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Research Article

Phosphorus-induced change in root hair growth is associated with IAA accumulation in walnut

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Abstract

Walnut, an important non-wood product forest tree, has free root hairs in orchards. Root hairs are specialized cells originating from the root epidermis that are regulated by plant hormones, such as auxins. This study was conducted to evaluate the effect and mechanism of phosphorus stress on root hair growth of walnut (*Juglans regia* L.) seedings by auxin (IAA) biosynthesis and transport. Both low phosphorus (LP) and no phosphorus stresses (NP) heavily decreased plant height, leaf number, total root length, root surface, shoot and root biomass, and root nutrient contents. The LP treatment significantly increased root hair growth, accompanied with up-regulation of the positive regulation root hair growth gene *JrCPC* and down-regulation of the negative regulation root hair growth gene *JrTTG1*, while the NP treatment had opposite effects. The root IAA level, IAAO activities, IAA transport genes (*JrAUX1*, *JrLAX1*, and *JrPIN1*), and the biosynthesis genes (*JrTAA1* and *JrTAR1*) were increased by the LP treatment, while the NP treatment decreased all of them. Interestingly, the auxin biosynthesis gene *CsYUCCA1* was not affected, which suggested that P mainly affects root hair growth of walnut by regulating auxin transport, and then affects root nutrient absorption and plant growth.

Keywords: IAA; phosphorus; root hair; walnut

Introduction

Plant roots have a high degree of phenotypic plasticity, and their morphology is affected by various factors, including soil nutrients, such as phosphorus (P) (Wu *et al.*, 2013). P is one of the necessary macro-nutrients for plant growth and metabolism, in photosynthesis, energy accumulation, and respiration (Balyan *et al.*, 2016). Approximately 80 % of soil P is in organic forms (Jungk, 2001), or is fixed by soil organic matter, causing P immobility and deficiency in plants and soils (Zheng *et al.*, 2015). So, P deficiency or stress is widespread in the plant kingdom (Bargaz *et al.*, 2013). Plants have evolved at least two pathways to respond to P-deficient stress, the direct uptake pathway from the rhizosphere by root epidermal cells and root hairs, and the indirect uptake pathway via fungi (Richardson *et al.*, 2009). Many studies indicated that phosphorus affects

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the root morphology of plants. In trifoliate orange and tea, P stress can improve root morphological traits, such as increased root hair growth (Wu *et al.*, 2016; Shao *et al.*, 2018).

Root hairs are highly specialized cells originating from the root epidermis that play a role in water and mineral nutrients absorption (Zhang *et al.*, 2018). As a general rule, root hair formation can be modulated by environmental signals, especially inorganic P (Liu *et al.*, 2018). In sand culture, P stress significantly increased the root hair diameter of trifoliate orange, while the root hair density showed an opposite effect (Liu *et al.*, 2018). However, the density of root hairs was greatly increased under P deficiency in *Arabidopsis* (Williamson *et al.*, 2001). Cao *et al.* (2013) reported that the deprivation of P highly improved initiation and elongation of root hairs. The effective use of P increased root hair density and elongation to alter the root configuration (López-Bucio *et al.*, 2002). A study conducted by Zhang *et al.* (2016) showed that low P activated auxin signals to initiate root hair formation. In auxin signaling mutants *axr1*, *axr2*, and *aux1* of *Arabidopsis*, a P deficiency can restore defective root hairs (Schmidt and Schikora, 2001). So, the substrate P level heavily affects root hair growth by auxin signaling.

Auxins are an important factor affecting the occurrence and growth of root hairs (Zhang *et al.*, 2016). Auxins do not directly affect the fate of root epidermal cell determination but play a role as an organizing center for environmental/hormonal signaling in root hair growth (Lee and Cho, 2008; 2013). Auxins are primarily synthesized in shoots and then transported via stem vascular tissues into the root hair zone through the cortex or vascular bundle (Rigas *et al.*, 2013). Auxin synthesis and transport are controlled by many genes. Tryptophan aminotransferase related (*TARs*), auxin permease (*AUX1*), and flavin monooxygenase-like enzymes (*YUCCAs*) are the key genes during IAA synthesis (Mano and Nemoto, 2012). Pin-formed (*PINs*) auxin exflux carriers and the AUXIN RESISTANT 1/LIKE AUX1 (*AUX1/LAX*) auxin influx carriers are indispensable for auxin transportation (Tromas and Perrot-Rechenmann, 2010). Auxin changes are highly related with root hair features.

Walnut (*Juglans regia* L), an important non-wood product forest tree, has few root hairs (Huang *et al.*, 2020; Vahdati *et al.*, 2021). As stated above, a low substrate P level stimulates root hair initiation and growth of walnut, while the effect of low P on root hair growth is not clear. Hence, the aim of this study was to investigate the response of P stress on root-hair growth under two different P stresses conditions. Root hair growth, auxin concentration, relative expression of root auxin synthesized, and transportation genes were determined.

Materials and Methods

Experimental design

This experiment was a completely randomized block design, consisting of 3 treatments: the control treatment (with 1.0 mmol dm^{-3} of P, CK), the low P stress treatment (with 0.1 mmol dm^{-3} of P, LP) (Huang *et al.*, 2020), and the no P stress treatment (with 0.0 mmol dm^{-3} of P, NP). Each of the 3 treatments was replicated 6 times, for a total of 18 pots.

Plant culture

Seeds of walnut (*Juglans regia* L., cv. 'Qingxiang') were provided by the Walnut Technology Promotion Center, Baokang, Hubei, China. Seeds were sterilized with 75 % alcohol for 10 min, rinsed six times with distilled water, and stored in autoclaved (0.11 MPa, 121 °C, 1.5 h) sand. The next year, the stored seeds were germinated in a growth chamber with a 28 °C/20 °C Day/night temperature and relative humidity of 80%. A month later, two-leaf-old seedings of the same size were transferred into plastic pots (15 cm in depth, 16 cm in mouth diameter, and 10 cm in bottom diameter) containing 2.1 kg of autoclaved sand. Subsequently, all pots were placed in a greenhouse from March 20 to June 20, 2020, where the photosynthetic photon flux density ranged from 550 to 900 μmol m^{-2} s^{-1}, 28 °C/20 °C Day/night temperature, and 60 -95% relative humidity.

Variable analysis

Root morphology was scanned with an Epson Perfection V700 Photo Dual Lens System (J221A, Indonesia) and analyzed by WinRHIZO software (Regent Instruments Inc, Canada) to obtain the total root length and root surface area. Plant height, leaf number, and shoot and root biomass were determined by a ruler and electronic scales.

To observe root hairs, sixty, one-cm-long fresh lateral root segments per treatment were pre-fixed for 24 h in 2.5% glutaraldehyde, eluted with increasing alcohol concentrations (from 30 -100%), and dried with critical-point drying (CPD) (Zhang *et al.*, 2018). Root hair images were obtained by a Scanning Electron Microscope (SEM, model JSM-6390LV, JEOL Co., Japan) at ×400 magnification. Root hair density, length, and diameter were determined using Image J software (National Institutes of Health, MD, USA; Zhang *et al.*, 2016).

Roots were oven-dried at 75 °C, ground into 0.5 mm powder, digested by H_2SO_4-H_2O_2, and measured by an Electrochemical Analyzer (Smartchem 200, Scientific Instruments Limited, USA) for N content and by an ICP Specmometers (IRIS Advantage, Thermo, USA) for P, K, Ca, Mg, and Fe content.

Root endogenous indole-3-acetic acid (IAA) was extracted according to the protocol of Liu *et al.* (2018) and determined by High Performance Liquid Chromatography (HPLC), in which the mobile phase was a mixture of methanol and ddH_2O (2/3, v:v). Ten mm^3 of the extracted solution was detected for 1 h under 254 nm at a 0.8 cm^3 min^{-1} flow rate, with a column temperature of 30 °C. Root indole-3-acetic acid oxidase (IAAO) activity was determined according to Liu *et al.* (2018) with a minor modification. A 0.2 g root sample was homogenized with phosphate buffer (pH 6.0), and the absorbance was detected under 530 nm after being colored by sulfuric acid-$FeCl_3$ mixed solutions.

Table 1. The specific primers of relevant genes designed for real time quantitative PCR amplification

Gene name	Sequence (5'-3')-forward	Sequence (5'-3')-reverse
JrCPC	AAGGCACGAGAAGCGATGTC	GATTCTCAAAACAACCA
JrTTG1	GATATTTTTATTGGAAGATC	CACTTTGGCCTAACCGAA
JrAUX1	TGTTATGATTATGTTTCAAG	ACATGAATTTGAAATCTT
JrLAX1	AACAAGCATGGAAAATTCAT	CGTTTTGGCGGCATTCTTGA
JrPIN1	TCGAACTCCGTACCTCCTC	ATATCGATCATAAGATCG
JrYUCCA1	GTCCTCCATCCAAACCATCT	GTCTATGGTTGTATAATTGA
JrTAA1	TGCTGATGAAGGGCATCGT	ATTCTGGGCAACCAGTGAT
JrTAR1	TTGCCAAGTGTCCAAAT	TGAACGATTTTCACTATAG
Housekeeping gene: 18S rRNA	GGTCAATCTTCTCGTTCCCTT	TCGCATTTCGCTACGTTCTT

Total root RNA was extracted from a 0.1 g fresh sample using the RNA prep pure plant kit DP441, TIANGEN Biotech Co. Ltd, China), and reverse transcription was done with the PrimescriptTM RT reagent kit with the gDNA Eraser (RR047A, TaKaRa Bio INC, Japan). All the steps followed the manufacturer's instructions. The specific primers (Table 1) of relevant genes for qRT-PCR analysis were designed using Primer Premier 5.0 software (Palo Alto, USA), according to the Walnut genome database (http://aegilops.wheat.ucdavis.edu/Walnut/data.php). A housekeeping gene, *JrGAPDH*, was used as an endogenous control along with the target genes. The qRT-PCR system was as follows: 10 mm^3 SYBR GREEN PCR Master Mix, 6.4 mm^3 ddH_2O, 2 mm^3 cDNA, 0.8 mm^3 forward primer, and 0.8 mm^3 reverse primer. The qRT-PCR was done on the Bio-rad CFX connect-time system under the following conditions: 95 °C for 30 s, 40 cycles with 95 °C for 5 s, 60 °C for 10 s, and 72 °C for 30 s. The relative expression of genes was calculated by the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen, (2001).

Statistical analysis

Statistical analyses were performed with *SAS, version 8.1*, and significant differences between treatments were compared by the Duncan's multiple range tests at the $p = 0.05$ level.

Results

Plant growth performance

As shown in Figure 1, low P and no P stresses significantly decreased plant growth compared to the control treatment. Compared with the control treatment (CK), low P (LP) and no P (NP) stresses decreased plant height 25% and 45%, leaf number 18% and 55%, shoot biomass 11% and 43%, and root biomass 25% and 37%, respectively (Table 2).

Table 2. Effects of phosphorus on plant growth performance of walnut (*Juglans regia*) seedlings grown in sand culture

Treatments	Plant height [cm]	Leaf number	Shoot biomass [g(f.m.) plant^{-1}]	Root biomass [g(f.m.) plant^{-1}]
CK	34.0±2.4a	22.33±1.75a	5.05±0.19a	3.47±0.14a
LP	25.5±1.2b	18.28±1.52b	4.50±0.11b	2.62±0.06b
NP	18.6±0.1c	10.02±0.81c	2.88±0.19c	2.17±0.14c

Note: Means ± SD ($n = 6$) followed by different letters within a column are significantly different at $P < 0.05$. CK-1.0 mmol dm^{-3}, LP-0.1 mmol dm^{-3}, NP-0.0 mmol dm^{-3}. The same below.

Figure 1. Whole root morphology in walnut (*Juglans regia*) seedlings grown in the three phosphorus treatment conditions

Note: CK-1.0 mmol dm^{-3}, LP-0.1 mmol dm^{-3}, NP-0.0 mmol dm^{-3}. The same below

Root morphology

As shown in Figure 1, LP and NP treatments significantly decreased root growth compared to the control treatment. Compared with CK, LP and no NP treatments decreased total root length 50% and 55%

and root surface area 46% and 47%, respectively (Table 3). With regard to root hairs features, LP treated roots displayed greater root hair growth while the NP treatment had few root hairs (Figure 2). Based on Table 3, the root hair length and number of the LP treatment (50.13 μm and 55.12×10^4 plant^{-1}) were significantly higher than CK and NP treatments (40.21 μm and 10.97×10^4 plant^{-1}; 40.56 μm and 5.53×10^4 plant^{-1}), respectively. However, there was no significant root hair diameter difference between the 3 treatments. What is noteworthy is that the NP treatment had fewer root hairs but similar length and diameter of root hairs compared with the CK treatment (Table 3).

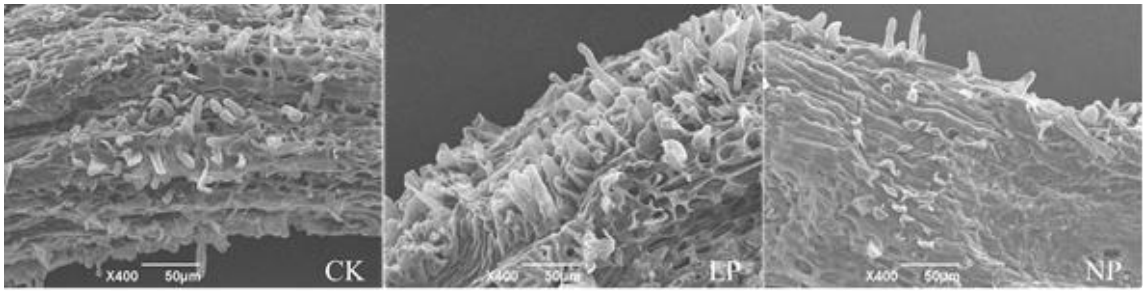

Figure 2. Whole root hair morphology in walnut (*Juglans regia*) seedlings grown in the three phosphorus treatment conditions

Table 3. Effects of phosphorus on root morphological traits of walnut (*Juglans regia*) seedlings grown in sand culture

Treatment	Total root length [cm]	Root surface area [cm^2]	Root hair growth		
			root hair length [μm]	root hair diameter [μm]	root hair number [$\times10^4$ plant^{-1}]
CK	155.95±11.28a	52.21±5.16a	40.21±3.23b	9.89±1.01a	10.97±1.01b
LP	77.61±7.15b	28.29±2.11b	50.13±3.11a	10.74±1.02a	55.12±3.13a
NP	70.30±6.83b	27.75±2.04b	40.56±2.01b	10.12±0.89a	5.53±1.12c

Root nutrient concentrations

Compared to the CK treatment, root N, P, K, Ca, Mg, and Fe contents were significantly decreased by 8.9, 51.7, 6.7, 2.9, 7.2 and 23.5% under the LP treatment, respectively (Table 4). So, the LP treatment significantly decreased root P and Fe contents, whereas no significant difference of root N, K, Ca, and Mg levels was observed between CK and LP treatments. Compared with the CK treatment, the NP treatment had no effect on root K and Ca content, but significantly reduced root N, P, Mg, and Fe contents by 14.8, 86.2, 15.4 and 29.1%, respectively (Table 4).

Table 4. Effects of phosphorus on root mineral nutrient contents of walnut (*Juglans regia*) seedlings grown in sand culture

Treatment	N	P	K	Ca	Mg	Fe
	[g kg^{-1}(d.m.)]					
CK	8.01±0.69a	0.87±0.04a	9.88±0.41a	7.95±0.29a	2.21±0.11a	2.89±0.25a
LP	7.30±0.22ab	0.42±0.02b	9.22±0.23a	7.72±0.31a	2.05±1.22ab	2.21±0.19b
NP	6.82±0.20b	0.12±0.01c	9.01±0.22a	7.56±0.29a	1.87±1.01b	2.05±0.14b

Root IAA concentration and IAAO activity

Compared with the CK treatment, the LP treatment dramatically increased root IAA concentration by 28.0%, whereas the NP treatment significantly decreased it by 20.1% (Figure 3). In addition, LP seedlings

possessed significantly higher root IAAO activity than CK seedlings (39.9%), but NP seedlings had dramatically decreased root IAAO activity by 19.8% (Figure 3).

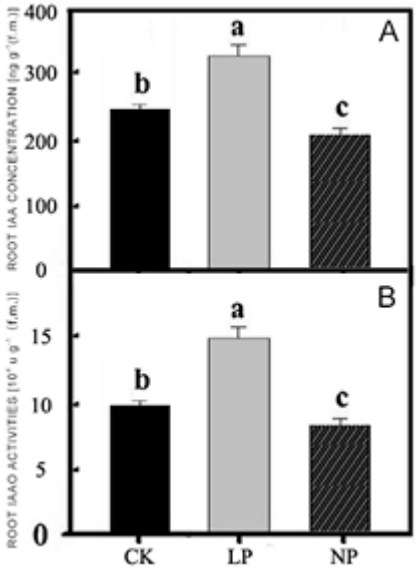

Figure 3. Effects of phosphorus on root IAA concentration (ng g^{-1}(f.m.): A) IAAO activity (10^4U g^{-1}(f.m.) B) walnut (*Juglans regia*) seedlings grown in sand culture

Relative expression of root hair responsive genes and root IAA-related genes

As shown in Figure 4, the LP treatment up-regulated the relative expression of the root hair responsive gene *JrCPC* by 2.6 folds whereas the NP treatment down-regulated it 28%, compared with the CK treatment. However, the LP treatment markedly decreased the root hair responsive gene *JrTTG1* expression level by 25%, whereas the NP treatment significantly increased it by 2.2 folds (Figure 4). Figure 5 shows the relative expression of root IAA-related genes. Compared with CK seedlings, LP seedlings had significantly higher expressions of J*rAUX1*, J*rLAX1*, J*rPIN1,* and J*rTAR1* whereas NP seedlings had markedly lower expressions of them (Figure 5). Interestingly, expression of J*rTAA1* in roots was remarkedly up-regulated only in the LP treatment (Figure 5). However, there was no significant change of expression of J*rYUCCA1* in the 3 treatments (Figure 5).

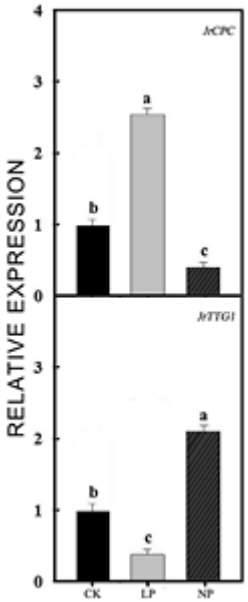

Figure 4. Effects of phosphorus on relative expression of root hair responsive genes in roots of walnut (*Juglans regia*) seedlings grown in sand culture

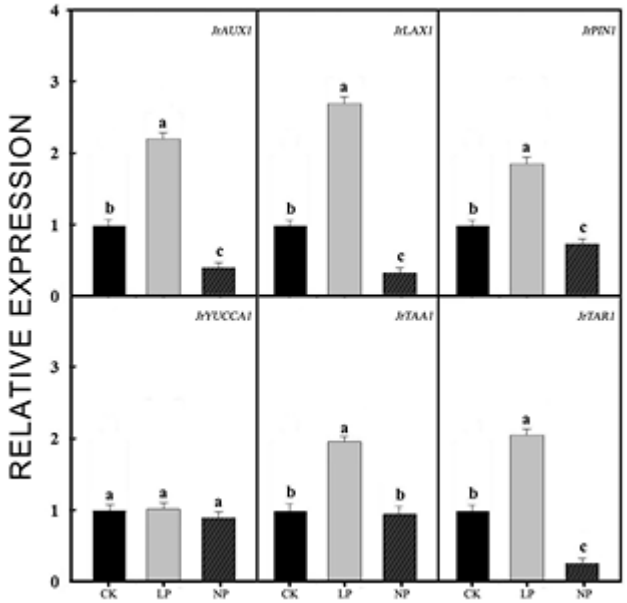

Figure 5. Effects of phosphorus on relative expression of IAA-related genes in roots of walnut (*Juglans regia*) seedlings grown in sand culture

Discussion

Phosphorus is considered to be one of the least available plant macronutrients in the soil. This mineral element plays an important role in plant growth and development and also is an essential part of nucleic acid synthesis, respiration, carbohydrate metabolism and nitrogen fixation, glycolysis, photosynthesis, signal transduction, energy transfer, membrane synthesis and stability, enzyme activation/inactivation, and redox reactions (Niu *et al.*, 2012, 2013; Kumar *et al.*, 2020). P stress has a significant limiting effect on crop yield and quality. It is estimated that 43 % of the world's arable land area is deficient in phosphorus, and 75 % of China's arable land is deficient (Li *et al.*, 2007). Earlier studies on tomato showed that the seedling growth under low phosphorus stress was severely inhibited (Jin *et al.*, 2021).

To adapt to P-deficient environments, plants increase the absorption of P from the soil through undergoing phenotypic changes in the root system architecture (RSA) such as favoring root hair growth through increased root hair density and length, and inducing lateral root growth (Williamson *et al.*, 2001; Vance *et al.*, 2003). In the present work, the low P and no P stress treatments significantly restrained the plant growth of walnut, compared with the control treatment, which was consistent with previous studies on citrus seedlings and walnuts (Salifu *et al.*, 2006; Wu *et al.*, 2015; Liu and Wu, 2017). With regards to root morphology, both low P and no P treatments decreased root biomass, total root length and root surface area, as similarly reported by Liu *et al.* (2018). However, the interesting point is that low P stimulated a large amount of root hair growth, but no P treatment inhibited root hair initiation and elongation. Studies demonstrated that root hairs in response to P deficiency were substantially longer than those in the presence of sufficient P levels (Williamson *et al.*, 2001; Zhu *et al.*, 2005; Cao *et al.*, 2013; Zhang *et al.*, 2016; Liu *et al.*, 2018; Huang *et al.*, 2020). In P-deficient soil, the length and density of root hairs significantly increase in *Arabidopsis*, with the root hairs constituting 91% of the total root's surface area (Bates and Lynch, 1996).

Root hairs can vastly facilitate the absorption of nutrients such N, P, K, Ca, Mg, and Fe from the soil. (Libault *et al.*, 2010; Wang *et al.*, 2016). In this work, low P resulted in denser and longer root hairs which increased the absorption of N, K, Ca, Mg, and Fe, suggesting that P plays a significant role in root hair growth and a moderate deficiency of P can help to absorb other nutrients (Narang *et al.*, 2000; Jungk, 2001; Huang *et al.*, 2018; Kohli *et al.*, 2020).

As can be seen from molecular biology, alterations of many genes are involved in root hair growth in walnuts. *CPC* (*CAPRICE*), the basic helix-loop-helix (bHLH) transcriptional gene, affects root epidermal cell conversion ion to root hairs (Savage *et al.*, 2013). The *cpc* mutant in *Arabidopsis* produces a reduced number of root hairs (Savage *et al.*, 2013). In this study, *JrCPC* was up-regulated in the low P treatment which had more root hairs, whereas it was down-regulated in the no P treatment with fewer root hairs. This result demonstrated that *CPC* is a positive regulator of root hair growth (Grierson *et al.*, 2015). The *TTG* (*TRANSPARENT TESTA GLABRA*) gene encodes a small protein with WD40 repeats, which is for the appropriate balance of target gene activation to achieve the proper pattern of root hair growth (Long and Schiefelbein, 2020). *ttg1* mutants can modify root hair pattern formation which results in explosive root hair growth in *Arabidopsis* (Long and Schiefelbein, 2020). Our study showed that the low P treatment markedly decreased the *JrTTG1* expression level whereas the no P treatment increased it significantly. This study implied that *TTG1* negatively regulates root hair formation and which is responsible for directing the fate of non-hair cells (Song *et al.*, 2011; Grierson *et al.*, 2015; Long and Schiefelbein, 2020).

Auxin has an important role in regulating root hair growth (Liu *et al.*, 2018). It was demonstrated that *AUXIN RESISTANT 1* (*AUX1*), *LIKE AUX1* (*LAX1*), *Pin-formed* (*PINs*), flavin-containing monooxygenase (*YUCCAs*), tryptophan aminotransferase of *Arabidopsis* (*TAAs*), and related genes of tryptophan aminotransferase (*TARs*) which regulate auxin transport in roots have a positive correlation with root hair growth (Rahman *et al.*, 2002; Ganguly *et al.*, 2010; Zhang *et al.*, 2018). *YUCCAs*, *TAAs*, and *TARs* are the key genes during IAA synthesis (Mano and Nemoto, 2012). *AUX1*, *LAX1,* and *PINs* are indispensable for auxin transportation (Tromas and Perrot-Rechenmann, 2010). In the present study, the low P treatment dramatically increased the root IAA concentration and had higher root IAAO activity, whereas the no P treatment significantly decreased it and had lower root IAAO activity. Furthermore, the low P treatment significantly up-regulated expression of root J*rAUX1*, J*rLAX1*, J*rPIN1,* and J*rTAR1,* whereas the no P treatment markedly down-regulated them. Interestingly, expression of J*rTAA1* in roots was remarkedly up-regulated only in the low P treatment. However, there was no significant change of expression of J*rYUCCA1* in the 3 treatments. Consequently, it was concluded that P regulates the root IAA level mainly by regulating IAA transport but not the biosynthesis pathway, which is consistent with previous studies (Liu *et al.*, 2018).

Conclusions

Phosphorus is essential to fruit trees for their development and growth, because of its important role in many metabolic processes of fruit trees. However, most part of phosphorus in soils of orchard is immobile and unavailable, and thus, many fruit trees often suffer from phosphorus stress which affects the biomass accumulation of fruit trees, the yield and quality of fruit. To adapt to phosphorus deficient soils, plants increase the absorption of phosphorus from the soil through undergoing phenotypic changes in the root system architecture (RSA) such as favoring root hair growth through increased root hair density and length. The aim of this study is to understand the effect and mechanism of phosphorus stress on root hair growth of walnut by auxin biosynthesis and transport. Sand culture experiment showed that both low phosphorus (LP) and no phosphorus stresses (NP) heavily decreased plant height, leaf number, total root length, root surface, shoot and root biomass, and root nutrient contents. The LP treatment significantly increased root hair growth, accompanied with up-regulation of the positive regulation root hair growth gene JrCPC and down-regulation of the negative regulation root hair growth gene JrTTG1, while the NP treatment had opposite effects. The root IAA level, IAAO activities, IAA transport genes (JrAUX1, JrLAX1, and JrPIN1), and the biosynthesis genes (JrTAA1 and JrTAR1) were increased by the LP treatment, while the NP treatment decreased all of them. Interestingly, the auxin biosynthesis gene CsYUCCA1 was not affected, which suggested that phenotypic mainly affects root hair growth of walnut by regulating auxin transport but not the biosynthesis pathway, and then affects root nutrient absorption and plant growth.

Authors' Contributions

Conceptualization: YJX and DJZ; Data curation: CYX; Formal analysis: XZD; Funding acquisition: YJX and XZD; Investigation: YJX and DJZ; Project administration: XZD; Supervision: YJX; Writing - original draft: YJX; Writing -review and editing: XZD and DJZ. All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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