythium aphanidermatum and Fusarium oxysporum (El Ghaouth et al., 1994; El Hassni et al., 2004) and also spore germination (Hadwiger and Beckman, 1980). Second, it acts as a potent inducer of plant resistance against pathogens (Benhamou and Thériault, 1992; El Ghaouth et al., 1994). It is thus used to increase the biosynthesis of secondary metabolites in various plant cells (Hadwiger and Beckman, 1980; Kohle et al., 1984; Akimoto et al., 1999; Uozumi and Kobayashi, 1994). Chitosan can trigger a set of defense responses, including gene activation (Doares et al., 1995; Chen et al., 2009), the oxidative burst (Orozco-Cardenas and Ryan, 1999; Rossard et al., 2006) and the production of secondary metabolites (Rabea et al., 2003; Kim et al., 2005; Lin et al., 2005).

To the best of our knowledge, only a few studies have investigated the effects of foliar applied chitosan on secondary metabolite production (Khan et al., 2003; Al-Tawaha et al., 2005), and none has focused on artemisinin biosynthesis in whole A. annua plants. Here, we show that foliar application of chitosan activates artemisinin biosynthesis in A. annua. The possible mechanisms are also discussed.

2. Materials and methods

2.1. Chemicals

Chitosan was purchased from Labsun (Beijing, China). HPLC grade methanol, n-hexane, trans-farnesol were purchased from Sigma–Aldrich (Shanghai, China). All other chemicals were standard commercial products of analytical grade from Beijing Chemical Company (Beijing, China). Artemisinin (purity >97%) and artemisinic acid (purity >95%) were provided by the Institute of Medicine, Chinese Academy of Medical Sciences. Dihydroartemisinic acid was donated by Dr. Bouwmeester (International Plant Institute, Netherlands).
2.2. Plant materials

The high-artemisinin-yielding A. annua L. SP18 used in the study has been described elsewhere (Wang et al., 2010). Fifteen days after rooting, the cloned plants were transplanted into peat soil and placed in a greenhouse with a 16-h light photoperiod at 100 μmol m⁻² s⁻¹, 25°C/18°C (day/night). Each pot contained one plant and pots were arranged at a density of five pots per square meter. The plantlets were watered every other day using tap water.

2.3. Determination of optimum concentration of chitosan

Chitosan was dissolved in 5% acetic acid, diluted in distilled water to various concentrations (50 mg l⁻¹, 100 mg l⁻¹, 150 mg l⁻¹, 200 mg l⁻¹, 250 mg l⁻¹ and 300 mg l⁻¹). The pH of chitosan solutions was adjusted to 6.5 with 2M NaOH. One month after transplanting, plantlets were separated into seven groups and arranged following a completely randomized block design with three replicates. Each group was surrounded with the same A. annua plants to guarantee the coherence of the experimental plants. One group was sprayed with distilled water (pH 6.5) and considered to be control plants, the remaining six groups were sprayed with chitosan solutions of various concentrations, using a hand-held sprayer. The number of spray shots per plant was kept constant, with each plant receiving approximately a total of 20 ml solution. Four days after treatment, leaves were harvested and dried at 40–45°C to a constant weight. The artemisinin content was determined with the method described by Han et al. (2006).

Dried leaf powder (0.1 g) was extracted with 20 ml of petroleum ether (boiling range 30–60°C) in an ultrasonic bath for 30 min. The supernatant was evaporated to dryness and redissolved with 5 ml methanol. Samples (1 ml) were incubated with 4 ml of 0.2% Tris–HCl (pH 7.5), 0.5 mM XTT and 100 μM potassium phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was used as an internal standard. The mixture was cooled to room temperature, then 5 ml of 0.08 M acetic acid was added. After filtering on a NC filter (0.45 μm), aliquots of reaction products (20 μl) were injected into a C18 reverse-phase column of 3.9 mm × 150 mm for HPLC analysis on the same day. Isocratic elution was carried out with 50% methanol (diluted in 0.01 M phosphate buffer, pH 7.0) as the mobile phase, at a flow rate of 1 ml min⁻¹; detection wavelength was 260 nm.

2.4. Determination of artemisinin, artemisinic acid and dihydroartemisinic acid

One month after transplanting, plantlets were separated into two groups and arranged following a completely randomized block design with three replicates. One group was sprayed with distilled water (pH 6.5); another group was sprayed with 100 mg l⁻¹ chitosan using the method described in Section 2.3.

Leaves were harvested at 0, 4, 8, 16, 24, 48, 96 h after chitosan treatment and dried in a forced-air oven at 40–45°C to a constant weight. Dry leaves were powdered using a pestle and mortar and stored at −20°C until extraction.

Artemisinin content was determined following the procedure described in Section 2.3. For artemisinic acid and dihydroartemisinic acid extraction, dried leaves (0.05 g) were extracted with 5 ml n-hexane in an ultrasonic bath for 20 min. Twenty microlitres of trans-farnesol (200 μg ml⁻¹) were supplemented as an internal standard. After being filtered through a NC filter (0.45 μm), the supernatant was evaporated to dryness in a hood, then, the residue was redissolved in 200 μl n-hexane. A DB-5 column (30 m × 0.25 mm × 0.25 μm) was used to determine the artemisinic acid and dihydroartemisinic acid contents using Agilent HP 6890N GC equipment, with helium as carrier gas (constant flow 1.0 ml min⁻¹), injection temperature 290°C, split ratio 10:1, and detector temperature 300°C. The oven was programmed at 100°C for 1 min, then increased to 150°C at a rate of 5°C min⁻¹, to 200°C at 2°C min⁻¹, and finally to 280°C at a rate of 15°C min⁻¹, and held at that temperature for 15 min. Authentic artemisinic acid and dihydroartemisinic acid were used to identify the two peaks.

2.5. Growth measurement

Growth was measured as dry mass and height of the A. annua plants. At 0, 2, 4, 8, 12 and 16 days after treatment with 100 mg l⁻¹ chitosan, all leaves of six treated plants and those of six control A. annua plants were harvested and weighed after being dried to a constant weight. For height measurement, six treated and six control A. annua plants were chosen and the length of the main stem was considered to be the height of A. annua. Heights of six treated and six control plants were recorded at 0, 2, 4, 8, 12 and 16 days after treatment.

2.6. RNA extraction and semiquantitative RT-PCR analysis

Young leaves of A. annua were sampled randomly at 0, 2, 4, 8, 16, 24, 36, and 48 h after treatment, immediately frozen in liquid nitrogen and stored at −80°C. Total RNA of samples taken from 3 individual plants was extracted using the RNeasy Plant Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The concentration and purity of RNA were determined by measuring A260 and A280. The quality of RNA was assessed by separation on 1.2% formaldehyde agarose gels. One microgram of total RNA was reversely transcribed using Transcript RT Kit (Tiangen Biotech, China) following the manufacturer’s protocol. The sequences of the primers used for PCR amplifications are listed in Table 1. Amplification was performed with initial denaturation at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, 90 s at 72°C, and a final extension at 72°C for 10 min. The housekeeping control gene β-actin was used as an internal reference gene. The amplification of each gene was repeated at least twice.

2.7. Reactive oxygen species determination

2.7.1. Determination of hydrogen peroxide

Leaves of treated and control A. annua were harvested at 0, 2, 4, 8, 16, 24, 48 and 96 h after treatment, frozen in liquid nitrogen and immediately stored at −80°C after fresh weight determination. Hydrogen peroxide concentration was determined with the method of Mukherjee and Choudhuri (1983). Concentration of H₂O₂ in the sample was calculated according to a calibration curve drawn up with a standard H₂O₂ solution.

2.7.2. Determination of superoxide anion

Superoxide anion production was measured according to the method of Able et al. (1998) with some modifications. A. annua leaves (0.5 g, FW) were homogenized with 1.0 ml of 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 12,000 × g for 15 min at 4°C. The reaction mixture, in a final volume of 3.0 ml, contained 50 mM Tris–HCl (pH 7.5), 0.5 mM XTT and 100 μl of supernatant. The reduction of XTT was determined by measuring the absorbance at 470 nm in a spectrophotometer (UV-1700, SHIMADZU, Japan). The quantity of O₂⁻ was determined using the molar extinction coefficient (ε) 2.16 × 10⁴ M⁻¹ cm⁻¹.

2.7.3. Antioxidant enzyme assay

A. annua leaves (0.5 g, FW) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone and centrifuged at 12,000 × g for 15 min at 4°C. The supernatant was collected for analysis of soluble protein.
concentration and antioxidant enzymes activities. Protein concentrations in soluble enzyme extracts were determined using the Bradford assay (Bradford, 1976).

Activities of SOD (EC 1.15.1.1), POD (EC 1.11.1.7), CAT (EC 1.11.1.6) and APX (EC 1.11.1.11) were determined according to the methods of Giannopolitis and Ries (1977), Macadam et al. (1992), Aebi (1984), and Nakano and Asada (1987), respectively.

2.8. Statistical analysis

Except for gene expression analyses, all experiments were conducted twice, each time in triplicate. Significant differences between the control and treatment were analyzed using Student's t test with SPSS 11.5 software.

3. Results

3.1. Chitosan improved the contents of artemisinic metabolites

Foliar application of chitosan at 100 mg l\(^{-1}\) increased artemisinin content (Fig. 2). Following 100 mg l\(^{-1}\) chitosan treatment, the artemisinin content started to increase after 4 h and reached its maximum amount (53% increase) at 48 h (Fig. 3A). The content of dihydroartemisinic acid was significantly higher than that of the control at 8 h after chitosan treatment, and increased by 72% at 24 h, then declined at 48 h (Fig. 3C). There was no clear change in the content of artemisinic acid after chitosan treatment (Fig. 3B).

Foliar application of chitosan did not influence the growth of A. annua over a 16-day investigation period, no significant differences in dry weight and height of A. annua were observed (Fig. S1).

3.2. Chitosan activated genes related to artemisinin biosynthesis

To determine if chitosan affects the transcription of genes of the artemisinin biosynthetic pathway, we used RT-PCR analysis to measure the relative transcriptional levels of the following genes: HMGR, DXS, DXR, ISPH, FPS, ADS, CYP71AV1, and DBR2. Transcriptional levels were measured in plants treated with chitosan and water (control). The expression of ADS, the first committed gene of artemisinin biosynthesis, was transiently and significantly induced at 2 h and returned to its initial level at 8 h (Fig. 4). The expression of genes downstream of ADS in artemisinin biosynthesis was also influenced by chitosan treatment; the expression level of CYP71AV1 increased from 16 h and continued to increase until 48 h, whereas the expression of DBR2 began to increase at 4 h and declined by 24 h (Fig. 4).

The early pathway genes HMGR and FPS showed differences in expression after exposure to chitosan (Fig. 4). Compared with the control plant, the expression level of HMGR increased significantly at 2–48 h after chitosan treatment. In contrast to HMGR, the expression level of FPS increased only slightly at 16 h after chitosan treatment. DXS, DXR and ISPH, the three key genes of plastidic MEP pathway of IPP biosynthesis were also induced by chitosan: the expression level of DXS was increased at 2 h and declined after 8 h; DXR was clearly and immediately induced at 4 h; and ISPH was induced strongly at 16 h and peaked at 24 h after chitosan treatment (Fig. 4).

3.3. Chitosan promoted ROS production

To investigate the mechanisms by which chitosan improved artemisinin production, the reactive oxygen species content was assessed. The results showed a rapid and significant increase in O\(^2-\) content upon chitosan treatment and reached the highest level at 8 h; this was three times higher than control values (Fig. S5A). The H\(_2\)O\(_2\) content increased from 2 h and peaked at

<table>
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<th>Gene</th>
<th>Accession number</th>
<th>Primer (5′–3′)</th>
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<tr>
<td>ISPH</td>
<td>EU332141</td>
<td>Forward ATGCCCTTCTGGACGTAAACCTCCTCTG</td>
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<tr>
<td></td>
<td></td>
<td>Reverse CTCAGCACCATTCAGGGGCCTCCACAGG</td>
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<tr>
<td>DXR</td>
<td>AF182287</td>
<td>Forward CCTAGATCATGCTTGGACGTAAACCTCCT</td>
</tr>
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<td></td>
<td></td>
<td>Reverse CTACATGACTCTATGACGTAAACCTCCT</td>
</tr>
<tr>
<td>DXS</td>
<td>AF182286</td>
<td>Forward CTACATGACTCTATGACGTAAACCTCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse CTACATGACTCTATGACGTAAACCTCCT</td>
</tr>
<tr>
<td>DBR2</td>
<td>EU704257</td>
<td>Forward CACCATGCTCACAAAACAAACCTCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse GCTCATAAGATGACGTAAACCTCCT</td>
</tr>
<tr>
<td>CYTP1AV1</td>
<td>DQ315671</td>
<td>Forward CTACATGACTCTATGACGTAAACCTCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse ATGGATCTCGTCGTAAACTGCCA</td>
</tr>
<tr>
<td>ADS</td>
<td>AF138959</td>
<td>Forward GGAATGGACCTTACAGAAAGAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse TCATATCAGTACAGTAAACCTCCT</td>
</tr>
<tr>
<td>FPS</td>
<td>AF112881</td>
<td>Forward CTACATGACTCTATGACGTAAACCTCCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse GAGCGTTTGAGCCTGGTGATTCTA</td>
</tr>
<tr>
<td>HMGR</td>
<td>AF142473</td>
<td>Forward ATGGATCTCGTCGTAAACCTCCT</td>
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<td>Reverse GCTCATAAGATGACGTAAACCTCCT</td>
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<td>U60495</td>
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<tr>
<td></td>
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<td>Reverse TCATATCAGTACAGTAAACCTCCT</td>
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Table 1 Sequences of primers used in gene expression analysis.
48 h (Fig. 5B). Accordingly, antioxidant enzymes activities were increased by chitosan (Fig. 6). Activities of SOD in chitosan-treated A. annua increased slowly and reached the maximum amount at 16 h. There was no significant difference in POD activity after chitosan treatment. Upon chitosan treatment, the activities of CAT and APX increased and reached the highest at 24 h (Fig. 6), which were 1.5 and 3.9 times higher than those of control, respectively. Beyond 24 h, the activities of CAT and APX in treated plants decreased slowly and returned to the level of control plants at 96 h.

4. Discussion

The effect of chitosan on artemisinin biosynthesis remains unclear, and conflicting findings have been reported. For example, its effect on artemisinin biosynthesis in suspension cultures (Baldi and Dixit, 2008) was different from that in hairy roots (Putalun 2009).
et al., 2007). The results presented here indicate that chitosan induced the transcription of most genes in the artemisinin biosynthesis pathway and increased artemisinin content in intact A. annua plants (Figs. 3 and 4). ADS and DBR2 expression are rapidly and transiently induced (Fig. 4), which could explain the increases in dihydroartemisinic acid and artemisinin content. The key genes of both C5 unit biosynthesis pathways are all induced by chitosan (Fig. 4), which is in agreement with recent results that both C5 units biosynthesis pathways contribute to the biosynthesis of sesquiterpenoids (Laule et al., 2003; Schuhr et al., 2003; Dudareva et al., 2005), and specifically to the biosynthesis of artemisinin (Schramek et al., 2010; Towler and Weathers, 2007).

It is interesting to note that the artemisinic acid content did not change significantly after chitosan treatment (Fig. 3); however, the artemisinic acid content has been shown to increase after SA treatment (Pu et al., 2009). This difference might be due to the A. annua strain used (SP18 versus 001). It has been found that the artemisinin biosynthesis pathway differs between the two A. annua strains used in this and the previous study; SP18 contains higher artemisinin and dihydroartemisinic acid levels, whereas 001 contains higher artemisinic acid and arteannuin B levels (Wang et al., 2009). In the artemisinin biosynthesis pathway, the conversion of artemisinic aldehyde to dihydroartemisinic aldehyde, which is then further converted to dihydroartemisinic acid, could be the other committed step (Zhang et al., 2008). The increase in DBR2 transcription following chitosan treatment may account for the increase in the concentrations of dihydroartemisinic acid and artemisinin. The FPS and CYP71AV1 transcriptional levels showed little change after chitosan treatment, as has been reported after SA treatment (Pu et al., 2009). These results indicate that FPS and CYP71AV1 may not be rate-limiting enzymes in artemisinin biosynthesis.

Chitosan was reported to trigger the oxidative burst, especially production of H2O2 and O2− (Orozco-Cardenas and Ryan, 1999; Rossard et al., 2006). In the present study, the ROS content in leaves of A. annua increased after foliar application of chitosan, which probably explains the reduction in dihydroartemisinic acid and the increase of artemisinin. This speculation is in agreement with the hypothesis that dihydroartemisinic acid can remove reactive oxygen by quenching singlet oxygen and yielding the stable end-product artemisinin. High levels of reactive oxygen produced in A. annua assist the conversion of dihydroartemisinic acid to artemisinin (Wallaart et al., 2000).

Chitosan applied to the soil at the time of sowing or at the seedling stage has previously been shown to markedly increase plant growth (Ohta et al., 1999, 2004). However, in our study, foliar application of chitosan had no effect on the growth of A. annua; this is in agreement with a previous report that foliar application of chitosan did not affect the growth of maize or soybean (Khan et al., 2002). There are several possible reasons for these different effects of chitosan on plant growth, including differences in application time and application methods, and the use of different plant species.

5. Conclusion

The results presented in this study demonstrate that foliar application of chitosan increased artemisinin concentration in A. annua by up-regulating the genes of artemisinin biosynthesis and by converting dihydroartemisinic acid to artemisinin using a burst of ROS. Thus, foliar application of chitosan for improving artemisinin yield is of great practical value and could be an effective method to reduce the costs associated with artemisinin production.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.indcrop.2010.10.001.

References


