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THE EFFECTS OF PHOSPHORUS DEFICIENCY ON THE MORPHO-PHYSIOLOGY AND EXPRESSION OF *LusWRKYs* IN *LINUM USITATISSIMUM* L.

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Abstract

Phosphorus (P) is a necessary factor for plant growth and development. It is easy to be combined by organic matter and minerals, which limits the effective utilization of plants. Flax is an important industrial crop that is often negatively impacted by P deficiency. Flax was used to explore adaptability of flax to P deficiency and mechanism of gene expression regulation. Flax seedlings were deal with four levels of P (0mM, 0.5mM, 1mM, and 2mM KH_2PO_4) for 15 days. Under P deficiency (0mM), biomass and shoot dry weight of flax decreased significantly, 36.36% and 61.11% lower than that of 1mM. P deficiency inhibited growth and development of flax. Under P deficiency (0mM), dry weight of root, root/shoot ratio, lateral roots number, length and surface area of total root were significantly increased, which increased accordingly by 75.00%, 29.73%, 14.29%, 28.66% and 33.33% compared with 1mM of P. P deficiency could induce root growth of flax to promote absorption of P. Contents of auxin (IAA), brassinolide (BR), ethylene (ETH) and acid phosphatase (ACP) of lateral roots were increased under P deficiency (0mM), which was accordingly by 48.29%, 61.11%, 58.26% and 55.88% higher than that of 1mM. Contents of gibberellin 3 (GA3), cytokinin (CTK) and abscisic acid (ABA) in lateral roots were decreased accordingly by 93.92%, 38.89% and 42.51% compared with 1mM P. Growth of lateral roots may be stimulated by regulating contents of hormones and acid phosphatase. Expression of genes *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* in lateral roots of flax seedlings was significantly increased under P deficiency (0mM), which indicated that these genes were involved in the regulation of P deficiency stress. This study comprehensively explained dependence of flax on P with regard to phenotype, physiology and molecular mechanism also provided ideas for efficient cultivation of flax and genetic improvement of P related traits.

Key words: *Linum usitatissimum*, Phosphorus deficiency, Gene expression, *LusWRKYs*.

Introduction

Phosphorus (P) is an essential mineral nutrient for plant growth and development. Lots of soil types are rich in P, but P is easy to be combined by organic matter and minerals (Fink *et al*., 2016). Plants could respond to P stress and use their own regulation to produce some tolerance responses in morphology and physiology. The response of plants to P stress could be divided into morphological and physiological aspects. Morphological changes help plants to absorb and store nutrients under nutrient stress. Physiological response help plants in improving the availability of nutrients in terms of biomass building, and change the adaptation and tolerance of plants to nutrient stress. Reducing of growth rate reduces the nutrient demand of plants, which is conducive to survival in the environment of nutrient deficiency. It is a regulatory mechanism for plants to adapt to nutrient deficiency. With the increase of nutrient supply, plant growth is significantly promoted. Plant growth is inhibited when the nutrient supply is excessive (Chapin, 1980).

Phosphorus is an inorganic element, necessary for plant growth. It participates in the metabolism of plants in many forms, such as photosynthesis, respiration, nucleic acid synthesis and membrane lipid synthesis (Theodorou *et al*., 1993; Buchanan *et al*., 2000). Growth rate and root shoot ratio of plants changed significantly under P stress (Cakmak *et al*., 1994; Eircsson *et al*., 1996). Aboveground / underground biomass ratio of *Picea asperata* Mast seedlings with normal P supply was twice as much as that under P deficiency, and almost there was no shoot growth under P deficiency (Proe *et al*., 1995). Growth of main roots was inhibited by P deficiency. Length and number of lateral roots, length and density of root hairs were increased under P deficiency (Willamson *et al*., 2001; Sanchez-Calderon *et al*., 2005). Special cluster roots were produced in some legumes (Gilbert *et al*., 2000). Morphological characteristics of roots of *Lupinus albus* were significantly changed under P deficiency, such as length of main roots was shortened, density and number of primary lateral roots were increased, formation of cluster roots was enhanced (Tang *et al*., 2013). P is a significant signal of root development after embryo, root hair extension in *Arabidopsis thaliana* was increased by P deficiency (Sanchez-Calderon *et al*., 2006). However, research on flax low P is limited.

Transcription factors (TFs) play a significant role in coping with P stress by regulating lots of genes participated in various metabolic pathways for maintaining P equilibrium of plants (Valdes-Lopez *et al*., 2008). WRKY (The name of WRKY comes from an important feature of its protein, that is, about 60 highly conserved WRKY domains.) TFs are involved in regulating all sorts of plant progress, for instance, biotic and abiotic stress, senility, germination of seed and others (Rushton *et al*., 2010; Chen *et al*., 2018). Lots of *WRKYs* were verified for involving in responding under low P, for instance, *OsWRKY74* (Dai *et al*., 2016), *AtWRKY75*

(Devaiah *et al*., 2007) and *AtWRKY6* (Chen *et al*., 2009). Tolerance to P deficiency was significantly enhanced by overexpression of *OsWRKY74*, while the transgenic lines down regulated by *OsWRKY74* were sensitive to low P (Dai *et al*., 2016). *AtWRKY75* is a positive TF of low P response *Arabidopsis thaliana*, inhibition of *AtWRKY75* causes damage of low P responding (Devaiah *et al*., 2007). *AtWRKY6* is negative regulation P deficiency response, *AtWRKY6* overexpression line is insensitive for P (Chen *et al*., 2009). At present, the special role and mechanism of WRKY in P deficiency stress are mainly reflected in *A. thaliana* and *Oryza sativa* research, while related research on flax is limited.

Flax is a very important industrial crop in the world. In recent years, flax industry is short of raw materials, the price is on the rise and market prospect is good. Flax growth period (75-85 days) is short. P deficiency is the limiting factor of flax growth and yield. At present, the research on P in flax is mainly focused on the effect of different fertilization levels and measures on yield (Wang *et al*., 2003; Zhu *et al*., 2006; Zhu *et al*., 2012). But these studies were mostly limited to field fertilization and yield measurement. The research on the physiology and potential molecular mechanism of flax under low P stress is very limited. Sofie is an excellent fiber flax variety introduced from Holland in 2015. Compared with other domestic and international varieties, Sofie has the advantages of high total fiber rate, good fiber quality, high raw stem yield, high fiber yield, strong disease resistance and early maturity. Transcriptome under potassium stress of Sofie has been studied (Huang *et al*., 2019). In this study, root growth indexes and hormone changes of Sofie under P deficiency were studied. It was clear that acceleration of lateral root growth was the basic physiological mechanism of flax response to low P stress. Hormone participated in reaction of flax to P deficiency. Four *LusWRKYs* genes were found in the WRKY TF gene family by analyzing expression of P deficiency gene, which might provide gene resources for molecular breeding. This study may provide a theoretical basis for the breeding of flax varieties with low P tolerance.

Materials and Methods

Plant materials: Flax variety Sofie was provided by the flax branch of the Chinese Flax Germplasm Improvement Center.

qRT-PCR primers: 21 *LusWRKYs* genes were annotated by TFs from different genes of flax transcription group. Among 32 members of WRKY TF family, 21 *LusWRKYs* genes were selected. Primers were designed according to 21 *LusWRKYs* genes sequences. Internal reference was *LusActin*.

Hydroponics conditions: Sofie seeds were seeded in a paper cup containing vermiculite (which had been sterilized for 20 minutes at 121℃), paper cup was placed on a plastic tray, each tray was poured with equal volume of water, each paper cup was seeded with 30 Sofie seeds. Seeded Sofie was placed in a plant incubator with 16 hours of light, 8 hours of darkness, 25℃ temperature and 70% relative humidity. After seedling emergence, then the same size Sofie plants were selected and transferred into plastic bottles. Pouring 1/2 of modified Hoagland medium until treatment. Sofie seedlings transferred to a tissue culture bottle containing 200mL 1/2 of modified Hoagland medium for 10 days when growing to 3 pairs of leaves (3-4cm). There were ten flax seedlings in every bottle. Medium was changed every 3 days. Hoagland medium includes 2mM KCl, 1mM KH_2PO_4, 5mM KNO_3, 4mM $Ca(NO_3)_2$, 1mM NH_4NO_3, 2mM $MgSO_4$, 0.1mM $FeSO_4$, 0.1mM EDTA-2Na, 0.1mM H_3BO_3, 0.13mM $MnSO_4$, 0.03mM $ZnSO_4$, 1μM Na_2MoO_4 and 0.1μM $CuSO_4$. After 10 days of growth, seedlings were transferred to the Hoagland medium containing 4 P levels of KH_2PO_4 (0, 0.5, 1 and 2mM) and KCl (3, 2.5, 2 and 1mM). Potassium (K) concentration was balanced by KCl. The medium was changed after every 3 days. Each treatment was repeated three times and air pump was used for ventilation. After 15 days of treatment, biomass, root and shoot dry weight, root/shoot ratio, lateral roots number, length and diameter of main root, total root length, IAA, BR, ETH, GA3, CTK, ABA and ACP were measured. After treatment, samples were taken at 0h, 6h, 12h, 24h, 2d, 4d, 6d, 8d, 10d and 15d. All samples were frozen at once with liquid nitrogen and then stored at -80℃ in order to extract total RNA. 21 *LusWRKYs* were tested by qRT-PCR. During the experiment, each sample is a cup, i.e., 10 Sofie plants.

Determination of root/shoot ratio: The seedlings were treated at 105℃ for 10 minutes, then 70℃ until constant weight. Sartorius BSA224s analytical balance was used to weigh dry weight of root and shoot. Biomass dry weight ratio between above- and below-ground was described for root/shoot ratio.

Determination of root parameters: Number of lateral roots of Sofie seedlings was observed manually. Length of main root of seedlings was measured with ruler (accuracy 1mm). Diameter, total root length and total root surface area were scanned with plant image analyzer instrument (Wanshen Series).

Determination of endogenous hormones contents: Contents of IAA, BR, GA3, ETH, CK, ABA and ACP in flax lateral roots were determined by ELISA Kit (Shanghai enzyme linked biotechnology limited company) on the basis of product specification.

qRT-PCR: Expression of 21 *LusWRKYs* was determined in Sofie lateral roots. Lateral roots of seedlings were used for extracting total RNA by Trizol (Thermo Fishe). First chain of cDNA was reversely transcripted by RT-PCR Kit (Takara). Real-time RT-PCR was fulfilled with SYBR® Premix Ex TaqTM II kit (Takara) in ABI7500 Real-time PCR instrument (Thermo Fishe). The list of sequences of primers used in qRT-PCR is presented in Table 1. Polymerase Chain Reaction (PCR) procedure: 94℃ 3min. 94℃ 15s, 55℃ 15s, 72℃ 45s, 40 cycles. Extending at 72℃ for 2 min. Relative expression level of objective gene was computed through $2^{-\Delta\Delta Ct}$. Primer Premier 5.0 software was used to design primers.

Table 1. Sequences of primers used in qRT-PCR.

Gene	Forward primers 5'-3'	Reverse primers 5'-3'
LusWRKY1	GGATGAGATTGATGAGGATGAACCT	CTACTCTAATCACTCCTACTTGGAC
LusWRKY2	CGACGATTCTAGGTGACGACATG	GAGCTGCTAAGATCCACTGCTGA
LusWRKY3	GAGTCCTGGTCCGATGACTCTGG	CTCAGGACCAGGCTACTGAGACC
LusWRKY7	TCTTCTTCCGCCGCCGTTGATGC	CGGCAACTACGGTTAGGACTGT
LusWRKY10	CTCCTTACCCGAGGAGTTATTACC	GAGCAATGGGCTCCTCAATAATGG
LusWRKY11	CAGCAACTCAAGGCTTGAAGAG	GTCGTTGAGTTCCGAACTTCTCG
LusWRKY13	GAGAGCCGAGGTTCAGTTTCAAGCA	CTCTCGGCTCCAAGTCAAAGTTC
LusWRKY15	GACTCCTCTCCCAACACCAGAC	CTGAGGAGAGGGTTGTGGTCTGT
LusWRKY20	GTGCTGCTTCTGGCGGAGCTA	CACGACGAAGACCGCCTCGATC
LusWRKY22	CTTCCAATCTCATCTGTCATGCA	GAAGGAAGGTTAGAGTAGACAGT
LusWRKY27	CGAACCCGACCCGTTTGCCTT	TGTTCGGAAAGAGCAGGCCAGTT
LusWRKY28	GCGTTCATGACGAAAAGCGAGGT	CCTTCTACCCATAGCTACCTCCT
LusWRKY32	GGAGACGCGTACGGAGGCTGGA	CCTCTGCGCATGCCTCCGACCTA
LusWRKY33	GCCTGATTCTCACCGCCACCGA	CGCCGAATAGGCTAGCTTAGCGAC
LusWRKY38	CTTCAGGTGCACCCATCAGAAGT	GAAGTCCACGTGGGTAGTCTTCA
LusWRKY39	GTTGGTCGTGGCAGATTCAG	CAACCAGCACCGTCTAAGTCT
LusWRKY40	TCTCCCTGTCAACAACAACAATG	ACCGCGGTTACCTCCAAGACTCT
LusWRKY48	CGATACTCCGATGATGACTCCG	GCTATGAGGCTACTACTGAGGCCG
LusWRKY49	CAAGACACACTAATCATCACCT	GTTCTGTGTGATTAGTAGTGGATGC
LusWRKY57	CACCACGACAACTTCAATCTCATC	GTGGTGCTGTTGAAGTTAGAGTAG
LusWRKY71	TCACTACTCCTCCTCCTGATTA	CTAATATCATTAATATTACCAGC
LusActin	GGTGTTATGGTTGGAATGGGTC	CCTCAGTGAGAAGTACAGGGTG

Statistical analysis

All the tests were repeated for three times. Data was described by mean ± SD. SPSS19.0 was used for statistical analysis. Single factor analysis of variance was used for comparison among groups. $p<0.05$ was significant difference.

Results

Influence of low P stress on growth and development of flax plants: Firstly, effects of P stress on biomass, root and shoot dry weight, root/shoot ratio of flax seedlings were analysed. (Fig. 1A), biomass dry weight of flax seedlings increased with increasing of P level and reached the highest value at 1mM P ($p<0.05$). Under P deficiency (0mM), biomass dry weight of flax seedlings decreased was by 36.36% compared with 1mM P ($p<0.05$). At the same time, shoot dry weight of flax seedlings was increased with enhancing of P level, while root dry weight was decreased with enhancing of P level, and peak value of both was 1mM P ($p<0.05$). Compared with 1mM P, shoot dry weight of flax seedlings under P deficiency (0mM) was decreased by 61.11%, while root dry weight was increased by 75.00% ($p<0.05$) (Fig. 1B and C). In addition, root/shoot ratio of flax seedlings was the highest under P deficiency (0mM) and decreased with enhancing of P level until 1mM P reached the lowest value ($p<0.05$). Root/shoot ratio of flax seedlings was increased by 29.73% ($p<0.05$) under P deficiency stress (0mM). Compared with 1mM P, the dry biomass of Sofie seedlings was decreased by 9.09% ($p<0.05$) at 2mM P (Fig. 1A). Compared with 1 mM P, the shoot dry weight of Sofie seedlings was decreased by 16.67% and the root dry weight increased by 25.00% ($p<0.05$) under 2mM P (Fig. 1B and C). In addition, the root/shoot ratio of Sofie seedlings was increased by 8.89% ($p<0.05$) compared with 1mM P at 2mM P (Fig. 1D).

Influence of low P stress on flax root growth: Because of influence of P stress on growth and development of flax seedlings, influence of different levels of P on flax root growth was further studied. (Fig. 2A), number of lateral roots was decreased dramatically with enhancing of P level and reached the lowest value at 2mM ($p<0.05$). Under P deficiency (0mM), number of lateral roots of flax seedlings was by 14.29% higher than that of 1mM P ($p<0.05$). At the same time, length and diameter of main root in flax seedlings raised with increase of P concentration until 1mM P reached the highest value ($p<0.05$). Under P deficiency (0mM), length and diameter of main root in flax seedlings were decreased by 17.26% and 22.22% respectively compared with 1mM P ($p<0.05$) (Fig. 2B and C). In addition, total root length and surface area was decreased remarkably with the increase of P concentration and reached the lowest value at 1mM P ($p<0.05$). Under P deficiency (0mM), total root length and total root surface area of flax were increased by 28.66% and 33.33% respectively compared with 1mM P ($p<0.05$) (Fig. 2D and E). Under the condition of 2mM P, the number of lateral roots of Sofie seedlings was decreased by 50.00% compared with 1mM P ($p<0.05$) (Fig. 2A). Under the condition of 2mM P, the main root length and diameter of Sofie seedlings were decreased by 4.21% and 16.67% ($p<0.05$) compared with 1mM P (Fig. 2B and C). In addition, the total root length and total root surface area of Sofie seedlings was increased by 16.59% and 12.78% respectively ($p<0.05$) compared with 1mM P (Fig. 2D and E).

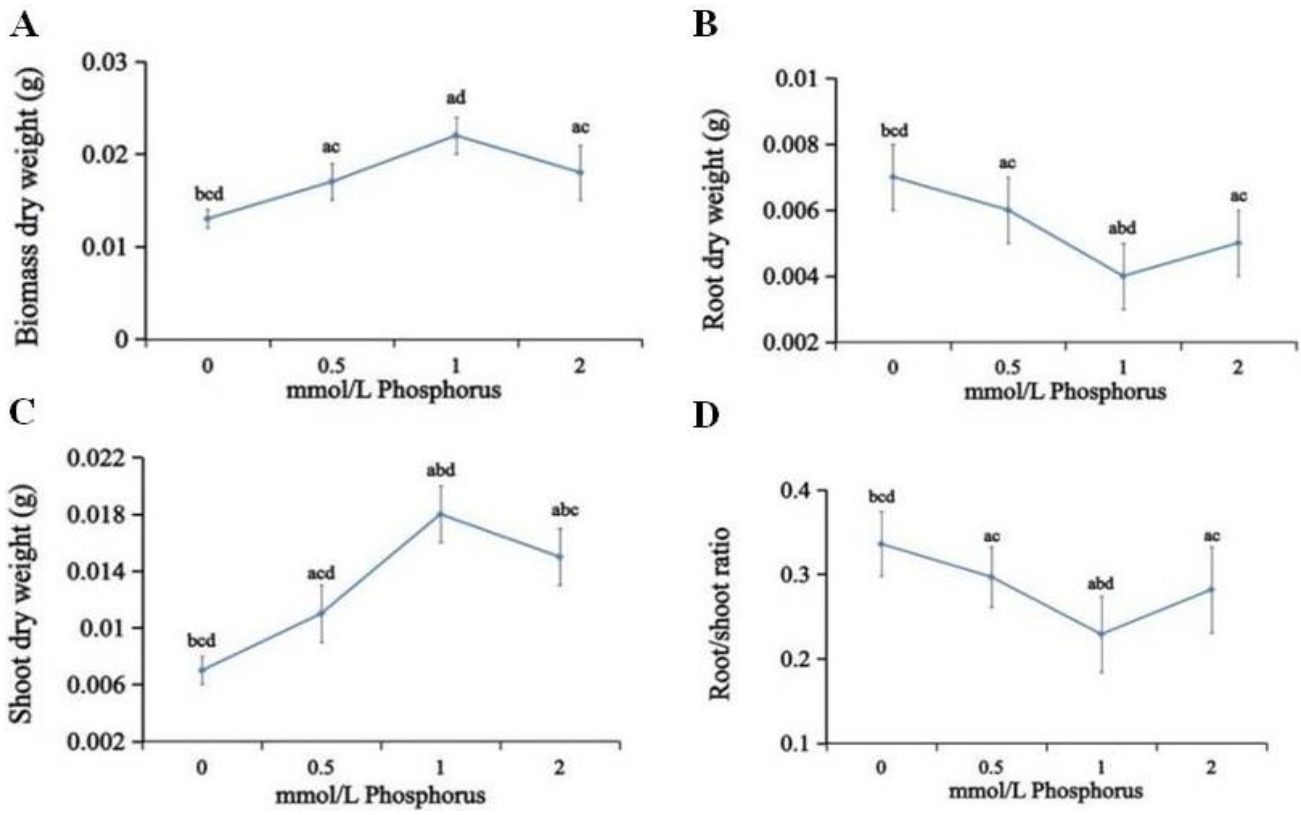

Fig. 1. Flax Sofie biomass and root/shoot ratio under different P concentrations
A) Biomass dry weight (g/plant) B) Root dry weight (g/plant) C) Shoot weight (g/plant) D) Root/shoot ratio
Note: a, b, c, and d represented significantly different at $p<0.05$ when compared with 0, 0.5, 1, and 2 mM, respectively

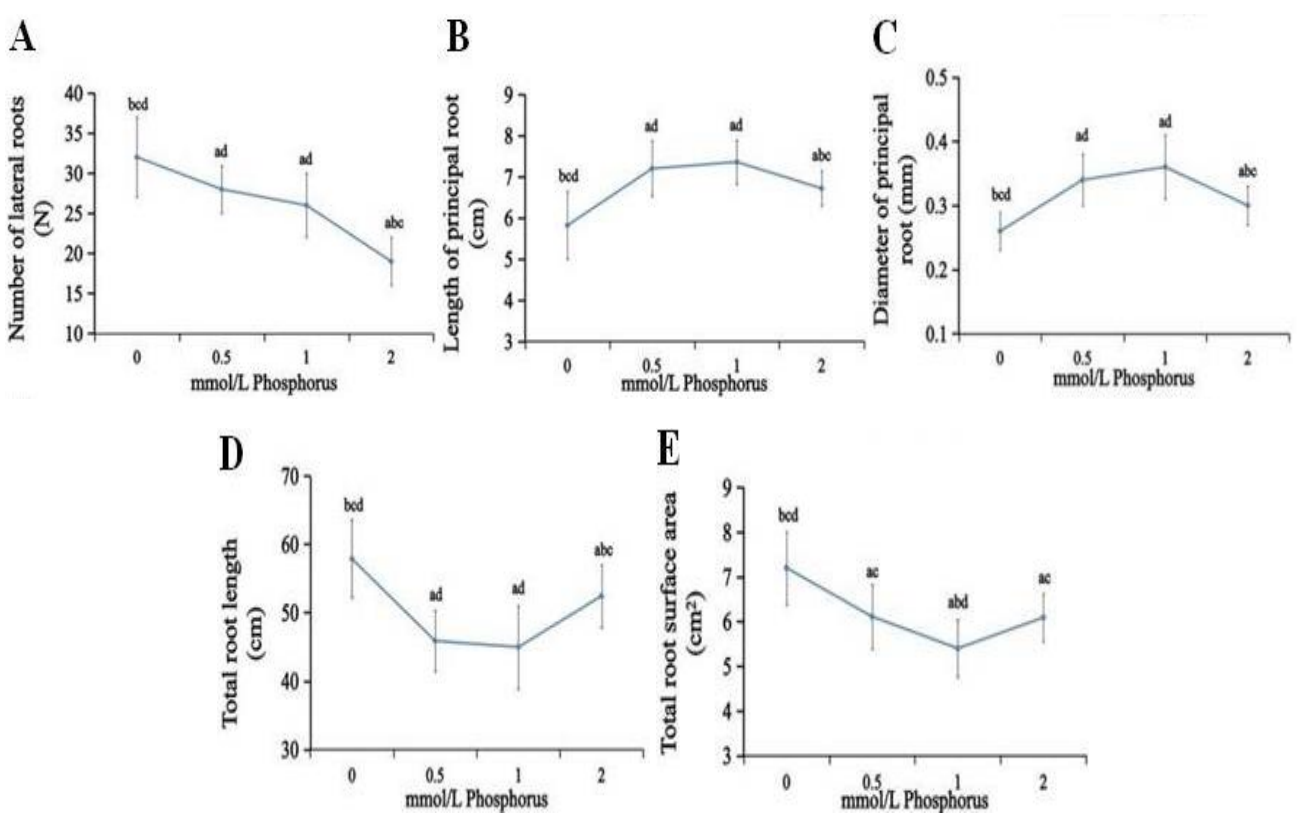

Fig. 2. Root of flax Sofie seedlings under different P concentrations
A) Number of lateral roots (Numbers) B) Length principal roots (cm) C) Diameter of principal root (mm) D) Total root length (cm) E) Total root surface area (cm^2)
Note: Same as Fig. 1.

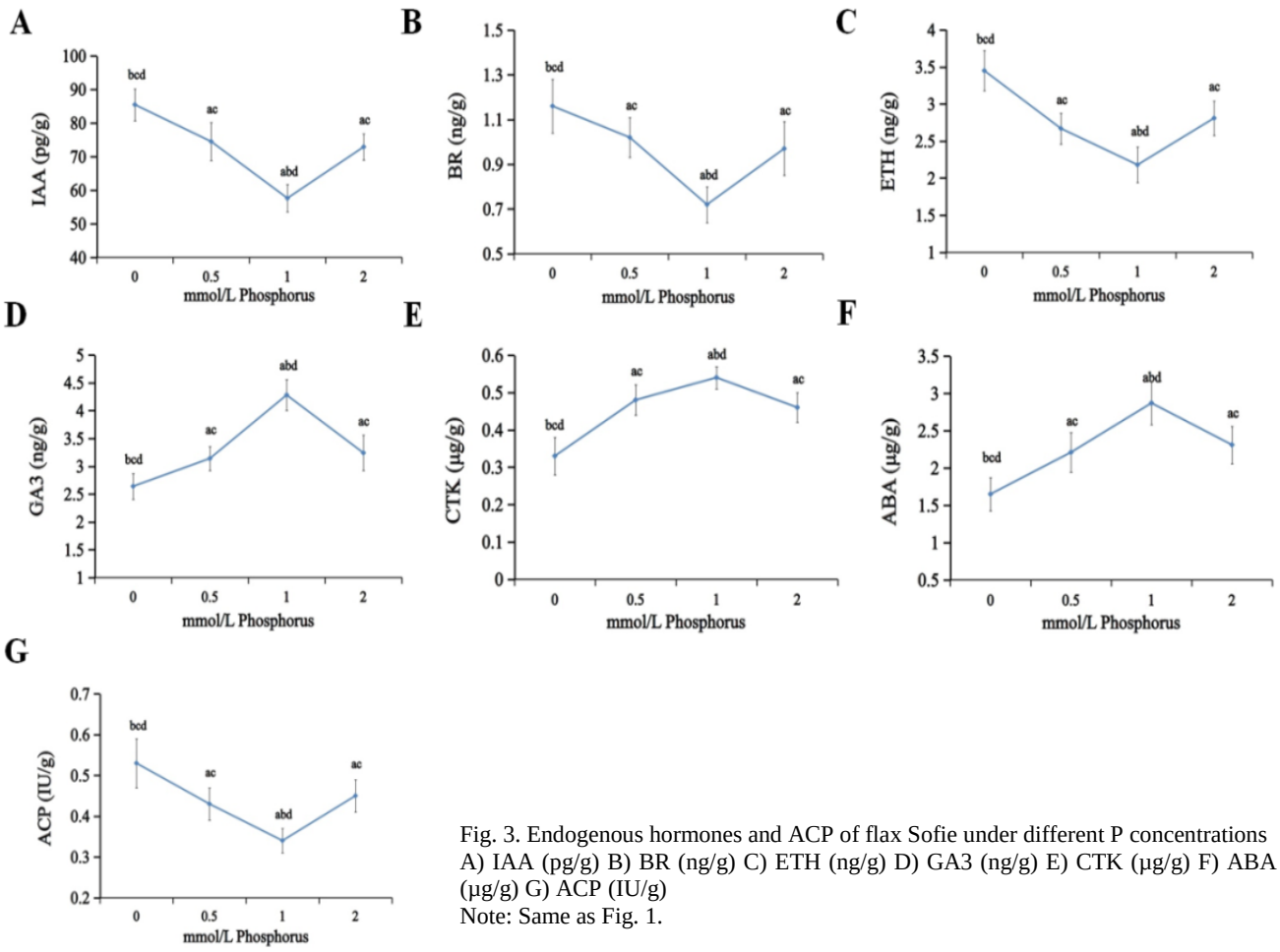

Fig. 3. Endogenous hormones and ACP of flax Sofie under different P concentrations A) IAA (pg/g) B) BR (ng/g) C) ETH (ng/g) D) GA3 (ng/g) E) CTK (μg/g) F) ABA (μg/g) G) ACP (IU/g)
Note: Same as Fig. 1.

Influence of low P stress on contents of endogenous hormones and ACP of flax lateral roots: For revealing basic physiological mechanism of low P in flax lateral roots, contents of endogenous hormones and ACP were evaluated. Contents of IAA, BR and ETH were decreased dramatically with enhancing of P level and reaching the lowest value at 1mM P (p<0.05) (Figs. 3A, 3B and 3C). Contents of IAA, BR and ETH in flax seedlings were the highest under P deficiency (0mM), which were 48.29%, 61.11% and 58.26% higher than that of 1mM P (p<0.05). On the contrary, contents of GA3, CTK and ABA in flax seedlings were increased dramatically with enhancing of P level (p<0.05). Under P deficiency (0mM), contents of GA3, CTK and ABA in flax seedlings were the lowest, 93.92%, 38.89% and 42.51% lower than 1mM P respectively (p<0.05) (Fig. 3D-F). In addition, contents of ACP in flax seedlings were decreased dramatically with enhancing of P level and reached the lowest value at 1mM P (p<0.05). Contents of ACP in flax seedlings were increased by 55.88% (p<0.05) compared with 1mM P under P deficiency (0mM). It is suggested that growth of lateral roots may be stimulated by regulating contents of hormones and ACP. Compared with 1mM P, IAA, Br and eth contents of Sofie seedlings were increased by 26.50%, 34.72% and 28.90% (p<0.05), respectively (Fig. 3A-C). On the contrary, the contents of GA3, CTK and ABA in Sofie seedlings were decreased by 24.30%, 14.81% and 19.51% respectively (p<0.05) compared with 1mM P (Fig. 3D-F). In addition, at 2mM P the activity of acid phosphatase in Sofie seedlings was increased by 32.35% (p<0.05) compared with 1mM P (Fig. 3G).

Influence of low P stress on the expression of *LusWRKYs* gene in flax lateral root: For further exploring molecular mechanism of P deficiency in flax lateral roots, the expression of 21 *LusWRKYs* (*LusWRKY1*、*LusWRKY2*、*LusWRKY3*、*LusWRKY7*、*LusWRKY10*、*LusWRKY11*、*LusWRKY13*、*LusWRKY15*、*LusWRKY20*、*LusWRKY22*、*LusWRKY27*、*LusWRKY28*、*LusWRKY32*、*LusWRKY33*、*LusWRKY38*、*LusWRKY39*、*LusWRKY40*、*LusWRKY48*、*LusWRKY49*、*LusWRKY57* and *LusWRKY71*) were analyzed. Internal reference is *LusActin*.

Results of Fig. 4A, 4B, 4C and 4D qRT-PCR revealed that expression of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* in lateral roots of flax seedlings increased with the increase of stress time. From 24 hours to 6 days, the expression of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* increased significantly, but on the sixth day, the gene expression tended to be the same. In contrast, there was no significant change in the expression of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* in lateral roots of seedlings treated with 1mM P. In addition, the rest 17 *LusWRKYs* were not obviously affected by P deficiency.

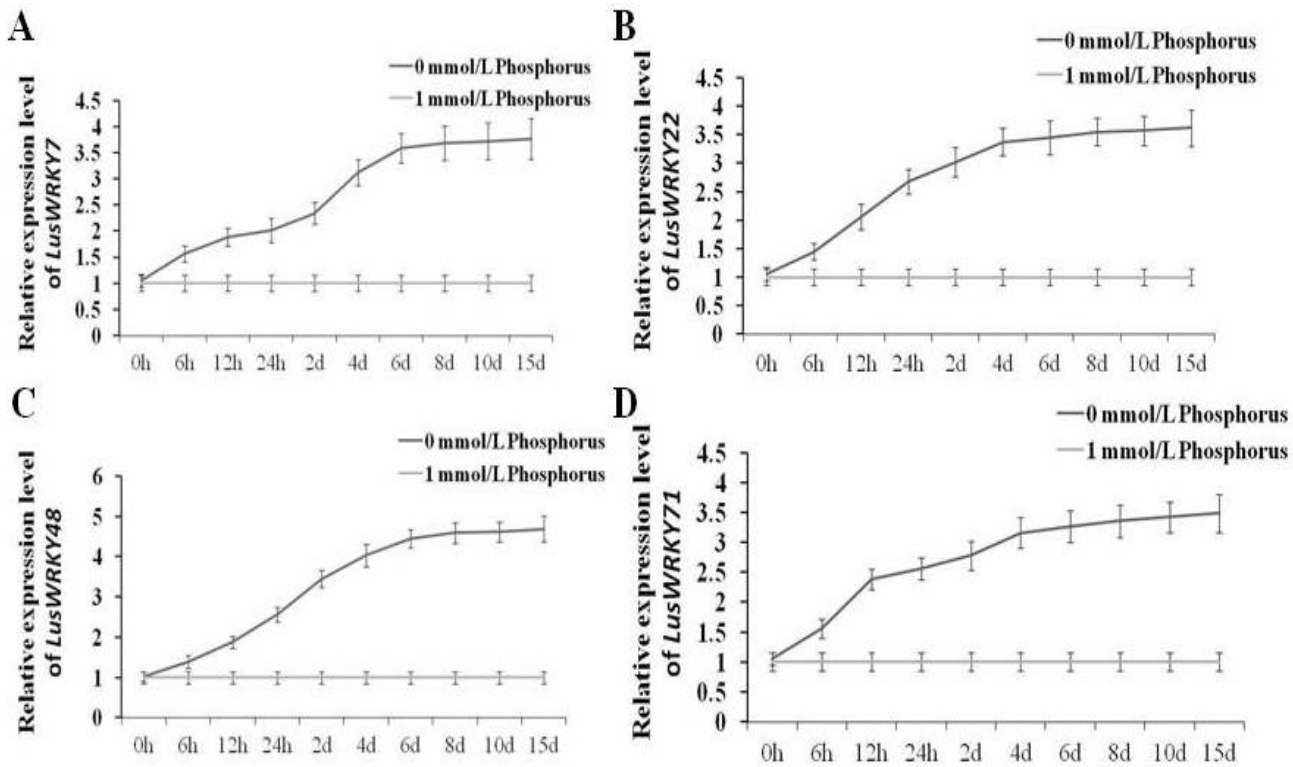

Fig. 4. Lateral root relative expression level of 21 *LusWRKYs*
A) *LusWRKY7* B) *LusWRKY22* C) *LusWRKY48* D) *LusWRKY71*

Discussion

Phosphorus deficiency is one of the biggest constraints on agricultural development (Zhu *et al*., 2018). Under P stress, growth rate of legumes decreased significantly (Cakmak *et al*., 1994), while spruce seedlings almost had no shoot growth (Proe *et al*., 1995). This study showed that P deficiency remarkably reduced biomass and shoot dry weight of flax seedlings. Because P is a significant factor of plant dry matter increase, P deficiency causes growth restraining of flax seedlings. P is indispensable for normal growth and development of flax.

Effect of low P on root growth has been confirmed in many plants, for example *L. albus* (Tang *et al*., 2013), *A. thaliana* (Sanchez-Calderon *et al*., 2006), *Glycine max* (Wang *et al*., 2010) and *Cucurbita pepo* (Cao *et al*., 2012). It is indicated that low P is a negative effect. Length of main root was significantly shortened under P deficiency. Number and density of lateral root in *L. albus* were increased by P deficiency (Tang *et al*., 2013). Root hair elongation in *A. thaliana* was increased under P deficiency (Sanchez-Calderon *et al*., 2006). Compared with the normal P supply ratio, maximum root length, root surface area and root volume of *G. max* were raised significantly under P deficiency (Wang *et al*., 2010). Twenty-one days of low P, root/shoot ratio and root tip of *C. pepo* were improved remarkably (Cao *et al*., 2012). Similar to results of previous studies, our studies showed that under P deficiency (0mM) length and diameter of main root, number of lateral root, total root length and surface area, dry weight of root and root/shoot ratio in flax were remarkably decreased compared with P supply (0.5-2mM). Plants usually change root morphology to adapt to limited nutrient resources. Under P deficiency, growth of fixed root (main root) was inhibited and growth of absorbed root (lateral root) was promoted in plants. Under P deficiency, normal growth of flax seedlings and absorption capacity of P was improved by promoting lateral root expansion.

Plant hormones play an important role in regulating growth of plant (Santner *et al*., 2009). Stimulation effect of P deficiency on lateral root growth is tightly related to hormone signal (Ma *et al*., 2003; Jiang *et al*., 2007; Meng *et al*., 2013). Research showed that free IAA concentration in P deficient plants roots was significantly higher than P sufficient plants, while rooting formation in *Lupinus albus* during P deficiency was stimulated by IAA in the roots (Meng *et al*., 2013). In *A. thaliana*, Bioactive GA was decreased and della protein was accumulated in roots, moreover increase of root hair length was stimulated under P deficiency (Jiang *et al*., 2007). *A. thaliana* root elongation was reduced with inhibition of ETH production by aminoethoxyethylene glycine or inhibition of ETH by 1-methylcyclopropene when P is sufficient (Ma *et al*., 2003). In our studies, IAA, ETH and BR concentrations in flax seedlings were significantly higher than those of P supply plants, while the GA3, CK and ABA concentrations were lower. Our results are consistent with those of previous studies. It is indicated that IAA, ETH, Br, GA3, CK and ABA signals participated in adjustment in lateral root growth during P deficiency. Mechanism of endogenous hormones on lateral root growth is very complicated. Morphological changes of lateral roots in *A. thaliana* probably attributed to redistribution and sensitivity changes of IAA (Pérez-Torres *et al*., 2008). During the condition of low P, IAA

in *A. thaliana* was redistributed, level of root free IAA was changed and accumulation of local IAA was increased by polar IAA transport process. Finally, root morphology in *A. thaliana* was changed (Nacry *et al*., 2005). In P deficient plants, sensitivity of IAA was enhanced by increasing TIR1 expression, Aux/IAA protein degradation was accelerated, and ARF TFs that genes participated in emergence and formation of lateral roots were activated was released (Pérez-Torres *et al*., 2008). We speculated under low P lateral root expansion of flax seedlings was stimulated by affecting sensitivity and redistribution of different endogenous hormones. Due to different action mechanism of different hormones under P deficiency, further research is needed.

ACP is key enzyme of organophosphorus hydrolysis (Mehra *et al*., 2017). Under P starvation *Solanum lycopersicum* ACP gene *LEPS2* was induced to express (Baldwin *et al*., 2001). Under low P stress ACP activity in *Pinus massoniana* (Xie *et al*., 2005), *Cunninghamia lanceolata* (Liang *et al*., 2005) and *Eucalyptus* (Feng *et al*., 2008) increased significantly, and ACP was secreted to rhizosphere. Results showed that ACP level in lateral roots of flax seedlings increased significantly under P deficiency stress. In plants, plasma membrane H^+-ATPase was activated, proton release was promoted, and cytoplasmic acidification was caused under P deficiency. Extracellular acidification caused by P deficiency created the best environment for ACP (Sujkowska *et al*., 2006). Under P deficiency increase of ACP meet the requirement of P during growth of flax.

Transcription regulation is one of significant regulatory mechanisms for plant responding to low P (Wang *et al*., 2014). WRKY TF is an important regulator of biotic and abiotic stress and plays an important effect in the responding of low P. There were 72 WRKY gene family members in *Arabidopsis thaliana*, 4 members are determined to participate in responding to low P, containing *AtWRKY75* (Devaiah *et al*., 2007), *AtWRKY6* (Chen *et al*., 2009), *AtWRKY45* (Wang *et al*., 2014) and *AtWRKY42* (Su *et al*., 2015). Research has shown that *AtWRKY*75 (Devaiah *et al*., 2007) and *AtWRKY45* (Wang *et al*., 2014) are positive TFs to P deficiency response, but *AtWRKY42* (Su *et al*., 2015) and *AtWRKY6* (Chen *et al*., 2009) are negative regulators of P deficiency response in *Arabidopsis thaliana*. In this study, 21 *LusWRKYs* were analyzed during low P. Real-time RT-PCR results showed expression of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* in lateral roots of flax seedlings was remarkably increased under P deficiency (0mM). In this study, *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* were positive regulatory responses to P deficiency. It is reported in the previous research that in *A. thaliana*, *AtWRKY75* plays an active TF of P acquisition in the absence of P (Devaiah *et al*., 2007). *AtWRKY45* adjusts P uptake through up regulating *PHT1;1* (Wang *et al*., 2014). In our studies, we found that under P stress expression of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* was up regulated in flax. These factors may have similar functions to *AtWRKY75* and *AtWRKY45*. We speculated that under P deficiency P uptake of flax was promoted by regulating expression of these factors in lateral roots.

Regulatory role of WRKY in hormones has been identified in *A. thaliana* and *O. sativa*. Many WRKY family members participated in IAA regulation, such as *OsWRKY72* (Song *et al*., 2010), *AtWRKY23* (Grunewald *et al*., 2012), *AtWRKY57* (Jiang *et al*., 2014), *AtWRKY46* (Ding *et al*., 2015) and *GbWRKY1* (Xu *et al*., 2012). *OsWRKY45* (Aya *et al*., 2014) participated in CTK regulation. *AtWRKY2* (Jiang *et al*., 2009), *AtWRKY8* (Chen *et al*., 2013), *AtWRKY18*, *AtWRKY40*, *AtWRKY60* (Chen *et al*., 2010) and *OsWRKY45* (Tao *et al*., 2011) participated in ABA regulation. However, WRKY plays a limited role in BR, GA and ETH. In view of the significant increase of *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* in flax seedlings under P deficiency stress, we speculated that under P deficiency stress related hormone response genes were regulated by up regulating four TFs, thereby increasing concentrations of IAA, BR and ETH, reducing concentrations of GA3, CTK and ABA, and finally stimulating lateral root growth of flax seedlings. In addition, four TFs might also be involved in the regulation of ACP in P deficient environment. However, the specific regulatory genes and related mechanisms still need to be confirmed in the future.

Conclusions

This study revealed the biological mechanism of the adaptation of flax to low P stress. Biomass dry weight, and shoot dry weight of flax seedlings were significantly decreased under P deficiency. Root dry weight, root/shoot ratio, number of lateral roots, total root length and total root surface area of flax seedlings were significantly increased under P deficiency which indicated that P deficiency significantly inhibited plant growth and development. Biomass distribution in the overground part of plant was reduced, and biomass distribution in the underground part of plant was increased in P deficient environment. Under P stress, growth of lateral roots was stimulated by regulating hormone levels of lateral roots (increasing IAA, BR and ETH, reducing GA3, CTK and ABA), moreover uptake of P elements in P deficient environment was promoted by increasing ACP. In the meanwhile, this study explored the molecular mechanism of adaptation of flax to P deficiency. *LusWRKY7*, *LusWRKY22*, *LusWRKY48* and *LusWRKY71* were involved in the regulation of P deficiency stress.

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