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ORIGINAL PAPER

Effects of artemisinin on root gravitropic response and root system development in *Arabidopsis thaliana*

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Abstract

Artemisinin, an antimalarial secondary metabolite produced in *Artemisia* species, also has been recognized as an allelochemical that inhibits the growth of several plant species. However, the phytotoxicity mechanism of artemisinin is not exhaustively deciphered up to now. In this research, the effects of artemisinin on *Arabidopsis thaliana* root gravitropic curvature and development were characterized. Exogenously applied artemisinin disturb the root gravitropic responses, inhibited the elongation of primary and lateral roots and root hairs in a concentration-dependent fashion, and prevented the formation of lateral roots and root hairs. Moreover, the number of starch grain and the distribution range of auxin in the root tip was reduced by artemisinin, and the redistribution of auxin was less sensitive to gravity stimulus when treated with artemisinin than that of control. The expression of auxin transporter PIN2 was partially suppressed by artemisinin. Together, the results demonstrated that the effects of artemisinin on root gravitropism and root system development were largely dependent on the reduction of starch grain and auxin levels, as well as the disordered lateral auxin redistribution.

Keywords *Arabidopsis thaliana* · Artemisinin · Auxin · Gravitropism · Root development · Starch grain

Abbreviations

DMSO	Dimethyl sulphoxide
ED50	Median effective dose
MS	Murashige and Skoog
PR	Primary root
LR	Lateral root
LRP	Lateral root primordia
ROS	Reactive oxygen species
RSA	Root system architecture

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Introduction

Artemisinin, a secondary metabolite produced by a traditional Chinese medicinal plant *Artemisia annua*, is used as the most effective current antimalarial agent (Zheng et al. 2016). In addition to its antimalarial activity, artemisinin also plays an important role in allelopathy (Jessing et al. 2014). Artemisinin could be released into the surroundings by *A. annua*, and then act as an allelochemical that inhibit the growth of neighboring plants (Lydon et al. 1997; Delabays et al. 2008; Panamanik et al. 2008). The mode of action of artemisinin as an allelochemical attracted attentions in recent years (Bharati et al. 2012; Yan et al. 2015b). However, more work is needed to decipher the mechanistic details of the phytotoxicity of artemisinin.

Plant roots could perceive gravity and then reorient their growth accordingly, this physiological process named root gravitropism, which contains four steps: perception, signal transmission, growth response, and the re-establishment of normal growth (Baldwin et al. 2013).

The perception of gravity is mediated by the sedimentation of starch-filled amyloplasts within columella cells of the root cap, which then provides directional cues to the root (Sato et al. 2015). Auxin, one of the most important phytohormones, plays critical roles in the process from gravity sensing to the response (Sato et al. 2015). Gravistimulation caused lateral transport of auxin and promote the formation of an auxin gradient across plant roots with accumulation of auxin at the bottom side where it inhibits cellular elongation (Hasegawa et al. 2017).

Root growth and development are of outstanding importance for the ability of plant to acquire water and nutrients from soil. In dicotyledonous plants, the development of root systems starts with the elongation of an embryonic primary root (PR) meristem, and the formation of a lateral root (LR) is a completely post-embryonic event (Giehl et al. 2013). The length of PR and LR, and the number of LRs represent the dominant feature of root system architecture (RSA) (Giehl et al. 2013). Polar auxin transportation in PR plays an important role during RSA. The levels and cell type-specific distribution of auxin, which are sustained by the auxin carriers such as PINs, are required for the activation of LR pre-branch sites and the promotion of LR initiation (Lavenus et al. 2013).

Natural compounds such as coumarin and flavonoid can affect root growth and gravitropic response in target plants (Santelia et al. 2008; Lupini et al. 2013). Previous studies showed that artemisinin possesses strong phytotoxic activities on root growth and development of several plant species (Duke et al. 1987; Lydon et al. 1997; Delabays et al. 2008; Panamanik et al. 2008; Yan et al. 2015b). In this study, the model plant *Arabidopsis* was employed to evaluate the detailed effects of artemisinin on root gravitropic responses and root system development. Furthermore, the grain starch sediment, auxin distribution and redistribution in *Arabidopsis* roots were also researched to reveal the phytotoxicity mechanism of artemisinin.

Materials and methods

Plant culture

After surface sterilized in 0.1% (w/v) $HgCl_2$ and rinsed with distilled water, seeds of *Arabidopsis* wildtype (Col-0) and transgenic lines (*DR5::GUS, DR5::GFP* and *PIN2::GFP*) were transported onto solid MS medium (0.7% agar) supplemented with 3% sucrose for a cold treatment (4 °C for 48 h), then germinated at standard condition (22 °C with a 16/8 h day/night photoperiod).

Root gravitropism assay

Root gravitropism assay was carried out according to Zou et al. (2012) with modification. Artemisinin (Meryer, Shanghai, China) was dissolved in dimethyl sulphoxide (DMSO, Solarbio, Beijing, China) and then added into the MS medium. The final concentrations of artemisinin were 1, 2, 5, 20 and 100 µM, respectively. A corresponding amount of DMSO (1/1000, v/v), same concentration to the artemisinin application medium, was used as the control. Col-0 seeds of were sowed onto the medium in square plates (10 × 10 cm) and then incubated vertically under standard condition. Then, images of the 5-day-old seedlings were collected with a digital camera (ZS7, Panasonic, Osaka, Japan). For gravistimulation, 4-day-old Col-0 seedlings were transferred into the medium and the root tips were marked with a marking pen at the corresponding positions of the plates. After being vertical cultured for 12 h, the roots were rotated 90° to initiate gravistimulation at time zero. Images were collected at 6, 12, 24, 48 and 72 h, respectively. Root elongation and bending angles were measured with Image J software (version 1.48, NIH, USA). Root length refers to the entire length of the root, and root elongation refers to the length of root growth after transfer to the treatment medium. The gravitropic angle is the angle of the root tip with respect to the gravity vector.

Root morphology measurements

Four-day-old *Arabidopsis* Col-0 seedlings were transferred onto the medium and then incubated vertically for 5 days. After treatments, the length of primary and lateral roots was recorded by Panasonic digital camera ZS7 and analyzed by Image J software (version 1.48, NIH, USA). Number of LRs (longer than 0.5 mm in length) were also recorded. *Arabidopsis DR5::GUS* lines were used to classify and count lateral root primordia (LRP) with different development stages according to Malamy and Benfey (1997). The emerged but shorter than 0.5 mm LR is also called LRP, and the density of LRP was determined by counting the LRPs per seedlings.

Determination of root hair length

Roots were grown vertically on artemisinin containing and control plates for 4 days, then examined using a stereomicroscope (Olympus, Osaka, Japan). Pictures of root segments (about 1 mm from the root tip) were taken every 24 h with a Panasonic digital camera ZS7. Root hair length were measured by Image J software (version 1.48, NIH, USA), and the density of root hair was determined by counting the root hair per length (mm).

GUS-histochemical analysis

Four-day-old *Arabidopsis DR5::GUS* seedlings were treated with various concentrations of artemisinin for 48 h, and 20 μM of artemisinin for 6, 12, 24, 48, 72, and 120 h, respectively. Then the seedlings were harvested and dealt with the method according to Yan et al. (2014), and then analyzed with an inverted microscope (37XB, Shanghai, China).

Confocal microscopy

Arabidopsis DR5::GFP and *PIN2::GFP* seedlings with 4-day-old were transferred onto the medium containing 20 μM artemisinin. *DR5::GFP* seedlings were incubated vertically for 12 h following with 90° rotation for 3 h. *PIN2::GFP* seedlings were incubated vertically for 6, 12, 24, 48 and 72 h, respectively. Fluorescent images of the root tips were captured with a laser-scanning confocal microscope (Olympus, Osaka, Japan).

Starch analyses

After treatments for 4 days, roots of *Arabidopsis* seedlings were stained in KI/I (10%/5%) solution for 10 min. The stained roots were cleared with chloral hydrate and then observed under bright-field microscopy (37XB, Shanghai, China).

Data analyses

For root gravitropism assay, root morphology measurements and of root hair length determination, at least 30 seeds or seedlings were used were per treatment with three replication. Then a representative repeat was choose to show the result. Data statistical analysis was conducted by SPSS software (version 16.0, SPSS Inc., USA). The differences in root growth and gravitropism after artemisinin treatments were analyzed by one-way ANOVA with an LSD test at probability levels of 0.05 and 0.01. The ED50 values of artemisinin on *Arabidopsis* root growth were estimated also using SPSS software.

Results and discussion

Artemisinin affects the root gravitropism of *Arabidopsis*

It has been demonstrated that some natural products affect root gravitropic response in plants (Santelia et al. 2008; Lupini et al. 2013; Yan et al. 2015a). In this study, the effect of artemisinin on *Arabidopsis* root gravitropic responses were determined (Fig. 1a, b). With the rise of artemisinin concentration, the root gravitropic angles of *Arabidopsis* seedlings first decreased and then increased after a 5-day growth (Fig. 1c). Additionally, the length of *Arabidopsis* root was reduced by artemisinin in a concentration-dependent manner (Fig. 1d).

Then, an experiment of gravistimulation with 90° reorientation was carried out to better understand the influence of

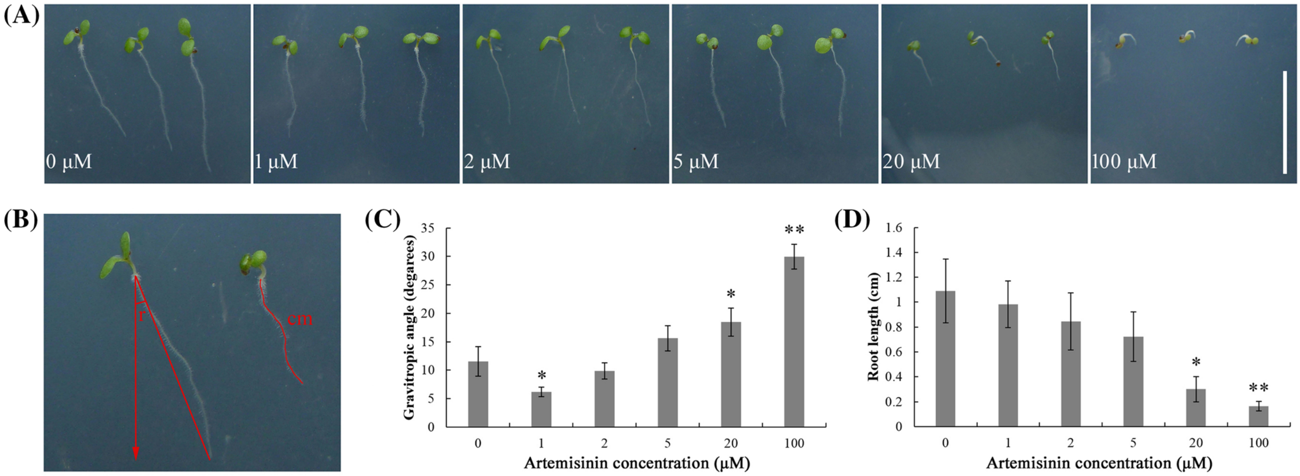

Fig. 1 Effect of artemisinin on *Arabidopsis* root growth and gravitropic angle after 5 days vertical growing. **a** Representative pictures of *Arabidopsis* roots after treatments with various concentrations of artemisinin. Bar = 1 cm. **b** Schematic diagram shows measurement of the gravitropic angle (r) and primary root length (in mm). **c** Gravitropic angles and lengths of *Arabidopsis* roots. Asterisks * and ** indicate statistically significant differences between treatments and control at $p < 0.05$ and $p < 0.01$, respectively ($n = 30$)

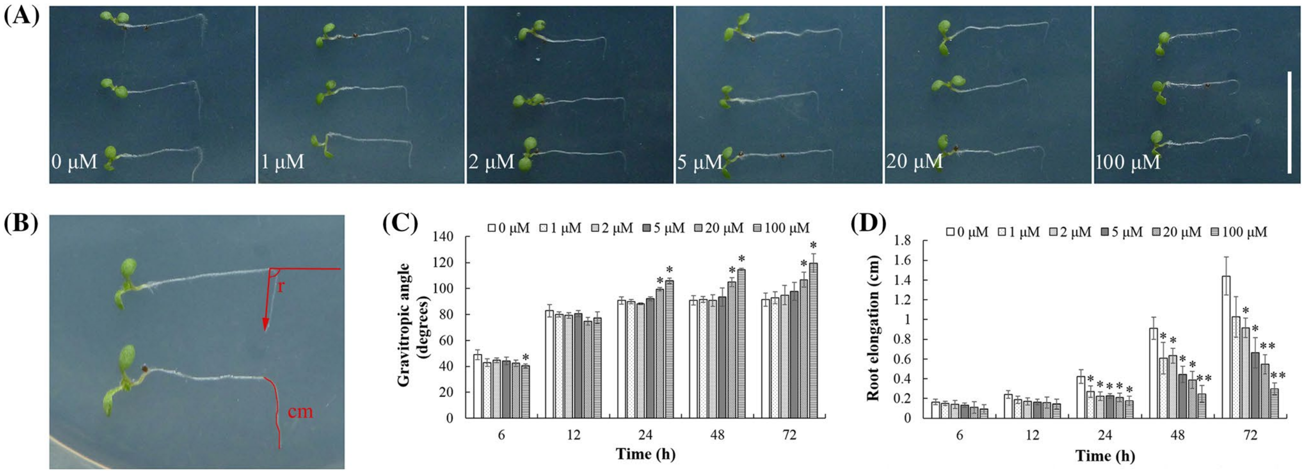

Fig. 2 Dynamics of root elongation and gravitropic curvature in *Arabidopsis* of various times and concentrations upon artemisinin exposure after 90° rotation. **a** Representative pictures of *Arabidopsis* roots after treatments with various concentrations of artemisinin for 48 h. Bar =1 cm. **b** Root elongation (in mm) refers to the new length established after transfer onto treatment medium, and the gravitropic angle is defined as the angle of the root tip with respect to the gravity vector (r). **c** Gravitropic angles and lengths of *Arabidopsis* roots. Asterisks * and ** indicate statistically significant differences between treatments and control at $p<0.05$ and $p<0.01$, respectively ($n=30$)

artemisinin on root gravitropism (Fig. 2a, b). Results showed that the gravitropic bending was first delayed relative to control within 12 h, and then gradually accelerated after 24 h in a concentration-dependent manner (Fig. 2c). Specifically, after treated with artemisinin at 20 and 100 μM for 24 h and longer, the root tips already shift to the other side of the direction of gravity (Fig. 2c). Meanwhile, root elongation of *Arabidopsis* treated with artemisinin was significantly inhibited after 24 h and longer (Fig. 2d).

In plants, the root gravitropism responses maybe influenced by the abnormal root elongation. In this study, artemisinin inhibited *Arabidopsis* root elongation in a concentration-dependent fashion. However, the gravitropism strengthened at 1 and 2 μM, and weakened with artemisinin concentration rising to 5 μM and higher (Fig. 1c). Moreover, after 90° reorientation, the gravitropic bending was initially delayed and accelerated later (Fig. 2c). These results suggested that the influence of artemisinin on the gravitropic bending showed a different variation trend with the root elongation, and was not a secondary effect of the inhibition of root length.

Artemisinin affects root system development of *Arabidopsis*

In the previous study, artemisinin was found to display significantly phytotoxic effect on lettuce seedlings, and the root growth is more sensitive to artemisinin than that of shoot (Yan et al. 2015b). In this study, the detailed effects of artemisinin on root growth and development was examined in the model plant *Arabidopsis*. Four-day-old *Arabidopsis* seedlings were treated with various concentrations of artemisinin for 5 days. Results showed that the elongation of the PR was inhibited by artemisinin in a concentration-dependent manner. The PR length was significantly shorter than control at 5 μM of artemisinin. When the concentrations were rising to 20 and 100 μM, the PR almost stop growing (Fig. 3a). Besides PR, the growth of LR were also affected by artemisinin. After treated with artemisinin, the LR density was lower than control. At 200 μM, there was almost none mature LR (more than 0.5 mm in length, Fig. 3b), indicating that artemisinin displayed strong inhibitory effects on mature LR formation. Similar with PR, the length of LR was shortened by artemisinin in a concentration-dependent manner (Fig. 3c). The ED50 values of artemisinin on PR length, LR length and LR density were 13.74, 4.08 and 4.33 μM, respectively (Table 1). Taken together, this results showed that artemisinin strongly inhibited the primary and lateral roots elongation. The length of a root is determined by the combination of three major cell biological processes: the rate of cell division, the rate of cell differentiation, and the extent of expansion and elongation of cells (Scheres et al. 2002). The previous study showed that artemisinin displayed significantly inhibitory effects on mitosis of every stage in lettuce root (Yan et al. 2015b). Therefore, the effects of artemisinin on *Arabidopsis* root elongation maybe involved in the arresting of the cell cycle.

DR5, an auxin-responsive promoter, has been used to infer the developmental stages of LRs (Ulmasov et al. 1997). In this study, the number of LRP was scored using dissecting microscope after staining of *DR5::GUS* lines. Compared to the control, artemisinin at 2 μM and higher concentrations

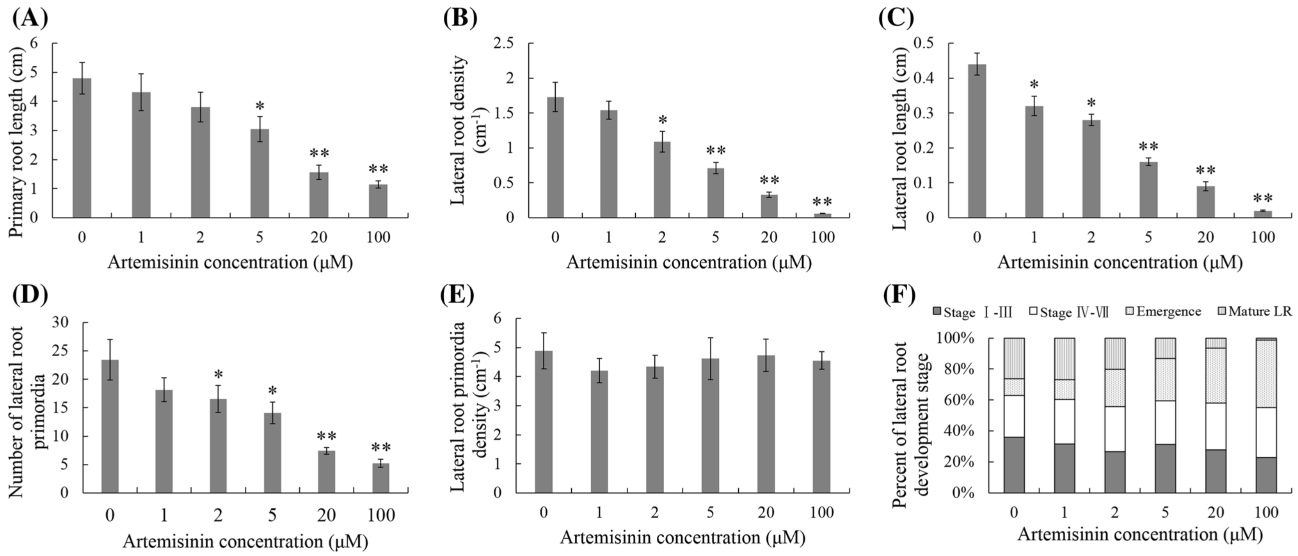

Fig. 3 Root system development of *Arabidopsis* after artemisinin treatments for 5 days. **a** Primary root length. **b** Lateral root density. **c** Lateral root length. **d** Numbers of lateral root primordia. **e** Lateral root primordia density. **f** Percent of Lateral root development stage. Asterisks * and ** indicate statistically significant differences between treatments and control at $p<0.05$ and $p<0.01$, respectively ($n=30$)

Table 1 The ED50 values (μM) of artemisinin on *Arabidopsis* root growth

PR length	LR length	LR density	Number of LRP	LRP density	Root hair length			Root hair density		
					24 h	48 h	72 h	24 h	48 h	72 h
13.74	4.08	4.33	16.52	> 100	4.25	1.86	2.47	4.85	4.88	4.76

significantly reduced the number of LRP (Fig. 3d) with an ED50 level of 16.52 μM (Table 1). However, the density of LRP was not influenced by artemisinin at any concentrations (Fig. 3e), indicating that the reduction of number of LRP was largely dependent on the inhibition of PR length by artemisinin. Furthermore, the LRP was calculated in each stage. The percentage of stage 1–7 was similar with that of control. However, with the rising of artemisinin concentration, the percentage of emergence LR was increased with the reduction of mature LR (Fig. 3f). These results suggested that artemisinin particularly prevent the development of emergence LRs to mature ones.

The development of root hair was further examined by treating with various concentrations of artemisinin after 24, 48 and 72 h, respectively (Fig. 4a). Results showed that the exposure of *Arabidopsis* seedlings to exogenous artemisinin led to a significant reduction in root hair density and length in comparison to control (Fig. 4b). This effect was apparent with the increasing of artemisinin concentration and the extension of the time; under low artemisinin concentrations (1 and 2 μM) at 24 h, no statistically significant effect was detected, while under higher concentrations and/or longer time, the root hair density and length were markedly inhibited by artemisinin (Fig. 4b, c). Interestingly, after all treated times, artemisinin displayed similar ED50 values on root hair density and relatively low ED50 levels (< 5 μM) on root hair length (Table 1).

Artemisinin inhibits auxin distribution in *Arabidopsis* roots

In *Arabidopsis* root development, a distal auxin maximum is related to the pattern formation (Sabatini et al. 1999). In this study, the changes of auxin level distribution in *Arabidopsis* roots by artemisinin were investigated using the *DR5::GUS* reporter line (Ulmasov et al. 1997). As shown in Fig. 5a, *DR5::GUS* gene expression in root tips of artemisinin-treated plants slightly decreased at 1 and 2 μM, and was largely decreased at 5 μM and higher concentrations. Under 20 μM of artemisinin, after 6 and 12 h of treatments, *DR5::GUS* gene expression in treated plants slightly decreased. However, a significant reduction of the GUS activity in the root tip was observed after exposed to artemisinin for 24 h and longer (Fig. 5b).

Auxin appears to play a critical role at every step of plant root development, including the elongation of PR and epidermal-derived root hairs, and the number of LPR (Rahman et al. 2002; Ishida et al. 2008; Peret et al. 2009). During root

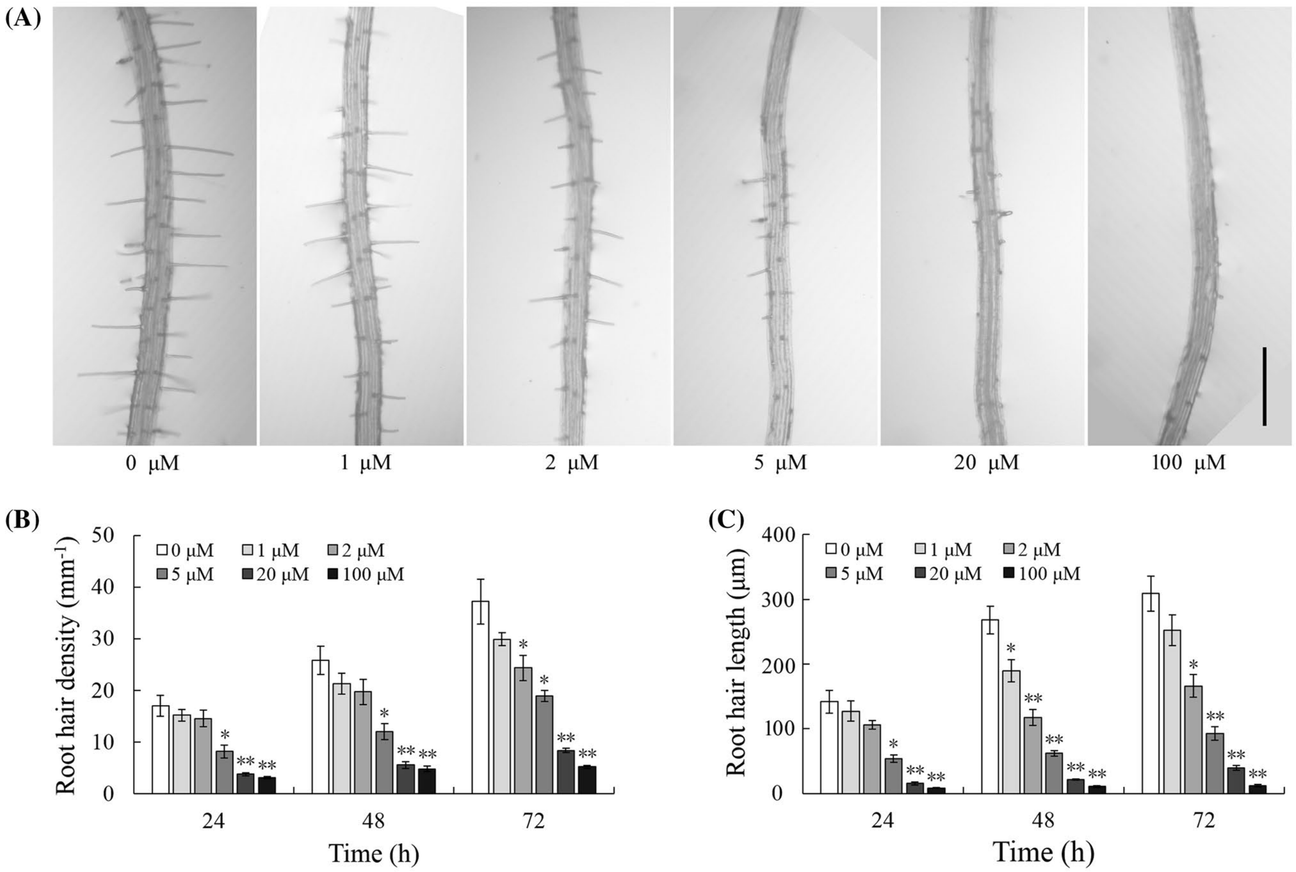

Fig. 4 Root hair development of *Arabidopsis* after artemisinin treatments. **a** Representative pictures of *Arabidopsis* root hair after treatments with various concentrations of artemisinin for 48 h. Bar = 250 μm. **b** Root hair density. **c** Root hair length. Asterisks * and ** indicate statistically significant differences between treatments and control at $p<0.05$ and $p<0.01$, respectively ($n=30$)

development, the level of auxin in different part is accurate and often shows dose-depend effects on the emergence and/or elongation processes (Lavenus et al. 2013; Overvoorde et al. 2010). Together with the results that artemisinin reduced auxin levels in *Arabidopsis* roots, suggesting that the altered auxin distribution in the radical tip may cause the arrest of PR, LPR and root hair elongation, as well as the LPR and root hair emergence.

Artemisinin inhibits auxin redistribution under gravitropic stimulus and the expression of PIN2 in *Arabidopsis* root tips

In gravitropism, the bending of the root is driven by the asymmetric redistribution between the upper and lower sides of the root, which was largely dependent on polar auxin transport (Peer et al. 2011). To examine auxin transport specifically with respect to artemisinin, *DR5::GFP* line were employed in this study. Consistent with previous reports (Ottenschlager et al. 2003), control roots expressed *DR5::GFP* asymmetrically, as evident from the enhanced expression in the outermost cell layer on the lower side of the root meristem (Fig. 6a). By contrast, in seedlings treated with artemisinin at 20 μM, *DR5::GFP* was expressed less symmetrically in more than 80% of roots (i.e. 42/50) (Fig. 6a).

Arabidopsis PIN2::GFP line was used to determine if artemisinin alters the expression of PIN2 protein. The seedlings were treated with artemisinin at 20 μM for 6, 12, 24, 48 and 72 h, respectively. Results showed that PIN2 promoter activity was reduced in the root tip after artemisinin treatments, and the reduction was more and more with the prolonging of cultured time (Fig. 6b).

In *Arabidopsis* roots, polar transport of auxin and the redistribution of auxin during gravotripic process are dependent upon the auxin flux-facilitators such as AUX1,

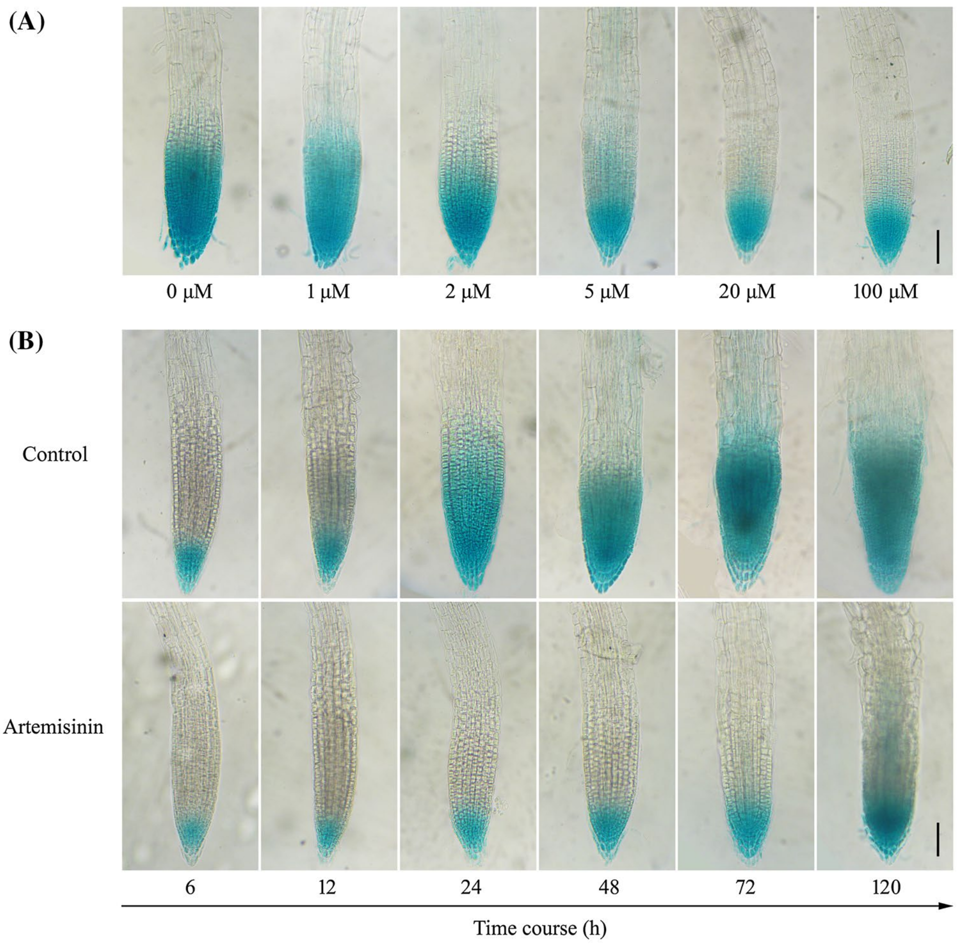

Fig. 5 DR5::GUS activity in *Arabidopsis* primary root tips after artemisinin treatments. **a** Representative pictures of *Arabidopsis DR5::GUS* root tips after treatments with various concentrations of artemisinin for 48 h. Bar = 100 μm. **b** Representative pictures of *Arabidopsis DR5::GUS* root tips after treatments with 20 μM of artemisinin for 6, 12, 24, 48, 72 and 120 h, respectively. Bar = 100 μm

PIN2, PIN3 and PIN7 (Petrásek and Friml 2009). Several factors have been shown affecting gravitropism through auxin transport. For instance, ellagic acid influenced root gravitropism through abnormal lateral auxin redistribution (Yan et al. 2015a). In this study, the asymmetric distribution of auxin between the upper and lower sides of the root was almost lost by artemisinin treatment under gravity stimulus. Therefore, the effects of artemisinin on root gravitropism were largely involved in the auxin redistribution. Moreover, the expression of PIN2 was markedly reduced by artemisinin, indicating that the effect of artemisinin on auxin redistribution at least partly dependent on its regulation of PIN2.

Root hair elongation is a result of increased auxin levels in the epidermal cells and auxin influx is an important factor for root hair development (Jones et al. 2009). This study suggested that artemisinin showed inhibitory effect on root growth and root hair elongation, which involved in auxin-efflux carriers. Hence, artemisinin treatment may alter auxin efflux, thereby accounting for the observed decreased length of the root hairs. Additionally, the root growth and development of recipient plant species could be affected by potential allelochemicals at the radicle level with morphological changes and damage (Aguilera et al. 2015). However, whether the structures of *Arabidopsis* root was damaged by artemisinin needed more research.

Artemisinin reduced starch grain levels in *Arabidopsis* root tips

In gravitropic response, the starch-dense amyloplasts within the columella cells of the root cap may play an important role in signal perception (Baldwin et al. 2013). In order to detect whether artemisinin affect starch content in *Arabidopsis* root, the morphology of columella amyloplasts was

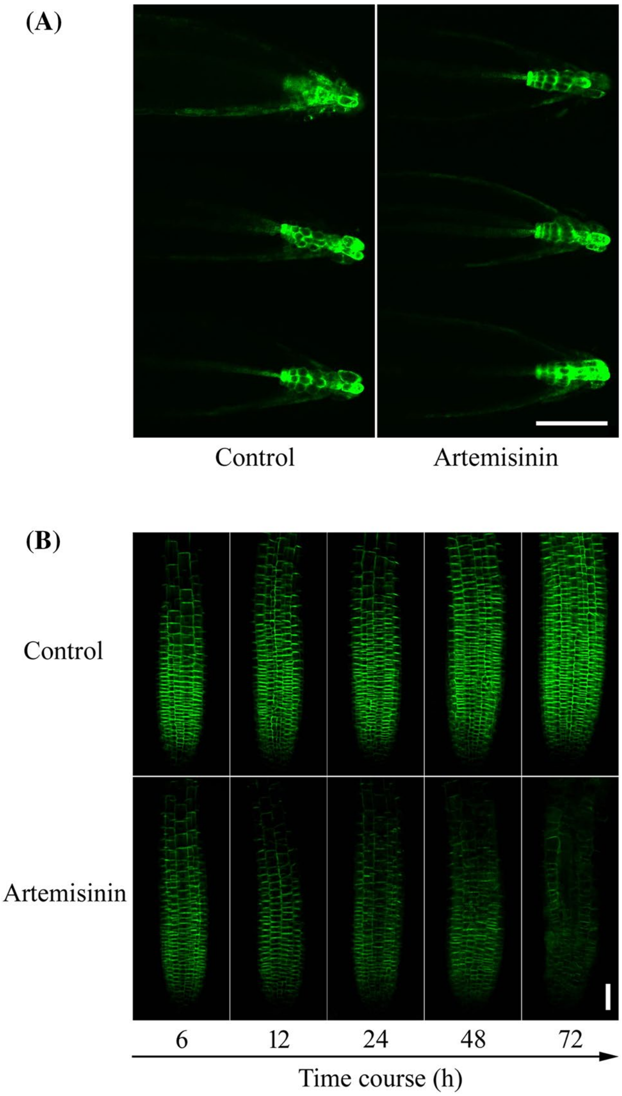

Fig. 6 **a** Fluorescence of DR5::GFP in *Arabidopsis* root tips after gravity stimulation. Three representative images are shown for each group. Bar =50 μm. **b** Fluorescence of PIN2::GFP in *Arabidopsis* root tips after gravity stimulation. Representative image is shown for each group. Bar = 100 μm

investigated. Under 1 and 2 μM of artemisinin, the starch levels in *Arabidopsis* root caps were similar as control (Fig. 7). While, the starch levels were slightly reduced by 5 μM of artemisinin after 48 and 72 h, respectively (Fig. 7). However, artemisinin treatments with the high concentrations (20 and 100 μM) notably reduced the starch levels after 48 h (Fig. 7).

Gravity perception is the first step in the response pathway of root gravitropism (Moulia and Fournier 2009). Previous studies have shown that starch-deficient and starch-excess mutants displayed insensitive and oversensitive responses to gravistimulation, respectively (Caspar and Pickard 1989; Vitha et al. 2007), indicating that the starch in plant root tip play an important role in the sensing of gravitropic signals in gravitropism. Some exogenous applied chemicals can affect the starch levels in plant. For instance, narciclasine, an alkaloid isolated from *Narcissus tazetta* bulbs, have strong inhibitory effects on starch granules in *Arabidopsis* roots (Na et al. 2011). In this study, the numbers of starch grain were dramatically reduced by artemisinin, which may depress the sensitivity of root cells to perceive the gravitropic stimulus. Therefore, the abnormal gravitropic response caused by artemisinin is at least partly depend on a defect in the gravity sensing process. Additionally, previous studies (Bharati et al. 2012; Yan et al. 2015b) showed that artemisinin possesses the ability to affect photosynthesis processes, which might be related to the cellular signaling molecular, reactive oxygen species (ROS). ROS distribution was showed to be involved in the gravitropic response induced by coumarin (Santelia et al. 2008). Therefore, the effects of artemisinin on root gravitropic response may be connected with ROS.

Conclusion

Partial reduction of root gravitropic responses and abnormal root system development in *Arabidopsis* seedlings were found after exogenous application of artemisinin. Further research showed that reduced grain starch and auxin levels and disordered auxin redistribution in root tips were induced by artemisinin, together with the reduced expression of the auxin-efflux carrier PIN2. These results indicated that the effects of artemisinin on root gravitropic responses and root system development may be largely dependent on its impact of the transportation and distribution of auxin, as well as the accumulation of grain starch in root tips.

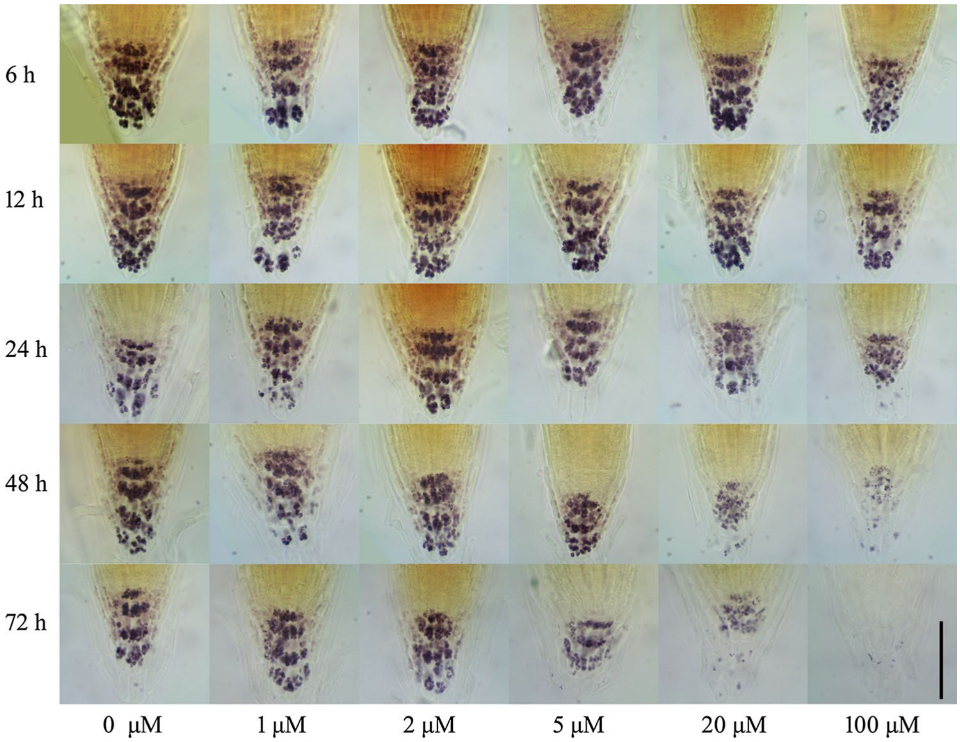

Fig. 7 Effect of artemisinin on starch grain in *Arabidopsis* root caps. Four-day-old *Arabidopsis* Col-0 seeds grown vertically in the medium supplemented with various concentrations of artemisinin for 6, 12, 24, 48 and 72 h, respectively. Root tips were stained with iodine-potassium iodide solution and observed under a light microscope. The representative images of are shown out of three separate experiments. Bar = 100 μm

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