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The promoting effects of alginate oligosaccharides on root development in *Oryza sativa* L. mediated by auxin signaling

Yunhong Zhang[a,b,*], Heng Yin[b], Xiaoming Zhao[b], Wenxia Wang[b], Yuguang Du[b], Ailing He[a], Kegang Sun[a]

[a] Institute of Plant Nutrition, Agricultural Resources and Environmental Science, Henan Academy of Agricultural Sciences, Zhengzhou 450002, China
[b] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China

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ABSTRACT

Alginate oligosaccharides (AOS), which are marine oligosaccharides, are involved in regulating plant root growth, but the promotion mechanism for AOS remains unclear. Here, AOS (10–80 mg/L) induced the expression of auxin-related gene (*OsYUCCA1*, *OsYUCCA5*, *OsIAA11* and *OsPIN1*) in rice (*Oryza sativa* L.) tissues to accelerate auxin biosynthesis and transport, and reduced indole-3-acetic acid (IAA) oxidase activity in rice roots. These changes resulted in the increase of 37.8% in IAA concentration in rice roots, thereby inducing the expression of root development-related genes, promoting root growth in a dose-dependent manner, which were inhibited by auxin transport inhibitor 2,3,5-triiodo benzoic acid (TIBA) and calcium-chelating agent ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA). AOS also induced calcium signaling generation in rice roots. Those results indicated that auxin mediated AOS regulation of root development, and calcium signaling may act mainly in the upstream of auxin in the regulation of AOS on rice root development.

1. Introduction

With an increasing population and economic improvement, the rapid increase in the demand for food, resources and energy has brought some serious problems. Therefore, the development of marine resources is inevitable. Alginates are quite abundant in nature since they occur as a structural component in marine brown algae (*Phaeophyceae*), comprising up to 40% of the dry matter; their commercial production started in 1927, which has now expanded to about 30,000 tons per year worldwide; 30% of this tonnage is devoted to the food industry, the rest being used in industrial, pharmaceutical, and dental applications (Ertesvåg, Valla, & Skjåk-Bræk, 2009). Therefore, it is significant to the development of alginates for the utilization of marine resources. Alginate oligosaccharides (AOS) are marine oligosaccharides generated from alginate, containing α-L-guluronate (G) and β-D-mannuronate (M), which are arranged in homopolymeric G blocks, M blocks, and random heteropolymeric G and M stretches (Nagasawa, Mitomo, Yoshii, & Kume, 2000). The increased studies have shown that AOS with degrees of polymerization 2–10 can increase the yield of crops (Xu, Iwamoto, Kitamura, Oda, & Muramatsu, 2003; Zhang, Wu, Geng, Hu, & Zhang, 2009), therefore, they may be used as a new type of plant growth regulator, which is very significant to the utilization of marine resources in agricultural production; however, the application is currently limited for want of a clear understanding of the functional mechanism of the action of AOS.

Root systems play important roles in providing the absorption area for plants to absorb water and other nutrients as well as to anchor the plants in the soil. Root morphology is not predetermined in plant development; each plant also integrates information from its environment into the "decisions" controlling root formation and growth (Malamy & Ryan, 2001). The biological activities of AOS in plant roots have been reported. For example, AOS promoted root growth in carrot and rice (Xu et al., 2003). AOS increased the mitotic index of root tip cells and lowered the rate of chromosomal aberrations in *Vicia faba* under cadmium stress (Ma, Zhang, Bu, & Wang, 2010). AOS increased the root length, root tips, root volume, root fresh weight, root absorption area and root activity at 10–50 mg/L in flowering Chinese cabbage (*Brassica campestris* L.), and the promoting effects were greater than other oligosaccharides such as oligoglacturonic acid under the same culture conditions (Zhang et al., 2009), which may be one of the main reasons that AOS improved the growth and yield of crops (Xu et al., 2003; Zhang et al., 2009). However, we have limited information about signal

* Corresponding author at: Institute of Plant Nutrition, Agricultural Resources and Environmental Science, Henan Academy of Agricultural Sciences, 116 Garden Road, Zhengzhou 450002, China. Tel.: +86 0371 65733514.
E-mail address: snowgirl23@126.com (Y. Zhang).

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transduction procedures by which AOS interaction with cells result in promoting root development.

Auxin plays a central role in regulating root growth, and mediates many aspects of the root development, including root elongation, later root (LR) formation, root hair and root growth direction (Casimiro et al., 2001; Sreevidya et al., 2010; Woodward & Bartel, 2005). For example, exogenous indole-3-acetic acid (IAA) and α-napthylacetic acid (α-NAA, an IAA homologue) promote cell division in the root pericycle, resulting in many LRs production, whereas auxin transport inhibitor 1-*N*-naphthylphtalamic acid (NPA) block LR initiation (Casimiro et al., 2001; Himanen et al., 2002). Proper output of auxin signaling depends on the interplay of the biosynthesis, transport, conjugation, and signaling of this class of small molecules (Woodward & Bartel, 2005). The prevalent form of auxin, IAA, is synthesized from indole precursors via tryptophan (Trp)-dependent synthesis or through Trp-independent pathways, and the *YUCCA* gene family of flavin monooxygenases is a rate-limiting enzyme in Trp-dependent pathways (Zhao et al., 2001). IAA is transported downward to the maturing stem and the roots by the influx carriers, auxin resistant 1/like aux 1(AUX/LAX), and the efflux carriers pin-formed (PIN) and P-glycoprotein (PGP), which play a major role in polar auxin transport (Woodward & Bartel, 2005). Once auxin arrives at destination, it binds to a pocket of the transport inhibitor response 1 (TIR1) auxin receptor, thus it causes AUX/IAA proteins degradation through the SCF^{TIR1} (SKP1, Cullin and F-box protein, in this case TIR1) complex, and thereby leads to auxin-induced gene expression changes (Mathesius, 2008).

Auxin homeostasis in plant is also affected with other endogenous signals including calcium (Ca^{2+}) and nitric oxide (NO) as well as environmental factors such as salinity, osmolality, drought, cold, and metal stress (Chen & Kao, 2012; Cousson, 2004; Pagnussat, Lanteri, Lombardo, & Lamattina, 2004; Woodward & Bartel, 2005). Recent studies have shown that oligosaccharides control processes involved in root growth and development under the regulation of phytohormones. Lipochitin oligosaccharides from *Rhizobium leguminosarum* bv. *viciae* enhanced auxin transport capacity in the roots of *Vicia sativa* subsp. *nigra* (Boot, van Brussel, Tak, Spaink, & Kijne, 1999), and promoted root growth and development in *Arabidopsis* at no more than 10 nM (Khan, Costa, Souleimanov, Prithiviraj, & Smith, 2011). Galactoglucomannan oligosaccharides (GGMOs) inhibited primary root elongation induced by indole-3-butytric acid (IBA) at low concentration (10^{-8} M) in mung bean (*Vigna radiata* (L.) Wilczek) (Kollárová, Vatehová, & Lišková, 2010). However, the regulation functions of various oligosaccharides on root development are different due to their different structures (Khan et al., 2011; Kollárová et al., 2010). In contrast, the promoting effects of AOS on root growth have been more reported (Xu et al., 2003; Zhang et al., 2013a,c; Zhang, Wang, Zhao, Du, & Wu, 2013b). Our recent studies showed that AOS induced NO generation in promoting the growth of roots in wheat (*Triticum aestivum* L.) (Zhang et al., 2013a,b,c), and NO interferes with auxin signaling through S-nitrosylation of the *Arabidopsis* TIR1 auxin receptor (Terrile et al., 2012). AOS also changed the concentration and distribution of IAA in flowering Chinese cabbage (Zhang et al., 2013a,b,c). However, little information is available about the interaction between AOS and auxin in induced root formation and development.

Rice (*Oryza sativa* L.) is the main human food crop, and its yield has increased considerably with the introduction of the green revolution. Therefore, in this study, we investigated the effects of AOS on rice root formation and development and explored the role of auxin in root development induced by AOS from many aspects including the biosynthesis, transport and signal transduction of auxin as well as its interaction with calcium signaling in AOS-induced root formation and development. The paper is for the purpose of better understanding the mechanisms by which AOS regulate plant growth from the aspects of the sensation and transduction of AOS signaling in cells, and provide a theoretical basis for AOS application in agriculture production.

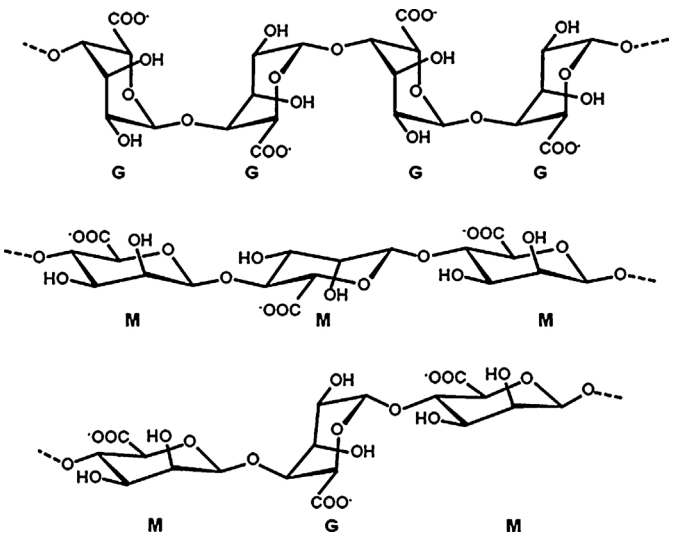

Fig. 1. Stereo-image of structure of alginate oligosaccharides, where G represents α-L-guluronate, and M represents β-D-mannuronate.

2. Materials and methods

2.1. Materials

The alginate (sample NO. 20100927004; viscosity, 1010 mPas; molecular formula, $(C_6H_7O_6Na)n$; molecular weight, 32,000–2,002,000) was offered by Qingdao Bright Moon Seaweeds Group Co., LTD. (Qingdao, Shandong, China) and degraded by an alginate lyase (purchased from Shanghai Nagase Trading Co., LTD., Shanghai, China) from *Sphingomonas sp.* at 45 °C for 10 h. Higher molecular weight component was precipitated by adding 80% (v/v) ethanol (4 volumes) and centrifuged at 8000 rpm for 15 min. The supernatant was freeze-dried and identified by the electrospray ionization mass spectrometry (ESI-MS), mainly composing of dimers, trimers and tetramers (the average molecular weight, 724; viscosity, 180 mPas) (Fig. 1). α-NAA, auxin transport inhibitor 2,3,5-triiodo benzoic acid (TIBA) and Ca^{2+} fluorescent indicator 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester (fura- 2/AM) were purchased from Beyotime Institute of Biotechnology, Beijing, China. Other reagents were analytical grade (Sinopharm Chemical Reagent Beijing Co., Ltd., Beijing, China), and all the solutions were prepared using deionized water (Resistivity > 16 MΩ cm at 25 °C).

The rice (*Triticum aestivum* L.) cultivar used in the experiment was Liangyou 287, a temperature sensing type two-line hybrid rice combinations, and was bred by Prof. Yong Zhou from Hubei University, Wuhan, China.

2.2. Plant culture and experimental design

Rice seeds were sterilized with 75% (v/v) ethanol for 5 min and washed extensively with distilled water. To obtain more uniformly germinated seeds, rice seeds were soaked in distilled water for 8 h at 25 °C in dark, and then transferred to a 20-cm Petri dish containing one sheet of filter paper moistened with distilled water for 5 days. Five-day-old seedlings were selected and then transferred to a 9-cm Petri dish containing with or without the auxin transport inhibitor TIBA (1.0 μM) or Ca^{2+} chelator ethylene glycol bis (2-aminoethyl) tetraacetic acid (EGTA, 0.4 mM) for 6 h, and then

Table 1
Polymerase chain reaction primers used in the study.

Gene	Primer sequence for cDNA isolation
OsActin	Forward 5′-GATGGTGTCAGCCACACTGTC-3′ Reverse 5′-ATGCTGCTAGGAGCCAGTGC-3′
QHB	Forward 5′-CAAGTCACGGAAGAACAA-3′ Reverse 5′-AACGGCTTACCAATAGAGT-3′
Cyc2;0s-2	Forward 5′-ACAGGCTGAACTGGATAA-3′ Reverse 5′-CTGGCTCTGACTCAAACA-3′
OsIAA11	Forward 5′-AGTTGTCCATGGCGTTCCA-3′ Reverse 5′-TGCTCTCCTTCAGCTGCTGAT-3′
OsARF1	Forward 5′-AGAGGTCAACCACGAAGA-3′ Reverse 5′-TCCGACAATAGTCCCAGT-3′
ARL1	Forward 5′-GAAGAAGAAGCGGCGGCCAAGATGAC-3′ Reverse 5′-CTTACGAGCGGTTAAGGTAAGCGTAGGCGA-3′
OsPIN1	Forward 5′-ACTACATCCTGGTGAACAT-3′ Reverse 5′-CACAAGAAGCCGCTGAAG-3′
OsYUCCA1	Forward 5′-GGCAGAGCAAGATTATCAGTC-3′ Reverse 5′-TCATCGGACGCCCTCAACGTCGC-3′
OsYUCCA5	Forward 5′-AGTAAGCGTCAATGGAGTT-3′ Reverse 5′-GCATAGCCAGAATTATCGT-3′

were cultured in AOS (at the desired concentration) or exogenous auxin (0.5 μM α-NAA), for 5 days. The roots of rice seedlings were harvested at different times after treatment to determine the root development-related indexes. Seedlings, grown in distilled water, were used as the controls. Each Petri dish contained 30 seedlings and each treatment was replicated three times. The plants were cultured at a day/night cycle of 12 h/12 h, 25 °C/20 °C, a relative humidity of 60% and a light intensity of 800 μmol/(m^2 s).

2.3. Root morphology

The morphology of rice roots was quantified by using WinRHIZO root analysis system (Regent instrument Inc., Quebec, Canada). For light microscopy, root samples from different treatment groups were fixed in 4% (v/v) paraformaldehyde in 0.1 M phosphate buffer solution (pH 7.4) for 24 h, dehydrated in an ethyl ethanol series, embedded in paraffin, sectioned at 6 μm using Leica microtome (RM2016, Shanghai Leica instruments Co. Ltd., Shanghai, China), stained with Safranin and Fast Green, and then observed under optical microscope (A1130259, Shanghai Wanheng Precision Instruments Co. Ltd., Shanghai, China) and photographed.

2.4. Total RNA extraction and semiquantitative RT-PCR

Using TRIZOL Reagent (Takara Biotechnology Co., LTD., Dalian, China) according to the manufacturer's protocol with slight modifications, total RNA was isolated from the rice roots in different treatment groups. The RNA quality was validated using electrophoresis and spectrophotometer. First-strand cDNA synthesis was conducted using TaKaRa RNA PCR Kit (AMV) Ver.3.0 (TaKaRa Biotechnology Co., Ltd., Dalian, China) from 1.0 μg total RNA according to the manufacturer's protocol.

Subsequently, PCR reaction with equal aliquots of cDNA samples was carried out using special primers with 2x Taq PCR MasterMix (Biomed technology Co., LTD., Beijing, China). The specific primers of the related genes were designed in Table 1. To standardize the results, the relative abundance of *OsActin* was determined and used as the internal standard. Amplified PCR products (10 μL) were electrophoresed on the 1% (w/v) agarose gels and monitored using the UVP GelDoc-It^{TM} 310 Imaging System. A negative control (mixing RNA with all the other reagents except RT enzyme during cDNA synthesis) was done and no visible band was seen in agarose gels, suggesting there was no genomic DNA contamination in the RNA samples. Experiments were repeated three times in each treatment and the same results were obtained.

2.5. Determination of auxin concentration

The concentration of auxin was determined by high-performance liquid chromatography (HPLC) (Sun, Hu, Tan, Liu, & Liu, 2010). Samples of frozen tissue (approximately 1.0 g each) were homogenized manually in chilled mortar in 3 mL of cold 80% (v/v) methanol containing 1 mM 2,6-di-tert-butyl-4-methylphenol (BTH, an antioxidant). The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C after 30 min treatment with 25 kHz and 400 W ultrasonic treatments in the dark. The supernatants were processed using a C18-Sep-Pak column (Waters, Milford, MA, USA), and then dried with N_2 and dissolved in 1 mL of the mobile phase that consisted of methanol-2% (v/v) acetic acid solution (55:45, v/v). The conditions for HPLC were as follows: Agilent TC-C18 column (250 mm × 4.6 mm, 5 μm) and mobile phase with a flow rate of 1.3 mL/min. The detection wavelength was 280 nm with a column temperature of 45 °C and an injection volume of 20 μL. Auxin concentration was calculated based on peak area according to the standard curve.

2.6. Determination of the activity of IAA oxidase

Sample containing approximately 1 g of frozen plant tissue each were homogenized manually in a chilled mortar in 6 mL of cold 0.02 M phosphate buffer (pH 6.0). The homogenate was centrifuged at 4000 rpm at 4 °C for 20 min. The resulting supernatant – the enzyme extract – was used for assaying the activity of IAA oxidase. The reaction mixture (samples of 10 mL each containing 2 mL 200 μg/mL IAA, 1 mL 1 mM $MnCl_2$, 1 mL 1 mM 2,4-dichlorophenol, 5 mL of 0.02 M phosphate buffer and 1 mL enzyme solution) was incubated at 25 °C for 30 min. 2 mL of the reaction solution was colored by adding 4 mL $FeCl_3$–H_2SO_4–H_2O (3–60–100) solution at 40 °C for 30 min, then the absorbance was measured at 530 nm. IAA oxidase activity is expressed in terms of micrograms of IAA decomposed per min per gram (fresh weight) of the tissue.

2.7. Observation of $[Ca^{2+}]_{cyt}$ by fluorescence microscope

$[Ca^{2+}]_{cyt}$ observation was performed by using Ca^{2+} fluorescent indicator fura-2/AM as described previously (Lu et al., 2010) with slight modifications. The rice roots were cut into 5 mm length and were incubated for 45 min in 20 mM Hepes buffer (pH 7.4, in dark) containing NaCl (115 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.8 mM), glucose (13.8 mM) and 1 μM fura-2/AM (Beyotime Institute of Biotechnology, Beijing, China). The roots were subsequently washed three times with Tris/KCl buffer (Tris 10 mM and KCl 50 mM, pH 7.2). After washing the excess dye with fresh Tris/KCl buffer, the roots were placed in Tris/KCl buffer containing different treatment solutions for 15 min, then Ca^{2+} signaling in roots was detected using a Nikon SMA 1500 stereoscopic fluorescence microscopy (IX71, OLYMPUS Co., Ltd., Japan) under the following settings: Excitation at 330–400 nm and monitoring emission at 510 nm. To extract quantitative data, pixel values were measured over root regions that were located manually on images and calculated using Images 1 .41 software (http://rsbweb.nih.gov/ij). The experiments were repeated at least three times in each treatment, and obtained the same results.

2.8. Statistical analysis

All experiments were repeated three times. All data obtained were subjected to analysis of variance, and the differences between the mean values were compared by Duncan's multiple range test at

an α-level of 0.05. Statistical analyses of the data were performed with SAS version 9.0.

3. Results

3.1. AOS promoted root formation and growth in rice

To examine the effects of AOS on root development, 5-day-old rice seedlings were treated with various concentrations of AOS (10–80 mg/L) for 5 days (Table 1, Fig. 2a). In comparison with the control, AOS significantly increased total root length and root surface area between 10 and 80 mg/L and improved plant development, with the optimal effects of 20 mg/L ($P < 0.05$). The numbers of root tips and adventitious roots, root volume and root fresh weight were also respectively increased by 4.1%, 15.6%, 43.8% and 30.0% at 20 mg/L. However, the length of the longest root and the number of root tips had a maximum response at 40 mg/L. These results indicated that AOS promoted the formation and elongation of rice roots and was dose-dependent.

Differences in the growth and morphology of rice roots were also reflected in their anatomy. To explore the regulation mechanism of AOS on root development, the microstructure and expression of development-related genes in rice roots, treated with AOS at 20 mg/L, were investigated (Fig. 2b and c). The number and size of stele cells in rice roots increased remarkably, which was confirmed by the increase in the expression level of cyclin-dependent kinase (*CDK*) gene *cyc*2; *os*-2 at 12, 24 and 96 h after the AOS treatment. In addition, the expression level of quiescent-center-specific homeobox (*QHB*) gene (a rice WUS-type homeobox gene), which is the marker gene of adventitious root primordia development, was also increased by 25.7% and 14.1% at 96 h and 120 h after the AOS treatment, which promoted the development of rice roots and facilitated the absorption of water and mineral nutrition.

3.2. Auxin signaling mediated AOS-induced root development in rice

To verify whether auxin was involved in root formation and development during AOS induction, auxin homeostasis in rice treated with AOS were investigated (Fig. 3). The results showed that AOS raised the expression level of auxin synthesis-related gene *OsYUCCA1* and *OsYUCCA5* by 15.0% and 26.3% respectively to accelerate auxin biosynthesis (Fig. 3a and b). However, IAA concentration in shoots was decreased by 9.9%, and its concentration in roots was increased by 37.8% after the AOS treatment (Fig. 3c), suggesting that AOS enhanced polar auxin transport in rice. Moreover, the activity of IAA oxidase in roots was reduced by 32.6% by AOS (Fig. 3d), which may contribute to the increases in IAA concentration in roots. These results indicated that AOS changed auxin homeostasis in rice; thereby it may regulate root development.

Many genes required in root development are reported to have connections with the auxin-signaling pathway, including the Aux/IAA family, the auxin response factor (*ARF*) gene family and certain hormone-related transporter (Liu et al., 2007; Woodward & Bartel, 2005; Xu, Zhu, Shou, & Wu, 2005). For example, *OsIAA11* gene, expressed in root tips, steles and LR caps, regulated LR development of rice by mediating auxin circulatory system (from base to tip and from top to base) (Jain et al., 2006; Zhu et al., 2012). *OsPIN1*, expressed in the vascular tissues and root primordial, played an important role in auxin-dependent adventitious root emergence (Xu et al., 2005). Therefore, the expression levels of several genes in auxin-signaling pathway were also analyzed in our experiments. These results showed that AOS raised the expression levels of *OsIAA11* and *OsPIN1* in rice roots to accelerate polar auxin transport (Fig. 4), thereby promoting the formation and development of rice roots, which further proved the above results of Fig. 3c. In addition, adventitious rootless1 (*ARL1*) gene, expressed in lateral and adventitious root primordial, tiller primordial, vascular tissues, scutellum and young pedicels, was involved in auxin-mediated cell dedifferentiation, and it promoted the initial cell division in the pericycle cells adjacent to the peripheral vascular cylinder in the stem (Liu et al., 2005). AOS induced the expression of *ARL1* gene in rice roots (Fig. 4), which also may be one of the reasons that AOS promoted the formation and development of rice roots. There are no obvious changes in the expression level of *ARF* gene in rice roots. These results indicated that auxin signaling mediated AOS-induced root development in rice.

To further verify the role of auxin in promoting root development by AOS, the related parameters of rice roots, treated with AOS after the treatment of auxin transport inhibitor TIBA, were analyzed (Fig. 5). Firstly, the role of TIBA in inhibiting polar auxin transport was validated, and it decreased IAA concentration ratio

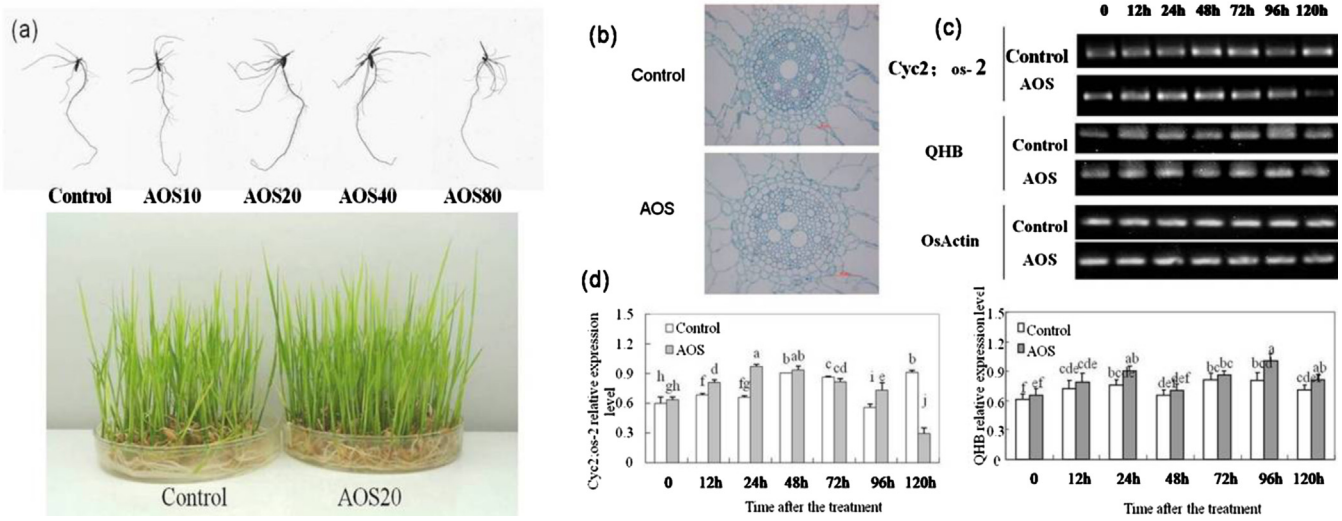

Fig. 2. Alginate oligosaccharides (AOS) promoted the root growth and development in rice. (a) Representative photographs of rice seedlings exposed to different concentration of AOS. Photographs of cross sections (b) and development-related genes expression (c) of rice roots treated with AOS (20 mg/L). Five-day-old rice seedlings were treated with AOS (10–80 mg/L) for 5 days. Experiments were repeated three times with similar results. Mean values ($n = 3$) with different lowercase letters are significantly different ($p < 0.05$) with Duncan's multiple range test. AOS, alginate oligosaccharides.

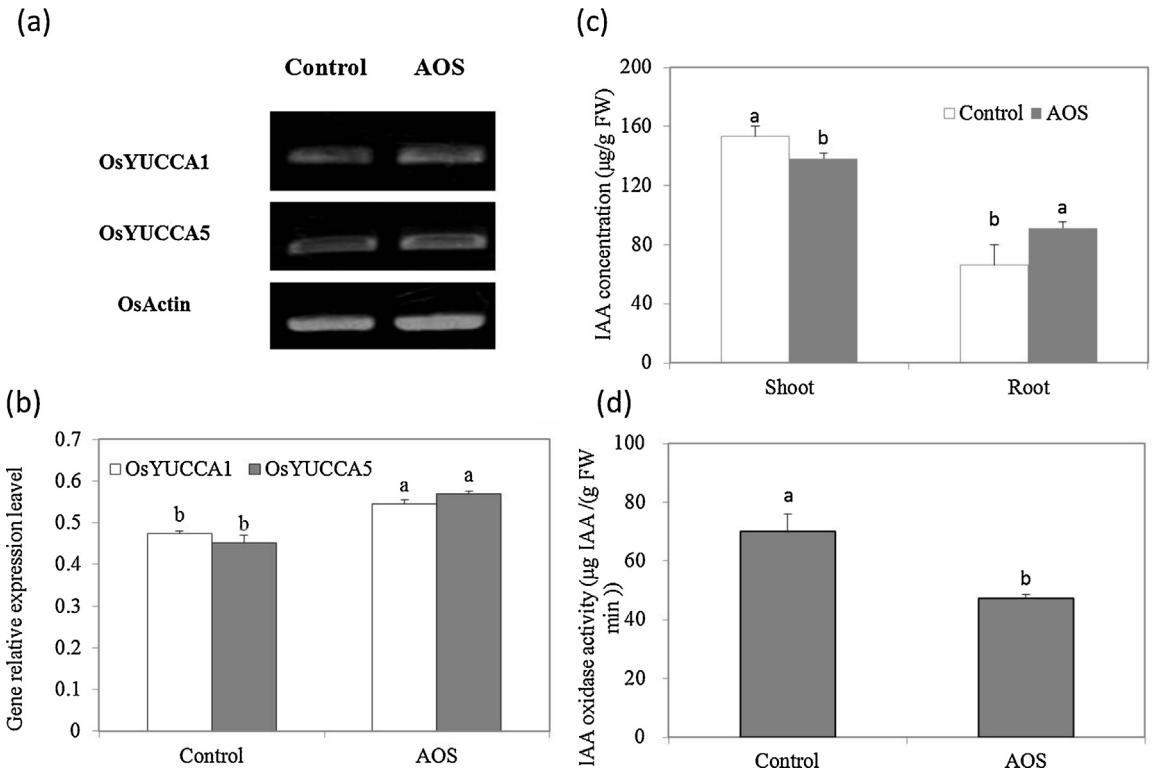

Fig. 3. Effects of alginate oligosaccharides (AOS) on auxin metabolism in rice. (a, b) The expression of auxin synthesis-related gene *OsYUCCA1* and *OsYUCCA5*; (c) Indoleacetic acid (IAA) concentration in rice; (d) IAA oxidase activity in rice roots. Five-day-old rice seedlings were treated with AOS (20 mg/L) for 5 days. Mean values ($n = 3$) with different lowercase letters are significantly different at $P < 0.05$. AOS, alginate oligosaccharides; IAA, indoleacetic acid.

of root and shoot compared to the control (Fig. 5b). Moreover, after the pretreatment of TIBA, the promotion effects of AOS on polar auxin transport and IAA concentration in roots were reduced significantly (Fig. 5a and b), and adventitious root numbers, shoot fresh weight and root fresh weight were decreased by 13.0%, 10.8% and 3.1% respectively, compared with those of the AOS treatment alone (Fig. 5c). These results further indicated that auxin signaling in roots was needed to promote root formation and development in rice induced by AOS.

3.3. *The role of calcium signaling in AOS-induced root development in rice mediated by auxin*

There are increasing evidences that auxin action is mediated by an intracellular change of calcium levels and that calcium acts as a second messenger during auxin-mediated cellular responses (Singla, Chugh, Khurana, & Khurana, 2006; Yang & Poovaiah, 2000). Therefore, we also studied the role of calcium signaling in AOS-induced root development in rice which was mediated by auxin (Fig. 6). The results showed that AOS induced Ca^{2+} signaling generation in rice roots, which had effects that were inhibited by adding the calcium-chelating agent, EGTA (Fig. 6a). Moreover, after the EGTA treatment, the up-regulation of AOS on auxin synthesis-related gene *OsYUCCA1* and *OsYUCCA5* were inhibited and the down-regulation of AOS on the activity of IAA oxidase was raised, which resulted in the decreases in IAA concentration in rice roots (Fig. 6b). However, the inhibitory and promoting effects of exogenously applied α-NAA on root elongation and adventitious root numbers had no obvious changes after the EGTA pretreatment (Fig. 6c). There results indicated that Ca^{2+} signaling might act mainly in the upstream of auxin in the regulation of AOS on rice root development. In addition, the applying of $CaCl_2$ to rice roots still increased the number of adventitious roots after the TIBA pretreatment, suggesting that Ca^{2+} signaling also might act as a downstream signal molecule of auxin in promoting the formation of adventitious roots in rice induced by AOS.

4. Discussion

The application of exogenous reagents to regulate plant root growth is an important agronomic measure in agriculture production. Although the positive effects of AOS on root induction and root elongation growth have been widely investigated, little is known about the mechanisms for regulating them. Root growth and development is a carefully orchestrated event regulated by both environmental and endogenous signals (Bengough & Mullins, 1990). Auxin plays a central role in regulating root growth (Woodward & Bartel, 2005). For example, the LR formation was induced by exogenous application of auxin or the over-expression of auxin synthesis genes (Muday & Haworth, 1993), but was inhibited in many mutants in the biosynthesis, transport and signal transduction of auxin (Fukaki, Nakao, Okushima, Theologis, & Tasaka, 2005). Moreover, NPA application in rice root collars (the junction between root and term) blocked the initiation and growth of adventitious and lateral roots in rice (Reed, Brady, & Muday, 1998; Zhou, Yin, Xu, & Xue, 2003). In this paper, our data demonstrated that AOS increased IAA concentration in rice roots (Fig. 3), thereby promoting the root formation and elongation (Table 2, Fig. 2), which had effects that were inhibited by auxin transport inhibitor TIBA (Fig. 5). These results indicated that auxin signals mediated AOS-induced root development in rice.

Proper output of auxin signaling depends on the interplay of the biosynthesis, transport, conjugation, and signaling of this class of small molecules (Sreevidya et al., 2010; Woodward & Bartel, 2005). Therefore, auxin homeostasis and its related transduction in rice treated with AOS were studied in our experiments (Figs. 3 and 4). The results showed that AOS induced the expression of *OsYUCCA1*

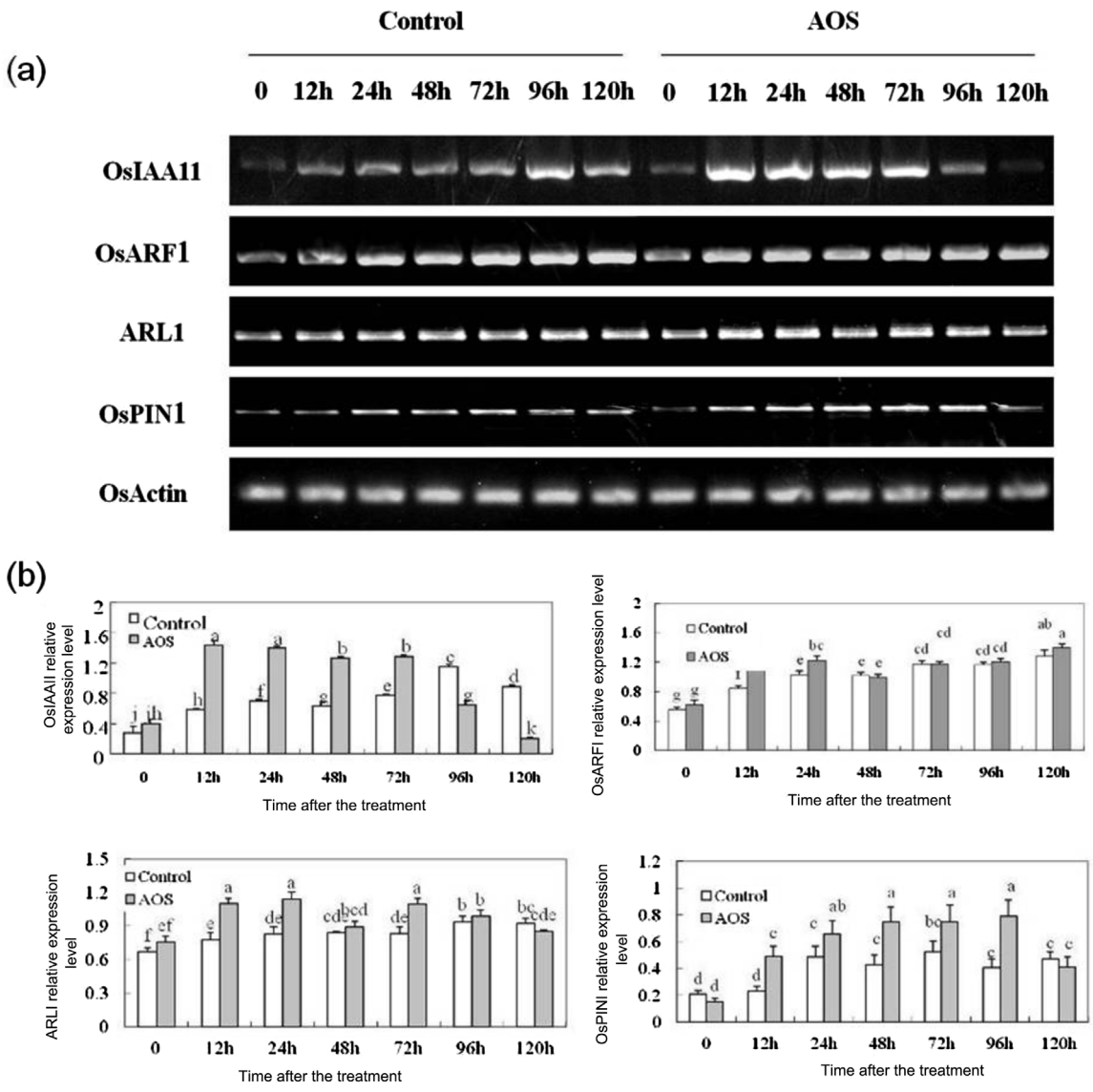

Fig. 4. Effects of alginate oligosaccharides (AOS) on the expression of the auxin-related genes in rice roots. Five-day-old rice seedlings were treated with AOS (20 mg/L) for 5 days. Mean values ($n = 3$) with different lowercase letters are significantly different at $P < 0.05$. AOS, alginate oligosaccharides.

and *OsYUCCA5* genes, a kind of the most important rate-limiting enzymes in Trp-dependent auxin synthesis in rice (Zhao et al., 2001), suggesting that AOS accelerate IAA biosynthesis in rice tissues. Moreover, polar auxin transport, which was accomplished by the auxin influx and efflux carrier proteins, was critical for the initiation and elongation of roots (Muday & DeLong, 2001). Our studies confirmed that AOS increased the expression level of *OsIAA11* and *OsPIN1* gene to promote the IAA transport to roots (Fig. 4). The activity of IAA oxidase in rice roots was also reduced after the AOS treatment (Fig. 3). These changes resulted in the increase in IAA concentration in rice roots (Fig. 3), thereby it may induce the expression of root development-related genes, finally promoting root growth (Table 2, Fig. 2). Some studies showed that the expression of *TaIAA1* gene in wheat is induced by exogenous auxin application. In our studies, the expression level of *OsIAA11* was increased significantly after the AOS treatment (Fig. 4), which may be due to the increase in

Table 2
Effects of alginate oligosaccharides (AOS) on the root morphology of rice. [a,b,c]

Treatment	Length of the longest root (cm)	Total root length (cm)	Root tips	The number of adventitious root	Average diameter of root (mm)	Root volume (cm^3)	Root surface area (cm^2)	Root fresh weight (mg/explant)
Control	9.86b	47.22b	97ab	9.00b	0.498b	0.105c	8.37b	74.12c
AOS10	11.03ab	60.62a	90b	9.67ab	0.547ab	0.138ab	10.09a	91.21ab
AOS20	10.80ab	61.38a	101a	10.40a	0.565a	0.151a	10.74a	96.34a
AOS40	11.13a	59.77a	103a	9.73ab	0.540ab	0.135b	10.05a	76.22c
AOS80	10.00ab	60.17a	85b	9.27b	0.544ab	0.136ab	9.92a	81.17bc

[a] Means within each column with different lowercase letters are significantly different ($P < 0.05$) with Duncan's multiple-range test.
[b] Data are presented as mean values ($n = 3$).
[c] AOS, alginate oligosaccharides.

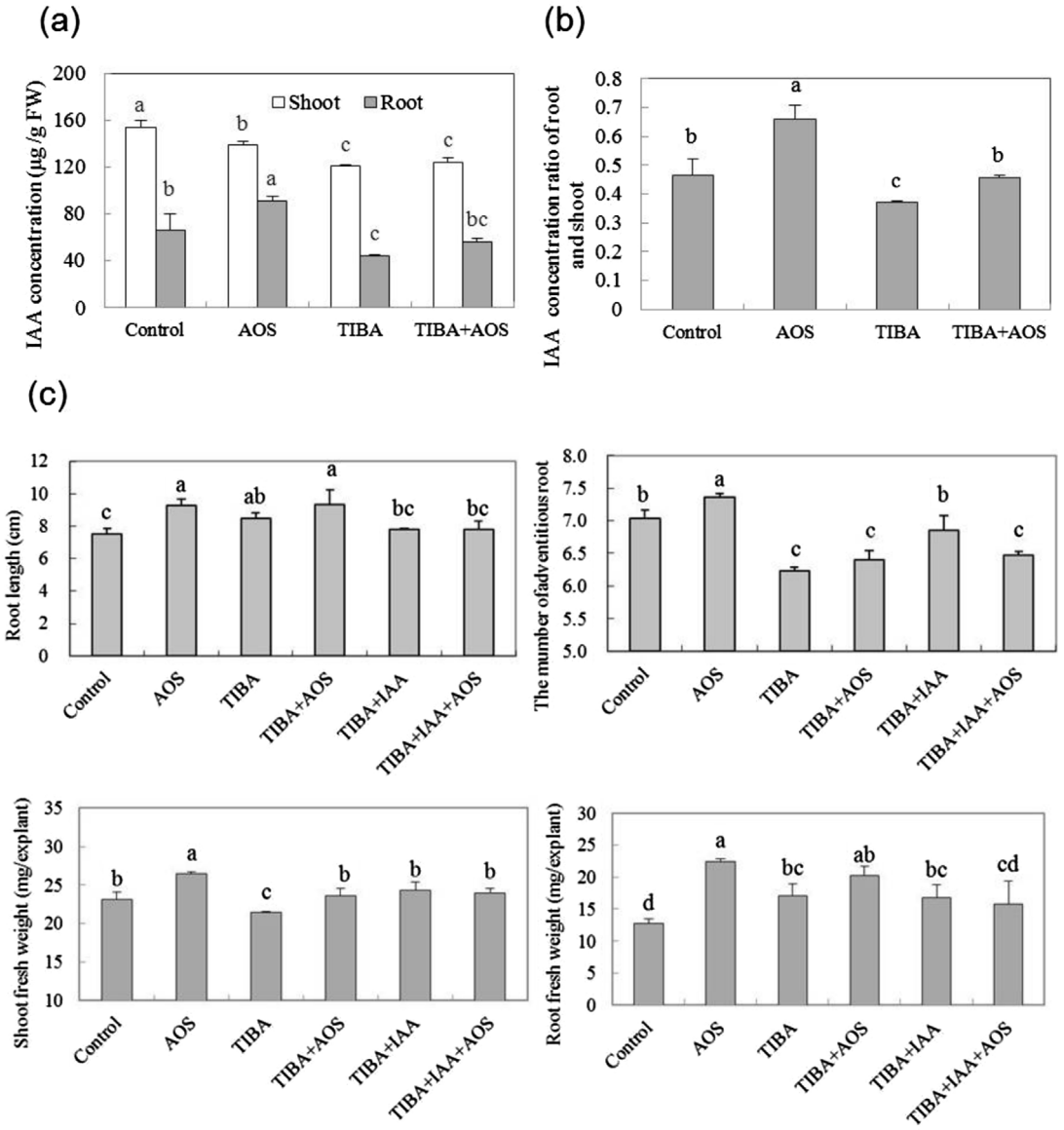

Fig. 5. Effects of auxin inhibitors 2,3,5-triiodo benzoic acid (TIBA) on root formation and growth of rice seedlings induced by alginate oligosaccharides (AOS). (a) Indoleacetic acid (IAA) concentration in rice. (b) IAA concentration ratio of root and shoot in rice. (c). The root morphology of rice. Five-day-old rice seedlings were pretreated with TIBA (1.0 μM) for 6 h and then treated with H_2O, AOS (20 mg/L), α-NAA (0.5 μM), AOS (20 mg/L) +α-NAA (0.5 μM) for 5 days. Means values ($n = 3$) with different lowercase letters are significantly different at $P < 0.05$. AOS, alginate oligosaccharides; α-NAA, α-napthylacetic acid; IAA, indoleacetic acid; TIBA, 2,3,5-triiodo benzoic acid.

IAA concentration in rice roots caused by AOS (Fig. 3). These results demonstrated that AOS changed auxin homeostasis in rice, and then activated auxin signaling to induce the development-related genes expression, resulting in the promotion of root development.

Although the effects of oligosaccharides on root induction and root elongation growth under the regulation of phytohormones have been investigated, the mode and site of their action remains unclear (Khan et al., 2011; Kollárová et al., 2010). An oligosaccharides receptor on plant cells has been proposed for studying the mechanisms (Ito, Kaku, & Shibuya, 1997; Wendehenne, Binet, Blein, Ricci, & Pugin, 1995), although no specific receptor for AOS has been presented. A calcium ions (Ca^{2+})-dependent configuration (known as the "egg-box") consisting of G-blocks in AOS was considered to be the active molecular species that initiated signal transduction pathways to activate several defence and developmental responses (Farmer, Moloshok, Saxton, & Ryan, 1991; Perić-Hassler & Hünenberger, 2010). Our recent studies confirmed that AOS caused the release of Ca^{2+} from extracellular and cytosolic sources to cytoplasm to activate nitrogen metabolism in flowering Chinese cabbage (Zhang et al., 2013a,b,c). The *TaIAA1* transcript levels in wheat was raised by applying Ca^{2+}, and this effect is reversed by the calcium-chelating agent, EGTA (Singla et al., 2006); Moreover, Ca^{2+} signaling was also reported to be a downstream messenger in the signaling pathway triggered by auxin to promote the formation of adventitious root and LR (Chen & Kao, 2012; Lanteri, Pagnussat, & Lamattina, 2006). Our data showed that AOS induced Ca^{2+} signaling generation in rice roots, and up-regulation of AOS on auxin synthesis-related gene was inhibited by the EGTA pretreatment. Moreover, exogenous application of $CaCl_2$ to roots still increased the number of adventitious roots after the TIBA pretreatment (Fig. 6c). These results suggested that Ca^{2+} acted both upstream and downstream of auxin in AOS-induced root development and formation in rice. In addition, NO mediates the auxin response leading to root formation through S-nitrosylation of the TIR1 auxin receptor (Terrile et al., 2012), accomplished through IP3-regulated Ca^{2+} channels in rice (Chen & Kao, 2012).

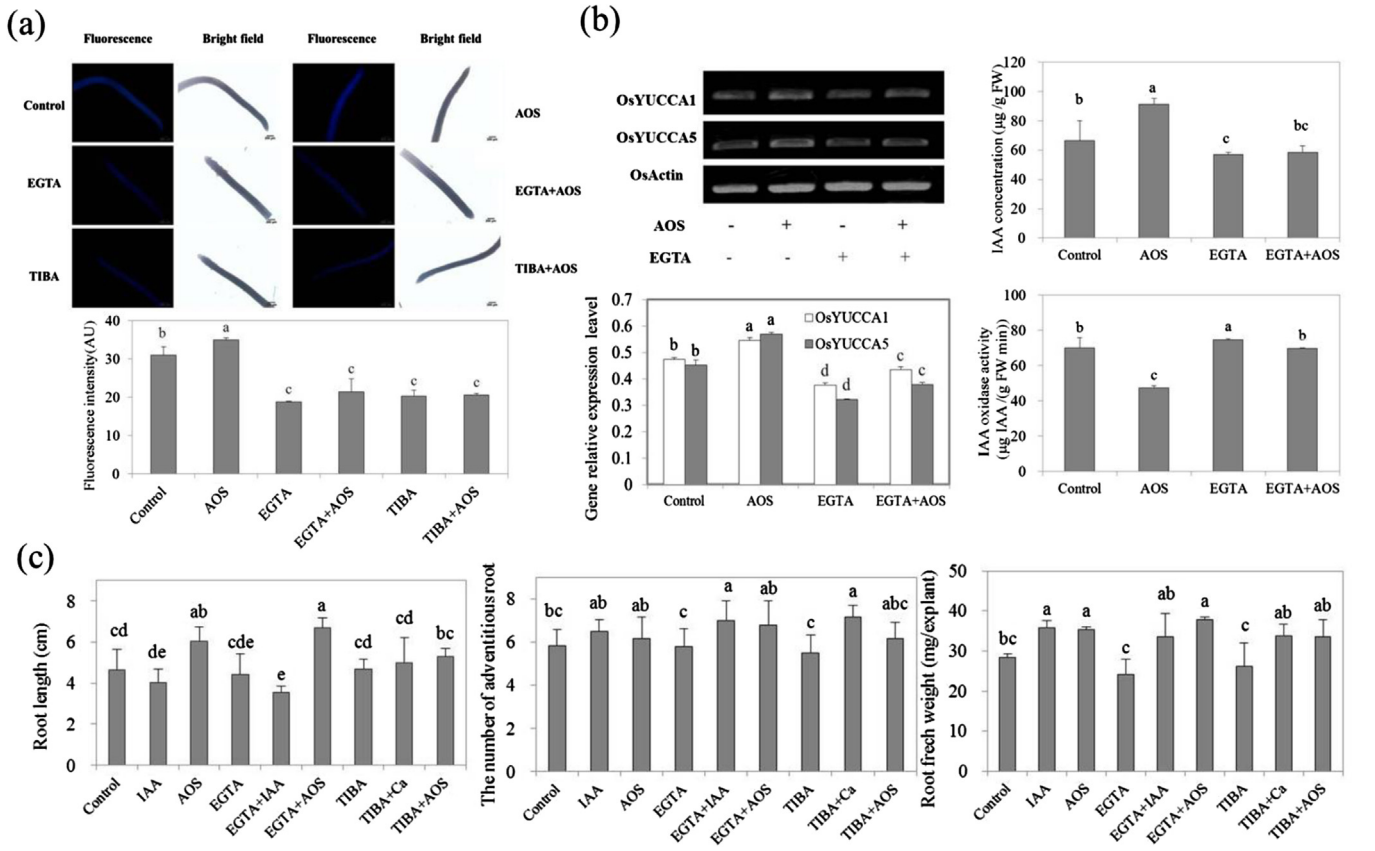

Fig. 6. The role of calcium signaling in alginate oligosaccharides (AOS)-induced root development in rice mediated by auxin. (a) Representative photographs of calcium signaling generation in rice roots (bar = 100 μm). Calcium signaling was visualized at approximately 5 mm from root tip. (b) The metabolic balance of auxin in rice. (c) The root morphology of rice. Five-day-old rice seedlings were pretreated with TIBA (1.0 μM) or EGTA (0.4 mM) for 6 h and then treated with H_2O, AOS (20 mg/L), α-NAA (0.5 μM), AOS (20 mg/L) or $CaCl_2$ (0.5 mM) for 5 days. Means with different letter are significantly different at $P < 0.05$. AOS, alginate oligosaccharides; α-NAA, α-napthylacetic acid; EGTA, ethylene glycol bis (2-aminoethyl) tetraacetic acid; TIBA, 2,3,5-triiodo benzoic acid.

NO also elevated cytosolic Ca^{2+} level by promoting Ca^{2+} release from intracellular of cADPR/ryanodine-sensitive Ca^{2+} channels in fava bean (*Vicia faba*) and tobacco cells (Garcia-Mata et al., 2003; Lamotte et al., 2004). Our recent studies showed that AOS induced NO generation in promoting root formation in wheat (*Triticum aestivum* L.) (Zhang et al., 2013a,b,c), which suggests that Ca^{2+} signaling may be a downstream messenger of NO in AOS-induced root formation mediated by auxin. However, there are still no definite and direct evidence to prove this so far. Moreover, it is necessary to study the clear roles of Ca^{2+} from extracellular and intracellular sources in AOS-induced root development mediated by auxin.

Based on the results obtained here, our data preliminary confirmed that auxin signaling was a novel mechanism by which AOS regulated plant root growth, and in which calcium signaling was involved . These results helps us to understand the mechanism by which AOS regulates root growth from the aspects of the sensation and transduction of AOS signaling in cells, and provides a theoretical basis for AOS application in agriculture production. However, not much is known about the complex molecular network operating during root development triggered by AOS. To obtain a more detailed picture of these processes, it is necessary to study the interaction of different signaling pathways induced by AOS using gene chip and laser scanning confocal microscope technique in a follow-up study. In addition, the structure of Ca-alginate in plants and the conditions under which it is formed are also needed to be observed, and the source of cytosolic Ca^{2+} induced by AOS in promoting root development should be investigated further.

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