Phosphate (Pi) Starvation Up-Regulated *GmCSN5A/B* Participates in Anthocyanin Synthesis in Soybean (*Glycine max*) Dependent on Pi Availability

Xiaohui Mo †, Mengke Zhang †, Zeyu Zhang, Xing Lu, Cuiyue Liang *† and Jiang Tian *

Root Biology Center, State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, China; xhmo@scau.edu.cn (X.M.); mengkezhang611@foxmail.com (M.Z.); jyxs@scau.edu.cn (Z.Z.); xinglu@scau.edu.cn (X.L.)

* Correspondence: liangcy@scau.edu.cn (C.L.); jitian@scau.edu.cn (J.T.)
† These authors contributed equally to this work.

Abstract: Phosphorus (P) is an essential macronutrient for plant growth and development. Among adaptive strategies of plants to P deficiency, increased anthocyanin accumulation is widely observed in plants, which is tightly regulated by a set of genes at transcription levels. However, it remains unclear whether other key regulators might control anthocyanin synthesis through protein modification under P-deficient conditions. In the study, phosphate (Pi) starvation led to anthocyanin accumulations in soybean (*Glycine max*) leaves, accompanied with increased transcripts of a group of genes involved in anthocyanin synthesis. Meanwhile, transcripts of *GmCSN5A/B*, two members of the COP9 signalosome subunit 5 (CSN5) family, were up-regulated in both young and old soybean leaves by Pi starvation. Furthermore, overexpressing *GmCSN5A* and *GmCSN5B* in *Arabidopsis thaliana* significantly resulted in anthocyanin accumulations in shoots, accompanied with increased transcripts of gene functions in anthocyanin synthesis including *AtPAL, AtCHS, AtF3H, AtF3′H, AtDFR, AtANS*, and *AtUFGT* only under P-deficient conditions. Taken together, these results strongly suggest that P deficiency leads to increased anthocyanin synthesis through enhancing expression levels of genes involved in anthocyanin synthesis, which could be regulated by GmCSN5A and GmCSN5B.

Keywords: phosphorus deficiency; soybean; *GmCSN5A/B*; anthocyanin synthesis

1. Introduction

Phosphorus (P) is one of essential mineral nutrients required for various biomacromolecules (e.g., nucleic acids and phospholipids), and a series of the biological processes, such as cell energy metabolism, signal transduction and protein modification in plants [1–3]. Inorganic phosphate (Pi) is the main form of phosphorus absorbed by plants, and is easily fixed in soils, mainly due to cation chelation and biological fixation by microorganisms [1,2]. Therefore, the amount of available Pi is approximately between 1 μM and 10 μM, which is suboptimal for crop growth [4,5]. Plants have deployed a set of mechanisms and physiological strategies in adaptation to P deficiency, including retardation of growth rates, enhancement of lateral root and root hair growth, accumulations of anthocyanin [6,7]. Meanwhile, plants have evolved the metabolic flexibility to improve P utilization efficiency, such as scavenging Pi from P-containing metabolites, replacing membrane P-lipids with sulfonyl-lipids [3,8]. Among them, anthocyanin accumulation in shoots or leaves of *Arabidopsis thaliana*, tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), *Medicago truncatula*, rice (*Oryza sativa*) and maize (*Zea mays*) was widely observed and suggested to protect chloroplasts from photo-inhibitory damage in plant responses to Pi starvation [7,9–13].
Anthocyanins are a kind of flavonoids, and exist in most plant tissues, including flower petals, leaves, stems, roots, and fruits [14]. Anthocyanins not only act as the pigments in tissues or organs (e.g., flowers, fruits, and leaves), but also protect plants from pathogen and insect invasion [15–17]. Meanwhile, the antioxidant activity of anthocyanins helps plants adapt to various abiotic stresses, such as nutrient deficiencies (e.g., nitrogen and phosphorus), and low temperature stress [18–20]. In Arabidopsis, biosynthesis of anthocyanins includes consecutive processes which are catalyzed by multiple enzymes including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (ANS), and flavonoid 3-O-glucosyl transferase (UFGT) [21,22]. Therefore, the synthesis of anthocyanins is tightly related to enzymatic activity of the enzymes, and transcription levels of their corresponding genes in plants [7,23]. For example, anthocyanin synthase (ANS) is the key enzyme in anthocyanin synthetic pathways, and ans-1/2 mutants accumulate less anthocyanin under high light stress in Arabidopsis [24].

Except for the functional identification of gene functions in anthocyanin biosynthesis processes, their upstream regulatory factors have been well characterized in plants [1,25,26]. Among them, the MYB-bHLH-WD40 (MBW) complexes are considered to play a critical role in controlling transcription levels of genes encoding anthocyanin biosynthesis [27–31]. In these complexes, a R2R3 MYB transcription factor PRODUCTION OF ANTHOCYANIN PIGMENTS1 (PAP1), also known as MYB75, functions as central regulator in anthocyanin biosynthesis in plants [30,31]. Therefore, it is reasonable to find that increased anthocyanin accumulations by Pi starvation were regulated by PAP1 in plants, such as Arabidopsis [29,31]. Recently, SPX4, the key regulator of Pi signaling network, has been found to cooperate with PAP1, and thus transform the signal of Pi deficiency to anthocyanin biosynthesis in plants [26].

Although the molecular mechanisms underlying anthocyanin synthesis are well demonstrated at transcription levels in model plants (i.e., Arabidopsis and rice), it remains largely unknown that protein modification might be involved in mediating the anthocyanin synthesis in plants. It is well known that a ubiquitin-proteasome-mediated protein degradation is involved in photomorphogenesis, floral organ formation and abiotic stress responses [32–34]. Among them, constitutive photomorphogenesis 9 (COP9) signalosome (CSN) was found to participate in protein degradation, mainly through interacting with Skp1-cullin 1-F-box (SCF) complexes, the largest family of ubiquitin E3 ligases [35]. Furthermore, loss-function of CSN5a, one of the eight CSN subunits, was found to enhance anthocyanin accumulations in Arabidopsis, strongly suggesting that AtCSN5A participates in anthocyanin synthesis [36–38]. However, the molecular mechanisms underlying CSN5A mediating anthocyanin metabolism in other plants, remain largely unknown especially in crops.

Soybean (Glycine max) is an important legume crop, and it provides a protein-rich food source in the world. However, phosphate starvation restrains soybean growth and yield in soils [39]. To deal with low Pi stress, the soybean has evolved a set of adaptive strategies to enhance Pi mobilization and acquisition in soil, such as the remodeling of root architecture, increased exudation of organic acids and root-associated purple acid phosphatase activities, formation of symbiosis with beneficial microbes (i.e., rhizobia and mycorrhiza fungi) [40–43]. However, few studies tried to investigate responses of soybean leaves to Pi deficiency in order to fully understand adaptive strategies of soybean to Pi deficiency. Therefore, in the study, we examined anthocyanin accumulations in both old and young soybean leaves, as well as transcripts of anthocyanin synthesis-related genes under Pi-sufficient and Pi-deficient conditions. Meanwhile, two Pi starvation up-regulated GmCSN5A/B members were functionally characterized, and were suggested to regulate anthocyanin synthesis tightly dependent on Pi availability through mediating transcription levels of several genes related to anthocyanin synthesis in plants.
2. Results
2.1. Effects of P Deficiency on Soybean Biomass and Anthocyanin Content

To investigate the effects of P deficiency on soybean growth, the fresh weight of leaves at two P treatments for 12 d was measured. The results showed that the fresh weight of young and old leaves under sufficient P conditions was 12.5% and 16.8% higher than that of P-deficient plants, respectively (Figure 1A,B). Anthocyanin content in both young and old leaves was determined at two P levels. As shown in Figure 1C, the anthocyanin content in young and old leaves of soybean seedlings grown under Pi-deficient conditions was about 1.2 and 1.3 times higher than those of plants grown on Pi-sufficient conditions, respectively. However, P concentration in young and old leaves was 72.3% and 77.2% lower at low P levels than that at high P levels, respectively (Figure 1D). These results suggest that Pi starvation led to increased accumulations of anthocyanin in soybean leaves.

Figure 1. Effects of P deficiency on soybean fresh weight, anthocyanin content and P concentration. (A) Phenotypes of soybean at two P levels. Bars = 5 cm. (B) Fresh weight of young leaves (YL) and old leaves (OL). (C) Anthocyanin content of YL and OL. (D) P concentration of YL and OL. Data are means of four replicates ± SE. Asterisks indicate significant difference between the two P levels in the Student’s t-test (* p < 0.05; ** p < 0.01; *** p < 0.001).

2.2. Effects of P Deficiency on Transcripts of Anthocyanin Synthesis Genes

In order to analyze the effects of Pi starvation on anthocyanin synthesis in soybean, the expression patterns of eight genes related to anthocyanin synthesis pathway were investigated in old leaves under two P treatments, including phenylalanine ammonia-lyase (GmPAL), chalcone synthase (GmCHS), chalcone isomerase (GmCHI), flavanone-3-hydroxylase (GmF3H),
flavonoid 3'-hydroxylase (GmF3'H), dihydroflavonol 4-reductase (GmDFR), anthocyanin synthase (GmANS) and flavonoid 3-O-glucosyl transferase (GmUF3GT) (Figure 2A). Results showed that transcripts of all genes were enhanced by more than 1-fold by Pi starvation, except F3H, which exhibited no response to P treatments (Figure 2B). Notably, the expression levels of GmDFR under low P conditions were 5.8-fold higher than those under high P conditions (Figure 2B).

2.2. Effects of P Deficiency on Transcripts of Anthocyanin Synthesis Genes

In order to analyze the effects of Pi starvation on anthocyanin synthesis in soybean, the expression patterns of eight genes related to anthocyanin synthesis pathway were investigated in old leaves under two P treatments, including phenylalanine ammonia-lyase (GmPAL), chalcone synthase (GmCHS), chalcone isomerase (GmCHI), flavanone-3-hydroxylase (GmF3H), flavonoid 3′-hydroxylase (GmF3'H), dihydroflavonol 4-reductase (GmDFR), anthocyanin synthase (GmANS) and flavonoid 3-O-glucosyl transferase (GmUF3GT) (Figure 2A). Results showed that transcripts of all genes were enhanced by more than 1-fold by Pi starvation, except F3H, which exhibited no response to P treatments (Figure 2B). Notably, the expression levels of GmDFR under low P conditions were 5.8-fold higher than those under high P conditions (Figure 2B).

Figure 2. Transcripts of eight genes in anthocyanin biosynthetic pathway in soybean leaves at two P levels. (A) The genes in anthocyanin biosynthetic pathway. (B) Relative expression of genes in anthocyanin biosynthetic pathway under Pi-sufficient (+P) and Pi-deficient (−P) conditions. Data are means of four independent replicates ± SE. Asterisks indicate significant difference between the two P levels in the Student's t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

2.3. Identification of GmCSN5A/B in Soybean Genome

Two putative GmCSN5 members in the soybean genome were identified by BLAST searching at http://www.phytozome.net, accessed on 10 February 2019, which were named as GmCSN5A (Glyma.04G075000) and GmCSN5B (Glyma.06G076000) based on its localization in soybean chromosomes. All the tested CSN5 proteins contained the conserved JAB_MPN domain (Figure 3). Phylogenetic analysis showed that CSN5 homologs in dicot plants significantly differed from monocot plants (Figure 3). Meanwhile, among dicot plants, CSN5 homologs in leguminous plants distinguished from other dicot plants, as reflected by CSN5 homologs from soybean, bean and Medicago, belonged to a subgroup. Interestingly, CSN5B was found to contain a transmembrane region, which was not identified in other CSN5 homologs (Figure 3).
Figure 3. Phylogenetic analysis of CSN5s in plant. The phylogenetic tree was generated ground on an amino acid alignment of CSN5 proteins from well-known species using the Mega 5.05 program. The gene loci on the Phytozome website are as follows: AtCSN5A (At1G22920), AtCSN5B (At1G71230), GmCSN5A (Glyma.04G075000), GmCSN5B (Glyma.06G076000), PvCSN5 (Phvul.009G100700), MtCSN5 (Medtr3g108790), SICSN5A (Solyc06g073150), SICSN5B (Solyc11g017300), OsCSN5 (LOC_Os04g56070), ZmCSN5 (GRMZM2G086801). At, Zm, Os, (Solyc11g017300), OsCSN5 (LOC_Os04g56070), ZmCSN5 (GRMZM2G086801). At, Zm, Os, (Solyc11g017300), OsCSN5 (LOC_Os04g56070), ZmCSN5 (GRMZM2G086801). At, Zm, Os, (Solyc11g017300), OsCSN5 (LOC_Os04g56070), ZmCSN5 (GRMZM2G086801).

2.4. Expression Patterns and Subcellular Localization of GmCSN5A/B

Expression patterns of GmCSN5A and GmCSN5B in leaves were investigated at two P levels using qRT-PCR. Results showed that transcripts of GmCSN5A in young and old leaves were significantly enhanced by low P stress, as reflected by 1.5-fold and 2.6-fold increases, respectively (Figure 4A). Similarly, transcripts of GmCSN5B in leaves were also up-regulated by P starvation, especially in old leaves with values 2.8-fold higher than those in sufficient P treatment (Figure 4B). These results suggest that GmCSN5A and GmCSN5B exhibited enhanced expression patterns in soybean leaves in response to P starvation.

Figure 4. Expression patterns and subcellular localization of GmCSN5A/B. (A) Relative expression of GmCSN5A. (B) Relative expression of GmCSN5B. Data are mean of four replicates ± SE. Asterisks indicate significant difference between the two P levels in the Student’s t-test (* p < 0.05; ** p < 0.01). (C) Subcellular localization of GmCSN5A and GmCSN5B. mCherry-PM represents plasma membrane localization indicated by the red fluorescence derived from AtPIP2A-mCherry. Bars = 20 μm.
To analyze the subcellular localization of GmCSN5A/B, the GFP (empty vector control), GmCSN5A-GFP and GmCSN5B-GFP plasmids were separately transiently expressed in tobacco leaf epidermal cells. Results showed that GmCSN5A-GFP fluorescence signals were detected in the cytoplasm and nucleus, and GmCSN5B-GFP fluorescence signals were detected in the whole epidermal cells, exhibiting similar patterns to the GFP fluorescence signals observed in cells transformed with the empty vector (Figure 4C). Therefore, it is suggested that GmCSN5A is mainly localized in the cytoplasm and nucleus, while GmCSN5B is localized in the plasma membrane, nucleus, and cytoplasm.

2.5. Overexpression of GmCSN5A/B Increases Anthocyanin Accumulations and Affects Photomorphogenesis in Arabidopsis

In this study, GmCSN5A and GmCSN5B were subsequently overexpressed in Arabidopsis to investigate their biological functions. Increased transcripts of GmCSN5A and GmCSN5B in transgenic Arabidopsis were validated via qRT-PCR analysis (Figure S1). Subsequently, wild type (WT) and transgenic Arabidopsis with overexpressing GmCSN5A/B were used to investigate the role of GmCSN5A/B in anthocyanin accumulations at two P levels (Figures 5 and 6).

![Image of experiment results](image-url)

**Figure 5.** Effects of GmCSN5A overexpression on anthocyanin accumulation in Arabidopsis. (A) The phenotypes. Bars = 1 cm. (B) Fresh weight. (C) Anthocyanin content. (D) P content. (E) Relative expression of eight genes in anthocyanin biosynthetic pathway. Seedlings were grown in peat soils for 8 d. Data are means of four independent replicates ± SE. Asterisks indicate significant difference between GmCSN5A overexpressing Arabidopsis and WT in the Student’s t-test (* p < 0.05; ** p < 0.01; *** p < 0.001).
Figure 6. Effects of GmCSN5B overexpression on anthocyanin accumulation in Arabidopsis. (A) The phenotypes. Bars = 1 cm. (B) Fresh weight. (C) Anthocyanin content. (D) P content. (E) Relative expression of eight genes in anthocyanin biosynthetic pathway. Seedlings were grown in peat soils for 13 d. Data are means of four independent replicates ± SE. Asterisks indicate significant difference between GmCSN5B overexpressing Arabidopsis and WT in the Student’s t-test (*p < 0.05; **p < 0.01; ***p < 0.001).

Wild type (WT) and GmCSN5A transgenic Arabidopsis were subjected to two P treatments for 8 d. Results showed that GmCSN5A overexpression resulted in enhanced Arabidopsis growth at two P levels. Fresh weight in two GmCSN5A overexpression lines (OX1 and OX2) was 12.3–25.3% and 39.6–40.1% higher than that in WT under P-sufficient and P-deficient conditions, respectively (Figure 5B). Furthermore, under low P conditions, anthocyanin content in GmCSN5A overexpression lines was increased by approximately more than 1.4-fold compared to those in WT (Figure 5C). For P content, no significant difference was observed between GmCSN5A overexpression lines and WT at two P levels (Figure 5D). To further investigate the roles of GmCSN5A in anthocyanin synthesis in responses to Pi starvation, transcripts of eight genes involved in the anthocyanin synthesis
pathway were analyzed in both WT and transgenic Arabidopsis, including \textit{AtPAL}, \textit{AtCHS}, \textit{AtCHI}, \textit{AtF3H}, \textit{AtF3′H}, \textit{AtDFR}, \textit{AtANS}, and \textit{AtUF3GT}. The results showed that expression levels of all of the tested genes were significantly increased in two transgenic lines with \textit{GmCSN5A} overexpression only under low P conditions, but not under high P conditions (Figure 5E).

Similarly, increases in fresh weight and anthocyanin content were also observed in \textit{GmCSN5B} overexpression lines compared to those in WT only under Pi-deficient conditions, not under Pi-sufficient conditions (Figure 6B,C). However, P content in \textit{GmCSN5B} overexpression lines was no different from that in WT at two P levels (Figure 6D). Furthermore, except CHI, expression levels of the tested genes were significantly increased in transgenic lines with \textit{GmCSN5B} overexpression under low P conditions (Figure 6E). These results together suggest that both \textit{GmCSN5A} and \textit{GmCSN5B} play important roles in anthocyanin synthesis in plant responses to low P stress. Meanwhile, hypocotyl length was significantly decreased in Arabidopsis with overexpressing \textit{GmCSN5B} after grown in the dark for 5 d (Figure S2). However, no difference in hypocotyl length was found between WT and plants with overexpressing \textit{GmCSN5A} (Figure S2).

3. Discussion

Phosphorus is one of essential macronutrients and participates in numerous biochemical and metabolic processes [1,2]. Among the adaptive strategies of plants to P deficiency, increased anthocyanin accumulations in shoot/leaves are generally considered to improve plant tolerance to P deficiency, and widely observed in plants including Arabidopsis, tomato, tobacco, and maize [9,10,13,26]. In this study, enhanced anthocyanin accumulations in soybean young leaves and old leaves were also found under Pi-deficient conditions, as reflected by the fact that their values were 1.2- and 1.3-fold higher than those plants in Pi-sufficient conditions (Figure 1C). Furthermore, it was observed that anthocyanin content in old leaves was 12.7% higher than that in young leaves by P starvation (Figure 1C), suggesting that more anthocyanins were synthesized in old leaves by P starvation because P might be transported out of old leaves to active tissues/organs in plants. Consistently, P concentration in old leaves was 33.2% lower than that in young leaves at low P levels (Figure 1D). Similarly, higher anthocyanin content, accompanied with lower P concentration was also observed in shoot/leaves of other plants under low Pi stress, such as Arabidopsis and Medicago [7,44]. Accompanied with anthocyanin accumulations, expression levels of several genes related to anthocyanin synthesis were significantly up-regulated by P starvation in soybean old leaves, including \textit{GmPAL}, \textit{GmCHS}, \textit{GmCHI}, \textit{GmF3′H}, \textit{GmDFR}, \textit{GmANS}, and \textit{GmUF3GT} (Figure 2). Meanwhile, it was widely observed that transcripts of one or more homologues related to anthocyanin synthesis were increased by P deficiency in plants, strongly suggesting that the regulatory pathway of P starvation responsive anthocyanin synthesis is well conserved in plants [7,12,23,26].

Consistently, we found that overexpressing \textit{GmCSN5A} and \textit{GmCSN5B} led to higher plant fresh weight, accompanied with more anthocyanin accumulations in plants under P-deficient conditions (Figures 5 and 6), partially suggesting that increased anthocyanin synthesis could contribute to enhancing plant tolerance to P deficiency. It is generally believed that anthocyanin accumulations play a role in scavenging reactive oxygen species (ROS), and protecting chloroplasts from photoinhibitory damage in plants under abiotic stress conditions, such as N and P deficiencies [18–20,45,46]. For example, mutation of \textit{ZmsSRO1e} in maize enhanced anthocyanin accumulations and ROS scavenging activity, and thus led to higher plant biomass, suggesting that increased anthocyanin accumulations resulted in plant adaptation to Pi starvation through avoiding ROS inhibition [46]. However, there should be further analysis into whether anthocyanin accumulations in Arabidopsis with \textit{GmCSN5A}/\textit{B} overexpression could inhibit excess ROS accumulations under low P conditions, which is widely observed in plants subjected to Pi starvation [47].

Functions of \textit{CSN5A} are generally considered to control plant photomorphogenesis in Arabidopsis, such as growth of hypocotyl and cotyledons [36–38]. For example, \textit{csn5}
mutants exhibited short hypocotyl and unexpanded cotyledons in seedlings grown in the dark [37,38]. However, hypocotyl length in Arabidopsis with overexpressing GmCSN5B, was significantly shorter than WT grown in the dark (Figure S2). These results strongly suggested that both increased and suppressed expression levels of CSN5 could result in the abnormal growth of plant hypocotyls. However, overexpressing GmCSN5A does not have any effect on the hypocotyl length and root length. It might be caused by differences in amino acids between GmCSN5A and GmCSN5B, as reflected by a transmembrane domain presenting in GmCSN5B, not in GmCSN5A (Figure 3), which merit further studies.

In addition to influencing hypocotyl growth, increased anthocyanin accumulations were observed in loss-function mutants of CSN5a, strongly suggesting that CSN5A participates in mediating anthocyanin synthesis in Arabidopsis [36–38]. CSN5 was suggested to regulate anthocyanin accumulation via interactions with one of SCF complexes, SCFCOI1 and its substrate, jasmonate ZIM-domain (JAZ) proteins, which negatively mediates the stability of a key transcription factor in anthocyanin synthesis, PAP1, and thus mediates expression levels of anthocyanin biosynthetic genes, including DFR and UFS3GT [32,38,48]. Furthermore, it is generally considered that the anthocyanin synthesis is affected directly by transcription levels of several genes encoding enzymes in anthocyanin biosynthetic pathway in plants [7,23]. For example, tt3, a mutant of DFR which is the rate-limiting enzyme in anthocyanin biosynthesis, decreases anthocyanin content under normal growth conditions or low N stress [49]. In this study, overexpressing GmCSN5A and GmCSN5B in Arabidopsis led to anthocyanin accumulations in Arabidopsis only under P-deficient conditions, as reflected by values more than 1.4- and 1.2-fold higher than those in WT (Figures 5 and 6), accompanied by increased transcripts of gene functions in anthocyanin synthesis, including AtPAL, AtCHS, AtF3H, AtF3′H, AtDFR, AtANS, and AtUFS3GT (Figures 5 and 6), strongly suggesting that GmCSN5A/B-mediated anthocyanin accumulations under Pi-deficient conditions. It has widely been observed that CSN5 exhibits non-identical functions by binding to different protein targets [35]. The functions of GmCSN5A/B mediating the anthocyanin synthesis in response to Pi starvation probably depend on their interacted proteins. Meanwhile, SPX4, a main regulator in P signaling network, has recently been suggested to transduce the Pi starvation signal to mediate anthocyanin synthesis in Arabidopsis through interacting with PAP1 [26]. It is speculated that GmCSN5A/B might be associated with SPX4-PAP1 complex to regulate anthocyanin accumulation in response to Pi starvation, which merits further analysis.

4. Materials and Methods

4.1. Plant Materials and Growth Conditions

Soybean genotype YC03-3 provided by Root Biology Center, South China Agricultural University, China was used in this study. The seeds were germinated for 4 d as described previously [50]. After germination, uniform seedlings were transplanted into nutrient solution containing 1500 µM KNO₃, 400 µM NH₄NO₃, 1200 µM Ca(NO₃)₂, 25 µM MgCl₂, 300 µM K₂SO₄, 500 µM MgSO₄, 0.3 µM (NH₄)₂SO₄, 0.5 µM CuSO₄, 1.5 µM MnSO₄, 1.5 µM ZnSO₄, 0.16 µM (NH₄)₆Mo₇O₂₄, 40 µM Fe-Na-EDTA, 2.5 µM NaB₄O₇, and 250 µM (+P) or 5 µM (−P) KH₂PO₄. The pH value of nutrient solution was adjusted to 5.8, and the nutrient solution was replaced every 7 d. After 12 d of P treatments, as described previously [51], soybean fresh weight, content of P concentration and anthocyanin in the youngest trifoliate leaves (i.e., young leaves) and primary leaves (i.e., old leaves) were measured. All experiments included four replicates.

Arabidopsis Columbia (Col-0) ecotype and tobacco (Nicotiana benthamiana) were used as background of transformation in this study. The seeds of Arabidopsis and tobacco were obtained from Dr. Jinxiang Wang at South China Agricultural University, China. Arabidopsis and tobacco plants were grown in a greenhouse at 22–24 °C and 60% relative humidity with a 16 h/8 h light/dark photoperiod.
4.2. Analysis of P Concentration and Anthocyanin Content

To determine P concentration, about 0.1 g of plant dry samples were ground into powder and digested with H\textsubscript{2}SO\textsubscript{4}-H\textsubscript{2}O\textsubscript{2}. P concentration in the extracts was measured according to the method described by Murphy and Riley [52].

For anthocyanin content measurement, about 0.1 g of fresh leaf samples were ground into powder by liquid N\textsubscript{2} and dissolved in extraction solution (0.1 M HCl mixed with 95% ethanol). The mixtures were incubated at 60 °C for 30 min. After centrifugation at 12,000 rpm for 15 min at 4 °C, the supernatants were collected and spectrophotometrically detected at 530 and 657 nm with a microplate spectrophotometer (Thermo Scientific, Waltham, MA, USA). Anthocyanin content was calculated as previously described [53].

4.3. Phylogenetic Analysis and Characterization of GmCSN5A/B in Plants

GmCSN5A (Glyma.04G075000) and GmCSN5B (Glyma.06G076000) were identified in soybean genome by BLAST searches using the AtCSN5A (At1G22920) and AtCSN5B (At1G71230) as the query sequences at the Phytozome website (http://www.phytozome.net, accessed on 10 February 2019). The phylogenetic tree was constructed using CSN5 proteins from soybean, Arabidopsis, common bean (Phaseolus vulgaris), Medicago, tomato, rice, and maize using MEGA 5.05 based on the Neighbor-Joining method [54]. Conserved domain analysis was performed at the SMART website (http://smart.embl.de/, accessed on 17 May 2020) as previously described [55].

4.4. Subcellular Localization Analysis of GmCSN5A/B

The subcellular localization of GmCSN5A and GmCSN5B was analyzed as previously described [43]. Briefly, the full-length of GmCSN5A and GmCSN5B was separately amplified and cloned into a pEGAD vector with eGFP at its C-terminus. The GFP and GmCSN5A/B-GFP plasmids and the plasma membrane marker AtPIP2A-mCherry were co-transformed into tobacco leaves by Agrobacterium-mediated transformation. The fluorescence signals were observed in a Zeiss LSM7 Duo confocal microscope (Zeiss, Oberkochen, Germany) at 488 nm for GFP and 543 nm for mCherry.

4.5. Functional Characterization of GmCSN5A/B in Arabidopsis

The full-length of GmCSN5A and GmCSN5B was separately amplified and cloned into pTF101s vector according to the method described previously [43]. The GmCSN5A- and GmCSN5B-pTF101s plasmids were transformed into Arabidopsis by Agrobacterium-mediated transformation as described previously [50]. Two homozygous T3 lines (OX1/2/3/4) were separately selected and used for functional analysis. The transcripts of GmCSN5A and GmCSN5B in transgenic Arabidopsis were determined by quantitative RT-PCR (qRT-PCR). To investigate the functions of GmCSN5A and GmCSN5B, both wild type (WT, Columbia-0) and transgenic Arabidopsis seeds were cultivated on peat soils (Jiffy, Zwijndrecht, Netherlands) supplied with or without phosphorus. After 8 or 13 d growth, Arabidopsis plants were separately harvested for fresh weight, P and anthocyanin content analysis. All experiments had four biological replicates.

Furthermore, to investigate the functions of GmCSN5A and GmCSN5B in photomorphogenesis, both WT and transgenic Arabidopsis were germinated on solid Murashige and Skoog (MS) medium in the dark for 5 d. The length of hypocotyls and roots was analyzed with Image J (National Institutes of Health, Bethesda, MD, USA). All experiments had 19 biological replicates.

4.6. RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from soybean and Arabidopsis plants using the RNAsolve reagent (OMEGA Bio-Tek, Norcross, GA, USA), and was then purified with RNase-free DNase I (Invitrogen, USA). About 1 µg of RNA was reversely transcribed to complementary DNA (cDNA) with MMLV-reverse transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using a SYBR Premix kit (Promega, Madison, WI, USA) on a Rotor-Gene
3000 real-time PCR system (Corbett Research, Mortlake, Australia). The GmEF-1a and AEF-1a were separately used as an internal control. Relative expression of genes was calculated as the ratio of expression of the tested genes to those of EF-1a as described previously [56]. Specific gene primers are listed in Table S1.

4.7. Statistical Analysis

All data were analyzed using Microsoft Excel 2019 (Microsoft Company, Redmond, WA, USA) and SPSS program (v21.0; SPSS Institute, Chicago, IL, USA) for calculating mean, standard error, and Student’s t-tests.

5. Conclusions

In summary, two CSN5 members in soybean were identified, and up-regulated by Pi starvation. Furthermore, overexpressing GmCSN5A and GmCSN5B in Arabidopsis resulted in significant increases in anthocyanin accumulation and fresh weight in shoot accompanied with increased transcripts of gene functions in anthocyanin synthesis including ATPAL, AtCHS, AtF3H, AtF3’H, AtDFR, AtANS, and AtUF3GT under P-deficient conditions. Taken together, these results suggest that Pi starvation responsive GmCSN5A and GmCSN5B may mediate anthocyanin accumulation in plants in responses to P deficiency.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms222212348/s1.

Author Contributions: Conceptualization, J.T. and C.L.; methodology, J.T.; validation, J.T., C.L. and X.L.; formal analysis, J.T., C.L. and X.M.; investigation, J.T., X.M., M.Z. and Z.Z.; data curation, X.M., M.Z. and Z.Z.; writing—original draft preparation, J.T., C.L. and X.M.; writing—review and editing, J.T. and C.L.; project administration, J.T. and C.L.; funding acquisition, J.T., C.L. and X.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (31872164), the Natural Science Foundation of Guangdong Province of China (2021A1515010826, 2020A1515110261), the Major Program of Guangdong Basic and Applied Research (2019B030302006), the Integrated Demonstration of Key techniques for the Industrial Development of Featured Crops in Rocky Desertification Areas of Yunnan-Guangxi-Guizhou Provinces (SMH2019-2021).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The sequences of GmCSN5A and GmCSN5B were acquired from http://www.phytozome.net, accessed on 10 February 2019. The data presented in this study are available in article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Chiou, T.J.; Lin, S.I. Signaling network in sensing phosphate availability in plants. Annu. Rev. Plant Biol. 2011, 62, 185–206. [CrossRef] [PubMed]
2. Ham, B.K.; Chen, J.; Yan, Y.; Lucas, W.J. Insights into plant phosphate sensing and signaling. Curr. Opin. Biotechnol. 2018, 49, 1–9. [CrossRef] [PubMed]
3. Dissanayaka, D.M.S.B.; Ghahremani, M.; Siebers, M.; Wasaki, J.; Plaxton, W.C. Recent insights into the metabolic adaptations of phosphorus-deprived plants. J. Exp. Bot. 2021, 72, 199–223. [CrossRef] [PubMed]
4. Smith, F.W.; Mudge, S.R.; Rae, A.L.; Glassop, D. Phosphate transport in plants. Plant Soil. 2003, 248, 71–83. [CrossRef]
5. Ticconi, C.A.; Abel, S. Short on phosphate: Plant surveillance and countermeasures. Trends Plant Sci. 2004, 9, 548–555. [CrossRef] [PubMed]
6. Raghothama, K.G. Phosphate acquisition. Annu. Rev. Plant Physiol. Plant Mol. Biol. 1999, 50, 665–693. [CrossRef] [PubMed]
7. Wang, Z.Q.; Zhou, X.; Dong, L.; Guo, J.; Chen, Y.; Zhang, Y.; Wu, L.; Xu, M. iTRAQ-based analysis of the Arabidopsis proteome reveals insights into the potential mechanisms of anthocyanin accumulation regulation in response to phosphate deficiency. J. Proteom. 2018, 184, 39–53. [CrossRef]
8. Vance, C.P.; Uhde-Stone, C.; Allan, D.L. Phosphorus acquisition and use: Critical adaptations by plants for securing a nonrenewable resource. New Phytol. 2003, 157, 423–447. [CrossRef]
9. Morcuende, R.; Bari, R.; Gibon, Y.; Zheng, W.; Pant, B.D.; Bläsing, O.; Usadel, B.; Czechowski, T.; Udvardi, M.K.; Stitt, M.; et al. Genome-wide reprogramming of metabolism and regulatory networks of Arabidopsis in response to phosphorus. Plant Cell Environ. 2007, 30, 85–112. [CrossRef]

10. Li, Y.; Gu, M.; Zhang, X.; Zhang, J.; Fan, H.; Li, P.; Li, Z.; Xu, G. Engineering a sensitive visual-tracking reporter system for real-time monitoring phosphorus deficiency in tobacco. Plant Biotechnol. J. 2014, 12, 674–684. [CrossRef] [PubMed]

11. Manna, M.; Islam, T.; Kaul, T.; Reddy, C.S.; Fartyal, D.; James, D.; Reddy, M.K. A comparative study of effects of increasing concentrations of phosphate and phosphite on rice seedlings. Acta Physiol. Plant. 2015, 37, 258. [CrossRef]

12. Sun, Y.; Mu, C.; Chen, Y.; Kong, X.; Xu, Y.; Zheng, H.; Zhang, H.; Wang, Q.; Xue, Y.; Li, Z.; et al. Comparative transcript profiling of maize inbreds in response to long-term phosphorus deficiency stress. Plant Physiol. Biochem. 2016, 109, 467–481. [CrossRef]

13. Tominaga-Wada, R.; Masakane, A.; Wada, T. Effect of phosphate deficiency-induced anthocyanin accumulation on the expression of Solanum lycopersicum GLABRA3 (SIGL3) in tomato. Plant Signal. Behav. 2018, 13, e1477907. [CrossRef]

14. Williams, C.A.; Grayer, R.J. Anthocyanins and other flavonoids. Nat. Prod. Rep. 2004, 21, 539–573. [CrossRef] [PubMed]

15. Winkel-Shirley, B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 2001, 126, 485–493. [CrossRef] [PubMed]

16. Zhang, Y.; Butelli, E.; De Stefano, R.; Schoonbeek, H.J.; Magusin, A.; Pagliarani, C.; Wellner, N.; Hill, L.; Orzaez, D.; Granell, A.; et al. Anthocyanins double the shelf life of tomatoes by delaying overripening and reducing susceptibility to gray mold. Curr. Biol. 2013, 23, 1094–1100. [CrossRef]

17. Khan, G.A.; Vogiatzaki, E.; Glauer, P.; Poirier, Y. Phosphate deficiency induces the jasmonate pathway and enhances resistance to insect herbivory. Plant Physiol. 2016, 171, 632–644. [CrossRef]

18. Stewart, A.J.; Chapman, W.; Jenkins, G.I.; Graham, I.; Martin, T.; Crozier, A. The effect of nitrogen and phosphorus deficiency on flavonol accumulation in plant tissues. Plant Physiol. 2001, 24, 1189–1197. [CrossRef]

19. Nakabayashi, R.; Yonekura-Sakakibara, K.; Urano, K.; Suzuki, M.; Yamada, Y.; Nishizawa, T.; Matsuda, F.; Kojima, M.; Sakakibara, H.; Shinozaki, K.; et al. Enhancement of oxidative and drought tolerance in Arabidopsis by overaccumulation of antioxidant flavonoids. Plant J. 2014, 77, 367–379. [CrossRef]

20. Viola, I.L.; Camoirano, A.; Gonzalez, D.H. Redox-dependent modulation of anthocyanin biosynthesis by the TCP transcription factor TCP15 during exposure to high light intensity conditions in Arabidopsis. Plant Physiol. 2016, 170, 74–85. [CrossRef]

21. Solfanelli, C.; Poggi, A.; Loretí, E.; Alpi, A.; Perata, P. Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. Plant Physiol. 2006, 140, 637–646. [CrossRef]

22. Sakuta, M. Diversity in plant red pigments: Anthocyanins and betacyanins. Plant Biotech. Rep. 2014, 8, 37–48. [CrossRef]

23. Yin, Y.; Borges, G.; Sakuta, M.; Crozier, A.; Ashihara, H. Effect of phosphate deficiency on the content and biosynthesis of anthocyanins and the expression of related genes in suspension-cultured grape (Vitis sp.) cells. Plant Physiol. Biochem. 2012, 55, 77–84. [CrossRef]

24. Zheng, X.T.; Chen, Y.L.; Zhang, X.H.; Cai, M.L.; Yu, Z.C.; Peng, C.L. ANS-deficient Arabidopsis is sensitive to high light due to impaired anthocyanin photoprotection. Funct. Plant Biol. 2019, 46, 756–765. [CrossRef]

25. Hsieh, L.C.; Lin, S.I.; Shih, A.C.; Chen, J.W.; Lin, W.Y.; Tseng, C.Y.; Li, W.H.; Chiou, T.J. Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing. Plant Physiol. 2009, 151, 2120–2132. [CrossRef] [PubMed]

26. He, Y.; Zhang, X.; Li, L.; Sun, Z.; Li, J.; Chen, X.; Hong, G. SPX4 interacts with both PHR1 and PAP1 to regulate critical steps in phosphorus-status-dependent anthocyanin biosynthesis. New Phytol. 2021, 230, 205–217. [CrossRef] [PubMed]

27. Borevitz, J.O.; Xia, Y.; Blount, J.; Dixon, R.A.; Lamb, C. Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis and the expression of related genes in suspension-cultured grape (Vitis sp.) cells. Plant Physiol. Biochem. 2007, 55, 849–869. [CrossRef] [PubMed]

28. Zhang, F.; Gonzalez, A.; Zhao, M.; Payne, C.T.; Lloyd, A. A network of redundant bHLH proteins functions in all TTG1-dependent pathways of Arabidopsis. Development 2003, 130, 4859–4869. [CrossRef] [PubMed]

29. Tohge, T.; Nishiyama, Y.; Hirai, M.Y.; Yano, M.; Nakajima, J.; Awazuhara, M.; Inoue, E.; Takahashi, H.; Goodenow, D.B.; Kitayama, M.; et al. Functional genomics by integrated analysis of metabolome and transcriptome of Arabidopsis plants over-expressing an MYB transcription factor. Plant J. 2015, 82, 218–235. [CrossRef]

30. Gonzalez, A.; Zhao, M.; Leavitt, J.M.; Lloyd, A.M. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. Plant J. 2008, 53, 814–827. [CrossRef]

31. Bhargava, A.; Mansfield, S.D.; Hall, H.C.; Douglas, C.J.; Ellis, B.E. MYB75 functions in regulation of secondary cell wall formation in the Arabidopsis inflorescence stem. Plant Physiol. 2005, 145, 1428–1438. [CrossRef] [PubMed]

32. Fang, S.; Ma, L.; Wang, X.; Xie, D.; Dinesh-Kumar, S.P.; Wei, N.; Deng, X.W. The COP9 signalosome interacts physically with SCFCOI1 and modulates jasmonate responses. Plant Cell 2003, 15, 1083–1094. [CrossRef] [PubMed]

33. Gusmaroli, G.; Figueuera, P.; Serino, G.; Deng, X.W. Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with Arabidopsis Cullin3-based E3 ligases. Plant Cell 2007, 19, 564–581. [CrossRef] [PubMed]

34. Wang, J.; Yu, Y.; Zhang, Z.; Quan, R.; Zhang, H.; Ma, L.; Deng, X.W.; Huang, R. Arabidopsis CSN5B interacts with VTC1 and modulates ascorbic acid synthesis. Plant Cell 2013, 25, 625–636. [CrossRef] [PubMed]

35. Jin, D.; Li, B.; Deng, X.W.; Wei, N. Plant COP9 signalosome subunit 5, CSN5. Plant Sci. 2014, 224, 53–61. [CrossRef] [PubMed]

36. Gusmaroli, G.; Feng, S.; Deng, X.W. The Arabidopsis CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development. Plant Cell 2004, 16, 2984–3001. [CrossRef]
37. Dohmann, E.M.; Kuhnle, C.; Schwechheimer, C. Loss of the CONSTITUTIVE PHOTOMORPHOGENIC9 signalosome subunit 5 is sufficient to cause the cop/det/fus mutant phenotype in Arabidopsis. Plant Cell 2005, 17, 1967–1978. [CrossRef]

38. Wei, S.; Li, X.; Gruber, M.Y.; Feyissa, B.A.; Amyot, L.; Hannoufa, A. COP9 signalosome subunit 5A affects phenylpropanoid metabolism, trichome formation and transcription of key genes of a regulatory tri-protein complex in Arabidopsis. BMC Plant Biol. 2018, 18, 134. [CrossRef]

39. Hou, E.; Luo, Y.; Kuang, Y.; Chen, C.; Lu, X.; Jiang, L.; Luo, X.; Wen, D. Global meta-analysis shows pervasive phosphorus limitation of aboveground plant production in natural terrestrial ecosystems. Nat. Commun. 2020, 11, 637. [CrossRef]

40. Wang, X.; Yan, X.; Liao, H. Genetic improvement for phosphorus efficiency in soybean: A radical approach. Ann. Bot. 2010, 106, 215–222. [CrossRef]

41. Liang, C.; Wang, J.; Zhao, J.; Tian, J.; Liao, H. Control of phosphate homeostasis through gene regulation in crops. Curr. Opin. Plant Biol. 2014, 21, 59–66. [CrossRef]

42. Li, X.; Zeng, R.; Liao, H. Improving crop nutrient efficiency through root architecture modifications. J. Integr. Plant Biol. 2016, 58, 193–202. [CrossRef]

43. Zhu, S.; Chen, M.; Liang, C.; Xue, Y.; Lin, S.; Tian, J. Characterization of purple acid phosphatase family and functional analysis of GmPAP7a/7b involved in extracellular ATP utilization in soybean. Front. Plant Sci. 2020, 11, 661. [CrossRef] [PubMed]

44. Qin, L.; Sun, L.; Wei, L.; Yuan, J.; Kong, F.; Zhang, Y.; Miao, X.; Xia, G.; Liu, S. Maize SRO1e represses anthocyanin synthesis through regulating the MBW complex in response to abiotic stress. Plant J. 2021, 105, 1010–1025. [CrossRef] [PubMed]

45. Khan, F.; Hussain, S.; Khan, S.; Geng, M. Seed priming improved antioxidant defense system and alleviated Ni-induced adversities in rice seedlings under N, P, or K deprivation. Front. Plant Sci. 2020, 11, 565647. [CrossRef]

46. Qi, T.; Song, S.; Ren, Q.; Wu, D.; Huang, H.; Chen, Y.; Fan, M.; Peng, W.; Ren, C.; Xie, D. The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. Plant Cell 2011, 23, 1795–1814. [CrossRef] [PubMed]

47. Khan, F.; Hussain, S.; Khan, S.; Geng, M. Seed priming improved antioxidant defense system and alleviated Ni-induced adversities in rice seedlings under N, P, or K deprivation. Front. Plant Sci. 2020, 11, 565647. [CrossRef]

48. Qi, T.; Song, S.; Ren, Q.; Wu, D.; Huang, H.; Chen, Y.; Fan, M.; Peng, W.; Ren, C.; Xie, D. The Jasmonate-ZIM-domain proteins interact with the WD-Repeat/bHLH/MYB complexes to regulate Jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. Plant Cell 2011, 23, 1795–1814. [CrossRef] [PubMed]

49. Liang, J.; He, J. Protective role of anthocyanins in plants under low nitrogen stress. Biochem. Biophys. Res. Commun. 2018, 498, 946–953. [CrossRef] [PubMed]

50. Peng, W.; Wu, W.; Peng, J.; Li, J.; Lin, Y.; Wang, Y.; Tian, J.; Sun, L.; Liang, C.; Liao, H. Characterization of the soybean GmALMT family genes and the function of GmALMT5 in response to phosphate starvation. J. Integr. Plant Biol. 2018, 60, 216–231. [CrossRef]

51. Mo, X.; Zhang, M.; Liang, C.; Cai, L.; Tian, J. Integration of metabolome and transcriptome analyses highlights soybean roots responding to phosphorius deficiency by modulating phosphorylated metabolite processes. Plant Physiol. Biochem. 2019, 139, 697–706. [CrossRef]

52. Murphy, J.; Riley, J. A modified single solution method for the determination of phosphate in natural water. Anal. Chim. Acta 1962, 27, 31–36. [CrossRef]

53. Xie, Y.; Tan, H.; Ma, Z.; Huang, J. DELLA proteins promote anthocyanin biosynthesis via sequestering MYBL2 and JAZ suppressors of the MYB/bHLH WD40 complex in Arabidopsis thaliana. Mol. Plant 2016, 9, 711–721. [CrossRef] [PubMed]

54. Xue, Y.B.; Xiao, B.X.; Zhu, S.N.; Mo, X.H.; Liang, C.Y.; Tian, J.; Liao, H.; Miriam, G. GmPHR25, a GmPHR member up-regulated by phosphate starvation, controls phosphate homeostasis in soybean. J. Exp. Bot. 2017, 68, 4951–4967. [CrossRef]

55. Bhadouria, J.; Singh, A.P.; Mehra, P.; Verma, L.; Srivastava, R.; Parida, S.K.; Giri, J. Identification of purple acid phosphatases in chickpea and potential roles of CaPAP7 in seed phytate accumulation. Sci. Rep. 2017, 7, 11012. [CrossRef] [PubMed]

56. Chen, Z.; Sun, L.; Liu, P.; Liu, G.; Tian, J.; Liao, H. Malate synthesis and secretion mediated by a manganese-enhanced malate dehydrogenase confers superior manganese tolerance in Stylosanthes guianensis. Plant Physiol. 2015, 167, 176–188. [CrossRef]