J Ginseng Res 44 (2020) 321–331

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: http://www.ginsengres.org

Research Article

Overexpression of ginseng patatin-related phospholipase *pPLAIIIβ* alters the polarity of cell growth and decreases lignin content in *Arabidopsis*

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ARTICLE INFO

Article history:
Received 3 October 2018
Received in Revised form
31 December 2018
Accepted 16 January 2019
Available online 23 January 2019

Keywords:
Auxin
Cell elongation
Lignin
Panax ginseng
Patatin-related phospholipase

ABSTRACT

Background: The patatin-related phospholipase AIII family (*pPLAIIIs*) genes alter cell elongation and cell wall composition in *Arabidopsis* and rice plant, suggesting diverse commercial purposes of the economically important medicinal ginseng plant. Herein, we show the functional characterization of a ginseng *pPLAIII* gene for the first time and discuss its potential applications.

Methods: *pPLAIIIs* were identified from ginseng expressed sequence tag clones and further confirmed by search against ginseng database and polymerase chain reaction. A clone showing the highest homology with *pPLAIIIβ* was shown to be overexpressed in *Arabidopsis* using *Agrobacterium*. Quantitative polymerase chain reaction was performed to analyze ginseng *pPLAIIIβ* expression. Phenotypes were observed using a low-vacuum scanning electron microscope. Lignin was stained using phloroglucinol and quantified using acetyl bromide.

Results: The *PgpPLAIIIβ* transcripts were observed in all organs of 2-year-old ginseng. Overexpression of ginseng *pPLAIIIβ* (*PgpPLAIIIβ-OE*) in *Arabidopsis* resulted in small and stunted plants. It shortened the trichomes and decreased trichome number, indicating defects in cell polarity. Furthermore, *OE* lines exhibited enlarged seeds with less number per silique. The *YUCCA9* gene was downregulated in the *OE* lines, which is reported to be associated with lignification. Accordingly, lignin was stained less in the *OE* lines, and the expression of two transcription factors related to lignin biosynthesis was also decreased significantly.

Conclusion: Overexpression of *pPLAIIIβ* retarded cell elongation in all the tested organs except seeds, which were longer and thicker than those of the controls. Shorter root length is related to auxin-responsive genes, and its stunted phenotype showed decreased lignin content.

1. Introduction

Lipid acyl hydrolases are a diverse group of enzymes that release fatty acids from acyl lipids. Patatin-related phospholipases A (pPLAs), which are homologous to the potato (*Solanum tuber*) tuber storage protein patatin, are major lipid acyl hydrolases that are involved in diverse functions of plant cellular biology, such as cell growth regulation, signal transduction, membrane remodeling in response to environmental stresses, and lipid metabolism [1–5]. Based on gene structure and amino acid sequence similarity, 10 members of the *pPLA* family have been classified into three groups: *pPLAI*, *pPLAII* (*α*, *β*, *γ*, *δ*, and *ε*), and *pPLAIII* (*α*, *β*, *γ*, and *δ*) in *Arabidopsis* [5,6]. Both *pPLAI* and *pPLAIIs* have been shown to be involved in the response of plants to auxin signaling, pathogens, and phosphate deficiency [7–10]. pPLAIIIs share less similarity to "patatin" which share evolutionary conserved esterase motif GxGxG [11] instead of GxSxG [5,6]. Recently characterized *pPLAIIIs* exhibit broad substrate specificity with different kinetics [12–14] but result in small and stunted growth pattern, along with reduced cellulose content by *pPLAIIIβ* overexpression [12] and increased seed oil content by *pPLAIIIδ* overexpression [14,15]. Activation tagging of *pPLAIIIδ* [16] also decreases cell elongation and stunted growth, similar to that by *pPLAIIIβ* overexpression. In recessive rice mutant *dep3*, deficiency of *OspPLAIIIδ* resulted in dense and erect phenotype with shorter and wider epidermal cells [17]. Constitutive overexpression of rice *pPLAIIIα* was

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https://doi.org/10.1016/j.jgr.2019.01.004
p1226-8453 e2093-4947/$ – see front matter

characterized in rice and was found to exhibit *pPLAIIIβ*- and *pPLAIIIδ*-like overexpression phenotypes, but the content of several lipid species was lower than that in the wild-type ones [18]. Overexpression of *pPLAIIIβ* from *Arabidopsis* increased fatty acids and lysophospholipids [12]. It explains that the changed lipid species may not be involved in the phenotypes reported for all isoforms. Among 4 isoforms of *pPLAIIIs* in *Arabidopsis*, *pPLAIIIα* and *pPLAIIIβ* are the most abundantly and ubiquitously expressed [12], which suggests that both genes are involved in more general functions than others. Thus, considering the additional functions of *pPLAIIIs* as described previously, further characterization of *pPLAIIIβ* from other plant organism, *Panax ginseng* Meyer, was carried out to reveal the molecular function of it.

To shed light on the regulation of pPLAIII, overexpression of *pPLAIIIβ* from ginseng was characterized in heterologous *Arabidopsis*. Since the ginseng genome sequence has been available, functional characterization in *Arabidopsis* can be an alternative way to reconcile the function of genes. Altered growth patterns observed in other *pPLAIII* lines were also observed with the overexpression of ginseng *pPLAIIIβ* but with reduced lignin content instead of reduced cellulose content reported in other *pPLAIII* overexpressing lines [12]. Larger seed and faster germination characteristics observed by the overexpression of ginseng *pPLAIIIβ* can be utilized as useful agricultural traits together with lowered lignin content, especially for use of ginseng root cultures as forage.

2. Materials and methods

2.1. Plant materials and growth conditions

Korean ginseng (*Panax ginseng* Meyer "Chun-Poong") roots provided by the National Institute of Horticultural and Herbal Science (NIHHS) of the Rural Development Administration in Eumseong, Korea, were used in this study. Columbia ecotype (Col-0) of *Arabidopsis thaliana* was used as a wild-type heterologous system. *Arabidopsis* seeds were surface-sterilized with 70% ethanol for 1 min and 20% bleach for 3 min and washed twice with sterile water. The sterilized seeds were sown on 1/2 Murashige and Skoon (MS) medium (Duchefa Biochemie, Haarlem, the Netherlands) supplemented with 1% sucrose, 0.5 g/L of 2-[N-morpholino] ethanesulfonic acid (MES), and 0.8% phytoagar; the pH was adjusted to 5.7 with 1N KOH. Once sown on plates, the seeds were cold-treated for 48 h at 4°C in dark and transferred to a growth chamber under long-day conditions (16 h light/8 h dark) at 23°C.

2.2. Sequence analysis

The nucleotide sequence of full-length *PgpPLAIIIβ* (1398 bp) was identified from ginseng genome database (http://ginsengdb.snu.ac.kr/blast/blast.php) by homology-based search using the pPLAIII enzymes from *Arabidopsis*. The amino acid sequence was analyzed using the ProtParam tool (https://web.expasy.org/protparam/; Swiss Institute of Bioinformatics) and other online programs (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The amino acid sequence alignment was performed using the BioEdit program (software version 7.1.9). A phylogenetic tree was constructed by the neighbor-joining method using the MEGA7 (software version 6.06) program.

2.3. RNA isolation and quantitative reverse transcriptase polymerase chain reaction

The total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions, with some modifications. Genomic DNA contamination was eliminated by treatment of DNase I (Takara, Japan) in a total reaction volume of 100 μL for 1 h before the washing step. The concentration of total RNA was measured using a Nano-MD UV-Vis spectrophotometer (Scinco, Seoul, Korea). To synthesize the first strand of cDNA, 4 μg of total RNA was reverse transcribed using RevertAid Reverse transcriptase (Thermo, USA). The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the Thermal Cycler Dice real-time PCR system (Takara, Shiga, Japan). The total volume of the reaction mixture was 20 μL. The thermal cycler conditions were as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s, and additional 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s for dissociation. At the end of qRT-PCR, a dissociation curve was generated to evaluate the generation of by-products. To determine the expression of genes, the threshold cycle (Ct) value of each sample was normalized using *β-actin* and calculated relative to a calibrator by the $2^{-\Delta\Delta Ct}$ method. Three independent experiments were performed for each primer set (Table S1). The gene-specific primers for *PgpPLAIIIβ* were 5′–GTA ATT TCC GAT ACC GGA G–3′ (forward) and 5′–TTT TCC CTT TCC ACC ACG–3′ (reverse). The primers for *Arabidopsis β-actin* were 5′–GTG TGT CTT GTC TTA TCT GGT TCG–3′ (forward) and 5′–AAT AGC TGC ATT GTC ACC CGA TAC T–3′ (reverse). The primers for ginseng *β-actin* (DC03005B05) were 5′–AGA GAT TCC GCT GTC CAG AA–3′ (forward) and 5′–ATC AGC GAT ACC AGG GAA CA–3′ (reverse).

2.4. Transgenic construct and in planta transformation

To characterize the function of *PgpPLAIIIβ*, full length of *PgpPLAIIIβ* (1398 bp) was amplified using ginseng Chun-Poong cDNA by PCR. The *PgpPLAIIIβ* cDNA was amplified using primers containing the SalI and SpeI sites as follows: 5′–AA GTC GAC ATG GCT TCT AAT CCC TCA–3′ and 5′–CG ACT AGT CTA GGT GGG TTT CTG GTT AGC–3′. The PCR product was cloned into the cloning sites of the pCAMBIA1300 vector driven by the cauliflower mosaic virus 35S promoter. The *PgpPLAIIIβ* overexpression construct was confirmed by nucleotide sequencing. The construct was transformed into *Arabidopsis* Col-0 using *Agrobacterium tumefaciens* C58C1 (pMP90) [19]. Transgene insertion was confirmed by the PCR of the transformants. Homozygous plants with a 3:1 segregation ratio were selected on plates containing hygromycin (50 μg/mL) for further analyses. For data analysis, Col-0 and empty vector lines were used as control of *PgpPLAIIIβ* overexpression lines.

2.5. Histochemical staining of lignin using phloroglucinol-HCl

To visualize the lignin content, stems of 7-week-old plants were cut into sections of width 100 μm using a razor blade. The sections were treated with saturated phloroglucinol in HCl and immediately observed under a light microscope. Mäule staining of syringyl (S) lignin was also performed with the stem sections by the following method. The stem sections were submerged in 0.5% potassium permanganate solution and washed with H_2O three times. After washing, 1 mL of 3% HCl was treated for 5 min, and then concentrated ammonium hydroxide solution was immediately added. The stained sections were observed under a light microscope.

2.6. Lignin content analysis

The lignin content in the stem was quantified by the acetyl bromide method [20]. The stems of 7-week-old plants were ground with liquid nitrogen and freeze-dried for 48 h. To obtain raw crude cell wall residue, 10 mg of dried material was treated

with 95% EtOH for four times and with distilled water twice sequentially. After 12 h of drying at 60°C, the product was dissolved in 25% acetyl bromide (% v/v in glacial acetic acid) and incubated at 70°C for 30 min. After incubation, 0.9 mL of 2 M NaOH, 3 mL of acetic acid, and 0.1 mL of 7.5 M hydroxylamine HCl were added sequentially and centrifuged at 4000 × *g* for 10 min. The supernatant was diluted 20-fold with glacial acetic acid. The absorbance of the sample was measured at 280 nm using a spectrophotometer.

2.7. Cellulose content analysis

The cellulose content was determined following a previously reported protocol [21]. Seven-week-old whole primary stems cut 50 mm above the ground were treated sequentially with 70% EtOH and acetone and then air-dried at 37°C. Thus, alcohol-insoluble residues were determined as the weight of cell wall material before acetic/nitric acid and 67% sulfuric acid treatments. Hemicellulose and lignin were removed by treating with acetic/nitric reagent. Crystalline cellulose is resistant to acetic/nitric reagent but gets disordered upon treatment with 67% H_2SO_4, producing monomeric sugars that can be measured by the colorimetric method at 620 nm using 0.3% anthrone as a dye. The cellulose content was calculated based on the standard curve of D-glucose.

3. Results and discussion

3.1. Isolation and identification of ginseng pPLAIII genes

There are four homologs of patatin-related phospholipases *pPLAIII (-α, -β, -γ,* and *-δ), AtpPLAIIIs*, in *Arabidopsis* [5,6]. To identify genes coding patatin-related phospholipase, expressed sequence tag clones showing amino acid sequence similarity with *pPLAIIIs* from *Arabidopsis* were selected from previously constructed expressed sequence tag libraries [22]. After rapid amplification of cDNA ends PCR, full-length complementary DNA (cDNA) sequences of *PgpPLAIIIs* (*PLAIII* from *P. ginseng*) were obtained. Using the Basic Local Alignment Search Tool (BLAST) tool, these *PgpPLAIIIs* were searched against the ginseng genome database constructed by the Seoul National University, Korea (http://ginsengdb.snu.ac.kr/blast/blast.php), and 10 more closely related *PgpPLAIII* genes (α, β, γ, δ, ε, ζ, η, θ, ι, κ, and λ) were identified (Fig. 1A). These findings suggest that the ginseng genome contains a family of 10 putative genes that are homologous with the known *pPLAIII* genes. PgpPLAIIIβ protein was grouped close to AtpPLAIIIs (pPLAIIIs from *A. thaliana*): 61.3%, 61.5%, 57% and 37% of amino acid identity with AtpPLAIIIα, AtpPLAIIIβ, AtpPLAIIIγ, and AtpPLAIIIδ, respectively (Fig. 1A). PgpPLAIIIβ showed the closest identity with two pPLAIII proteins, AtpPLAIIIα and AtpPLAIIIβ, with 1% difference. Thus, we decide to characterize *PgpPLAIIIβ* gene, which can lead us to speculate the relevant roles of functional ortholog of two abundantly expressed genes [12]. *PgpPLAIIIβ* is represented by a single gene encoding a protein of 465 amino acids with a predicted pI of 8.33 and molecular mass of 50.3 kDa using ProtParam [23].

The recently characterized pPLAIIIs contain noncanonical esterase box motif GX**G**XG instead of catalytic serine-containing motif GX**S**XG [5,6], but they still exhibit lipase activity with broad substrate specificity [12–14]. All PgpPLAIII proteins, including PgpPLAIIIβ, also contained the GX**G**XG motif (Fig. 1B) instead of the GX**S**XG motif. The Ser (S) in the GX**S**XG motif and the Asp (D) residue in the conserved DGG motif are recognized as critical amino acids in the catalytic S-D dyad [12]. This indicates that the putative S-D catalytic dyad is not present. However, the second residue of the putative catalytic S-D dyad, aspartate (D), was present in the DGG motif of *PgpPLAIII* genes (α, β, γ, δ, ε, ζ, and η), except in the *PgpPLAIII* genes (γ, θ, ι, κ, and λ) (Fig. 1B). The phosphate- or anion-binding element was replaced into DGGGXX**N** in the PgpPLAIII (α, β, and γ) protein, which was mostly conserved with DGGGXX**G** [12–14].

3.2. Organ-specific expression pattern of PgpPLAIIIβ

From the age of two years, ginseng plants have five leaves and the number of petioles increases with the number of years of cultivation (Fig. 2A, inset). To understand the expression patterns of *PgpPLAIIIβ*, all organs obtained from 2-year-old ginseng plants were analyzed by qRT-PCR (Fig. 2A). The expression of *PgpPLAIIIβ* was relatively higher in the leaves, with the highest expression in the first leaves, followed by a similar level of expression in the stem, petiole, and roots. So far, functional characterization of *pPLAIIIβ* has been performed only in *Arabidopsis* [12]. *AtpPLAIIIβ* has been reported to be expressed in all organs at various developmental stages with the highest in roots [12]. Thus, *PgpPLAIIIβ* seems to play major roles in the leaves than in the roots. In 4-year-old ginseng roots, the transcripts were evenly distributed in all parts of the root with the highest in rhizome (Fig. 2B). Rhizome is the most important organ in a perennial ginseng plant, which is the main initiation site for the annual growth. Thus, it suggests that *pPLAIIIβ* might play important roles for annual regeneration from the rhizome.

3.3. Overexpression of PgpPLAIIIβ reduced plant height and changed the polarity of cell elongation

Overexpression of patatin-related phospholipase *pPLAIIIβ* in *Arabidopsis* resulted in stunted and dwarf phenotypes with altered cell elongation patterns [12]. To analyze whether ginseng *PgpPLAIIIβ* also exhibits similar morphological characteristics, heterologous overexpression was carried out. Full-length cDNA encoding *PgpPLAIIIβ* was cloned into a binary vector and transformed into *Arabidopsis* using *Agrobacterium tumefaciens* [19]. Three selected lines following Mendelian segregation were chosen for the primary study. *PgpPLAIIIβ* was not expressed in Col-0 and vector control, indicating the specificity of primers used for qRT-PCR. The expression of *PgpPLAIIIβ* was on an average 110-fold higher in lines 5 and 6 and 18-fold higher in line 12 than that in the controls using samples from 4-week-old leaves (Fig. 3A). Generally, this expression patterns were kept except seedling stages, where line 12 showed the strongest transcript levels in 2-week-old seedlings and roots (Supplementary 1). The higher the expression of *PgpPLAIIIβ*, the more stunted and dwarfed the leaf phenotype was (Fig. 3B, Supplementary Fig. 2A). For further characterization, line 6, in which the expression of *PgpPLAIIIβ* was the highest in the leaf (Fig. 3A) and stem (Supplementary Fig. 2B), was chosen. The overexpressing lines driven by the 35S promoter (Fig. 3C, inset) expressed *PgpPLAIIIβ* in all the organs and presented extremely small plant height with reduced number of small rosette leaves (Fig. 3C, Supplementary Fig. 2A and C).

Besides basic pavement cells, leaf epidermis contains specialized single-cell layer called trichome. On adaxial and abaxial sides of cauline and rosette leaves, cell expansion pattern was observed in the pavement and stem cells of the overexpressing lines (Fig. 3D). Cell expansion was more apparent in the four-branched trichomes (Fig. 3D) with reduced length (Supplementary Fig. 3). Considering that the trichomes are predominantly three-branched on the adaxial surface of the rosette and cauline leaves of Col-0 [24], more branched trichomes indicate that the proteins regulating cell polarity or signaling molecules are altered.

Overall, overexpression of *OspPLAIIIδ* homolog from *Oncidium* also caused smaller and rounder flowers which is reminiscence to that of *PgpPLAIIIβ-OE*, and this phenotype was explained by the

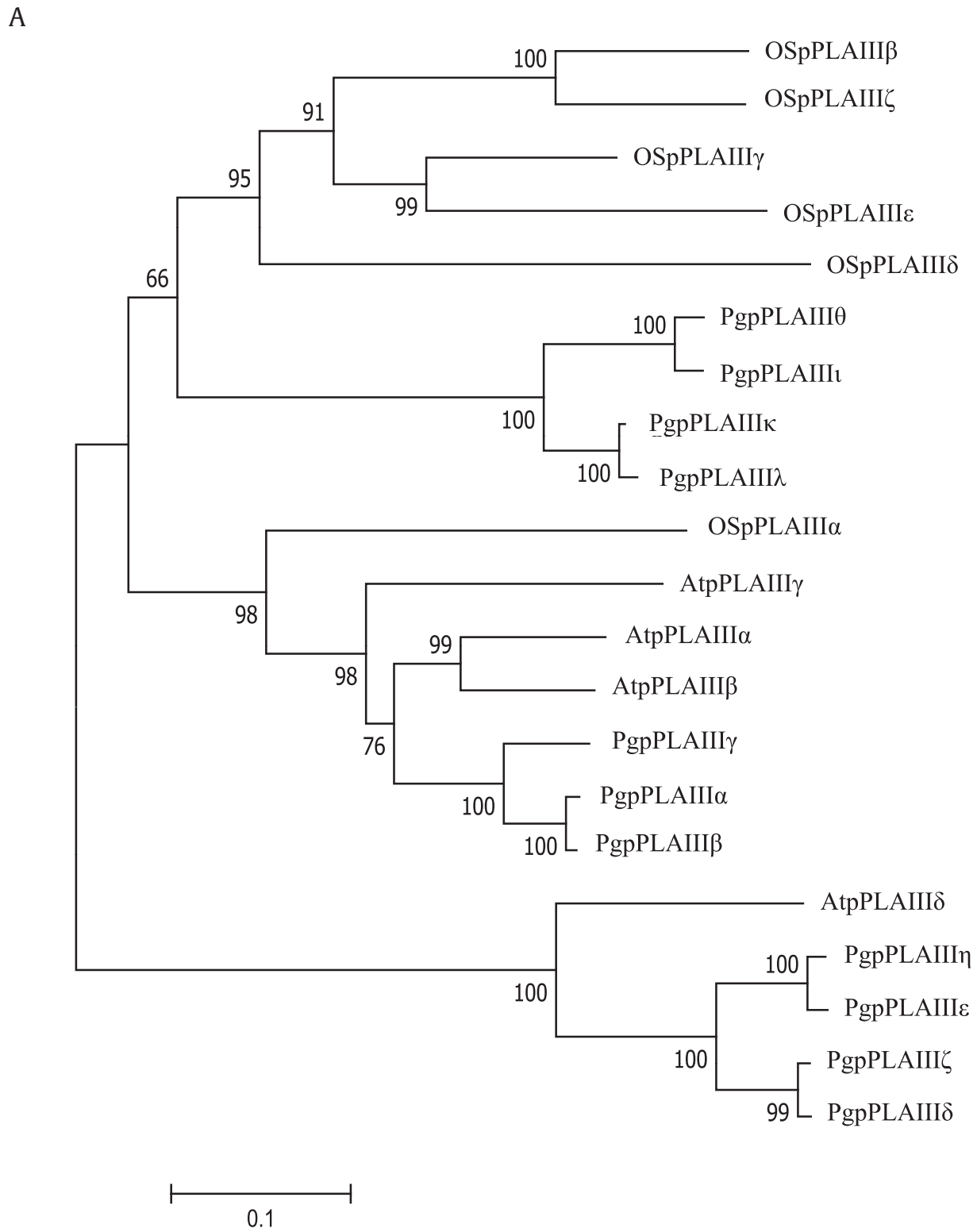

Fig. 1. Ginseng-derived PgpPLAIII proteins are closely related to other pPLAIII proteins. (A) Phylogenetic tree of PgpPLAIII proteins with the closest homologous proteins from *Arabidopsis* and rice. The phylogenetic tree was constructed using the ClustalX program (neighbor-joining method). *At, Arabidopsis thaliana; Os, Oryza sativa; Pg, Panax ginseng*. The GenBank accession numbers are AtpPLAIIIs: pPLAIIIα, (At2g39220), pPLAIIIβ (At3g54950), At4g29800 (pPLAIIIγ), and pPLAIIIδ (At3g63200) and OspPLAIIIs: OspPLAIIIα (LOC_Os03g14950), OspPLAIIIβ (LOC_Os03g43880), OspPLAIIIγ (LOC_Os03g57080), OspPLAIIIδ (LOC_Os06g46350), OspPLAIIIε (LOC_Os07g05110), and OspPLAIIIζ (LOC_Os12g41720). The bar represents 0.1 substitution per amino acid position. (B) Alignment of PgpPLAIIIβ protein with its closest homologs. The red-dotted box motifs represent the anion-binding box, esterase box, and catalytic dyad–containing motif.

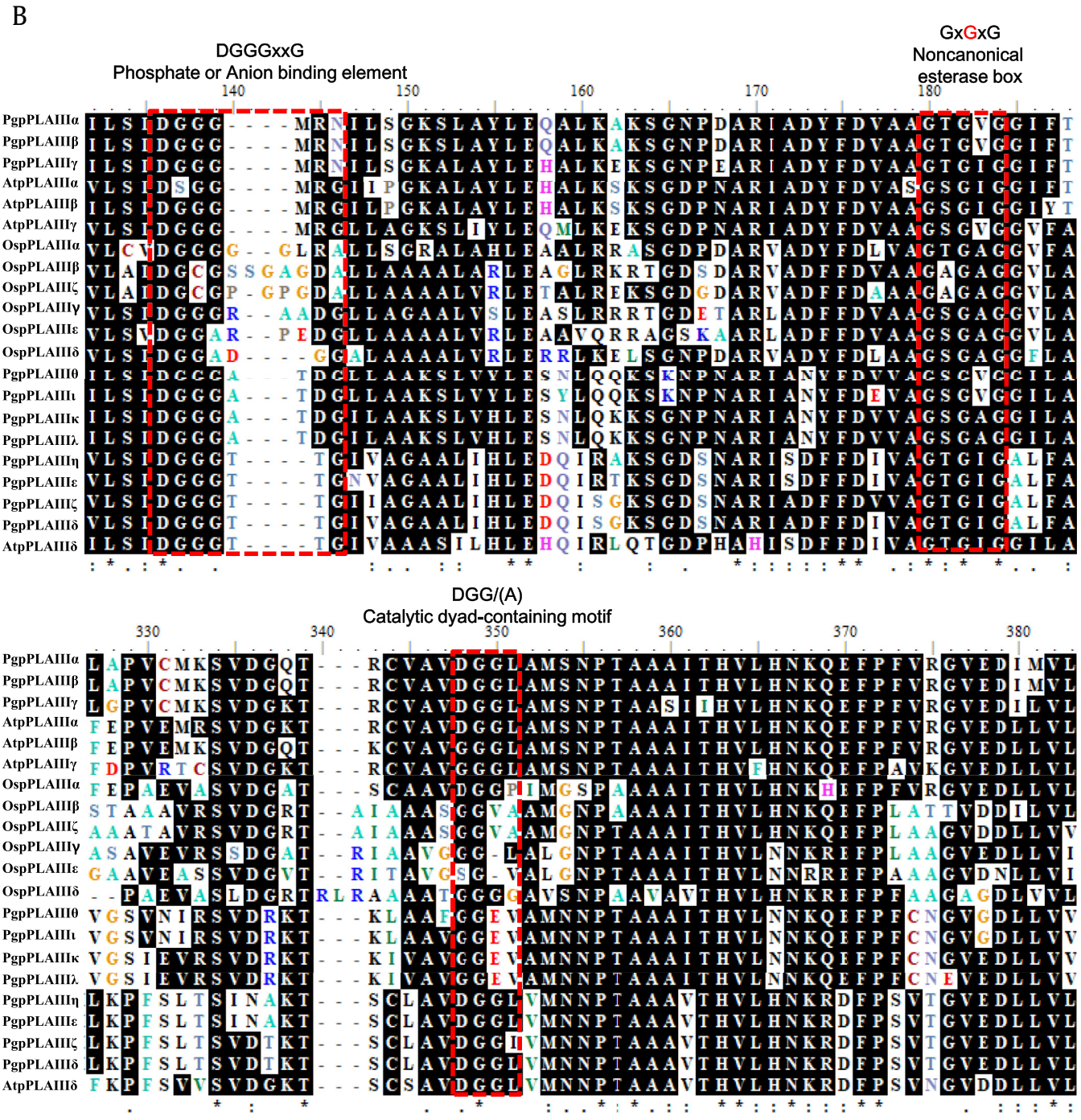

Fig. 1. (*continued*).

reduction of bioactive gibberellin A_3 (GA_3) with concurrent reduced transcription levels of GA biosynthetic genes *GA3ox1* and *GA20ox1* [13]. *GA 20-oxidase* (*GA20ox*) and *GA3ox* are involved in the last two steps of bioactive GA biosynthesis, and *GA2oxs* are responsible for the deactivation of bioactive GA. In *PgpPLAIIIβ-OE* lines, both *GA3ox1* and *GA20ox1* are transcriptionally reduced, but the *GA2ox2* is increased (Fig. 4), which indicates that more bioactive GAs are decreased. It suggests that the stunted plant height and smaller leaves are caused by the reduction of GA in one part.

3.4. Overexpression of PgpPLAIIIβ enlarges seed size and improved germination rate

Overexpression of rice *pPLAIIIa* in rice [18] and *Arabidopsis pPLAIIIδ* in camelina [15] increased seed width, but the length of the seeds was reduced or not changed significantly. The recessive rice mutant *dep3*, in which a part of *pPLAIIIδ* gene is deleted, showed smaller and rounder seeds, but more grain yield [17]. Thus, it seems likely that the modified seed size is host-dependently variable,

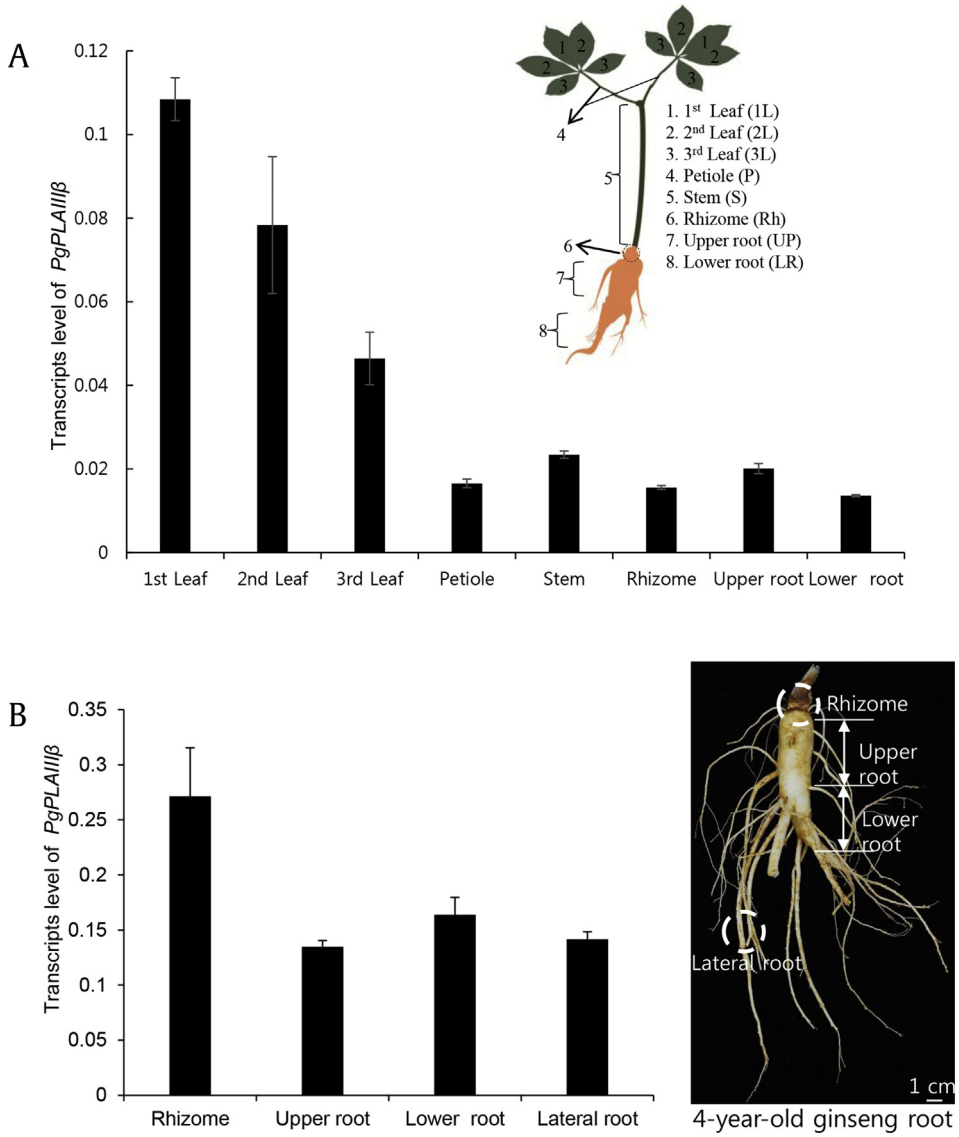

Fig. 2. Organ-specific expression pattern of *PgpPLAIIIβ*. (A) Differential expression pattern of *PgpPLAIIIβ* in 2-year-old ginseng plants by the qRT-PCR. Exact organ nomenclature for 2-year-old ginseng plants is indicated on the right inset. (B) Differential expression pattern of *PgpPLAIIIβ* in 4-year-old ginseng roots. The data are presented as mean ± SD of three independent replicates.
qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SD, standard deviation.

which suggests that the different number of isoforms existing in different plants might behave synergistically or antagonistically. In the present study, we found that the overexpression of *PgpPLAIIIβ* increased not only seed width but also seed length (Fig. 5A and B). To verify whether other endogenous gene expression of *pPLAIIIs* altered by the overexpression of *PgpPLAIIIβ* could cause the enlarged seed, qRT-PCR was performed (Supplementary Fig. 4). It explains that the endogenous *pPLAIII* isoforms are growth stage–differentially downregulated or upregulated, which suggests that the cross-talks between *pPLAIII* isoforms and other growth-controlling signals are developmentally linked to regulate each organ sizes. However, the number of seeds per silique decreased by 64% compared with the controls (Fig. 5C). Altered seed morphology prompted us to test the initial germination rate after seed imbibition. Within 20 h after seed imbibition, the initial germination rate increased by 1.5 times in the overexpression line compared with that in Col-0 (Fig. 5D). This indicates that heterologous expression of ginseng *PgpPLAIIIβ* gene changes the seed size and also enhances the initial germination rate.

3.5. *PgpPLAIIIβ-mediated reduced primary root length is via increased auxin-responsive gene expression*

To verify whether the stunted and dwarf phenotype observed in *PgpPLAIIIβ* overexpressing lines is also visible in underground parts of the plant, the root phenotype was analyzed (Fig. 6A). Eight-day-old seedling of each overexpressing line exhibited 15% (No. 6) and 20% (No. 12) shorter root length than that of Col-0 (Fig. 6B). However, the number of lateral roots was higher in the overexpressing lines than in the control (Fig. 6C). *Arabidopsis* seedlings grown on auxin-containing media developed shorter primary roots and more lateral roots than those of plants grown on media without exogenous auxin [25]. Thus, our data indicate the involvement of auxin-responsive genes in the growth of roots.

Several auxin response–related genes, such as *Aux/IAA*, *GH3*, and *SMALL UP RNA* (*SAUR*) genes, were quantified for the relative transcripts level in several overexpressing lines and compared with those in the control. The transcripts of *IAA2*, *IAA11*, and *GH3.5* did not change significantly (Fig. 6D). However, the relative transcripts

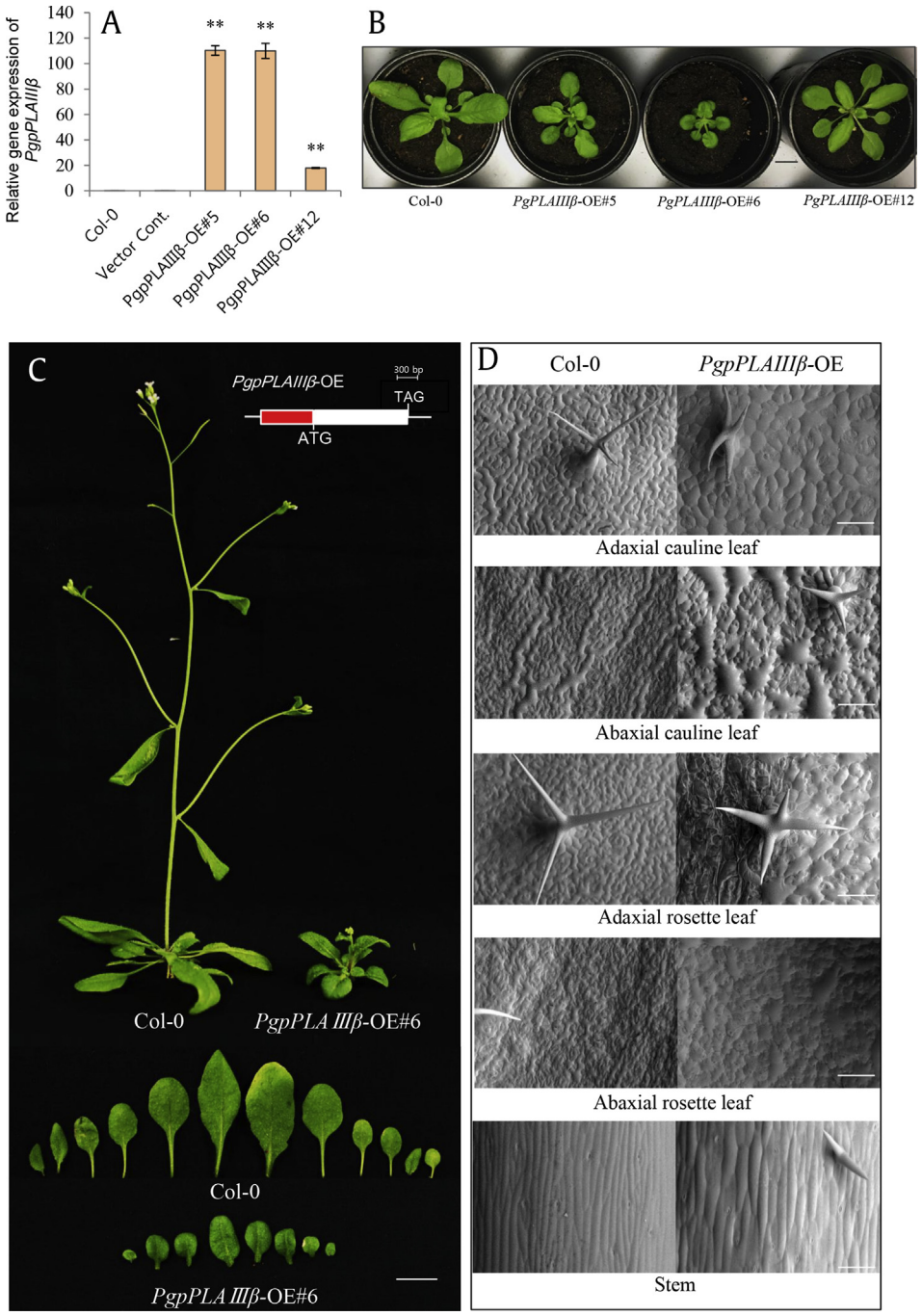

Fig. 3. Heterologous overexpression of *PgpPLAIIIβ* caused dwarf plant with altered cell polarity in the trichome. (A) Transcript level of *PgpPLAIIIβ* from 4-week-old leaves of three overexpressing lines. The data are presented as mean ± SE of three independent replicates at $P < 0.05$ (*) and $P < 0.01$ (**). (B) Transgenic plants were smaller than Col-0 plants. (C) Overexpressing lines exhibited stunted and dwarf phenotype with smaller and less number of leaves. Scale bar = 1 cm. Construction map of overexpression under the 35S promoter is depicted in inset. (D) Cell growth patterns are altered in the *OE* lines. Scale bar = 100 μm. All surface images were taken using a low-vacuum scanning electron microscope (JSM-IT300, JEOL Korea) at 10.8-mm working distance and 20.0 kV.
SE, standard error.

of *SAUR9* and *YUCCA8* (*YUC8*) and *YUCCA9* (*YUC9*) exhibited an overall increase (Fig. 6D) in the 8-day-old seedlings. However, these auxin response genes, especially the *YUCCA* genes, are modulated depending on the developmental stage (Fig. 6E). YUCCA, a flavin monooxygenase (FMO)–like enzyme, catalyzes tryptophan-dependent auxin biosynthesis [26]. Of the two *YUCCCA* genes—*YUCCA 8* and *YUCCA9*—which are reported to be involved in lignification [27], *YUCCA9* was significantly downregulated in the fully grown 7-week-old stems (Fig. 6E). Thus, the auxin-related short and stunted phenotype of *PgpPLAIIIβ* overexpressing lines suggest that it might also be linked with lignin biosynthesis.

3.6. Lignin content was decreased by constitutive overexpression of *PgpPLAIIIβ*

Plant cell walls are composed of a complex matrix of three organic compounds such as cellulose, hemicellulose, and lignin. Lignin is primarily involved in the maintenance of plant structural

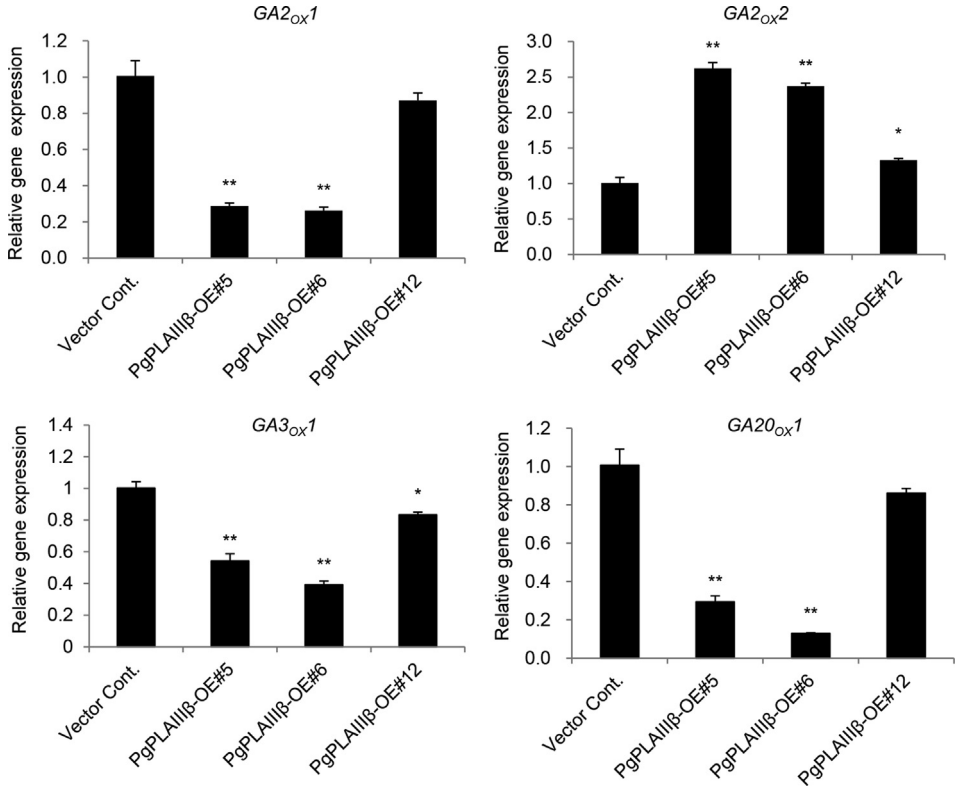

Fig. 4. Quantification of gibberellin oxidases by the overexpression of *PgpPLAIIIβ*. Relative gene expression patterns of four gibberellin oxidases (*GA2ox1*, *GA2ox2*, *GA3ox1*, and *GA20ox1*) in 6-week grown plant stems. The data are presented as mean ± SE of three independent replicates at $P < 0.05$ (*) and $P < 0.01$ (**).

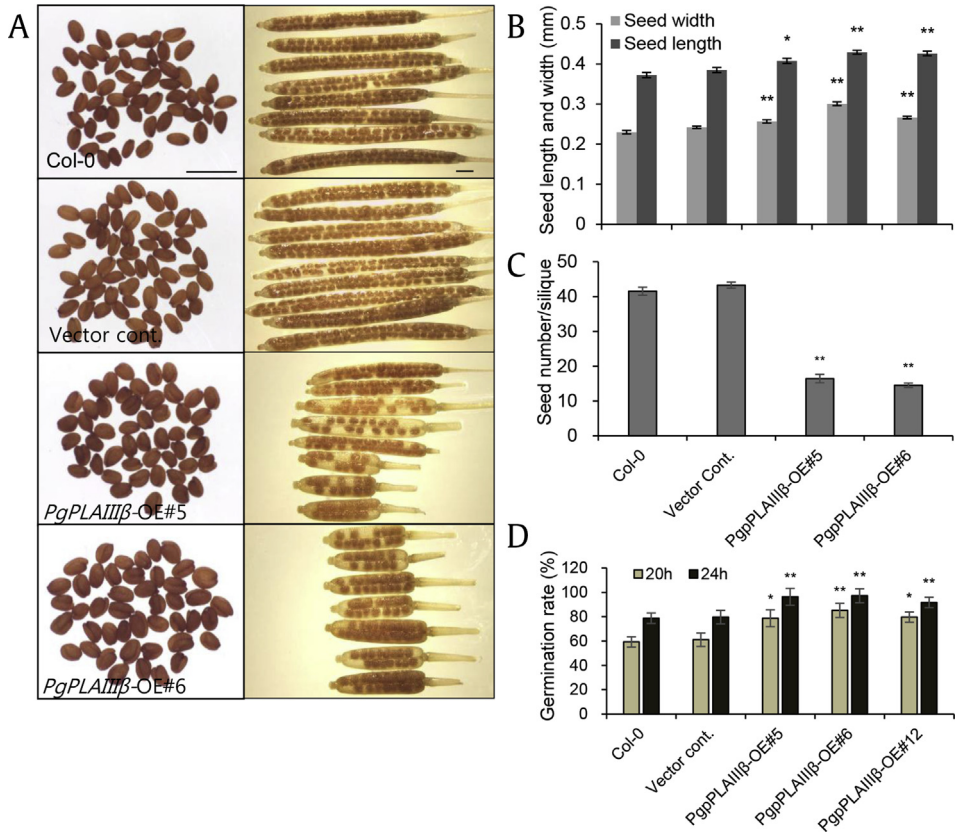

Fig. 5. Germination rate is regulated by the function of *PgpPLAIIIβ* with the increase in seed size. (A) The number of individual mature seeds increased but the number of siliques reduced in the *OE* lines. Scale bar = 1 mm. (B) Seed length and width were measured from mature seeds n = 35–51. Scale bar = 1 mm. (C) Seed number per each silique was reduced in *OE* lines. n = 19–27. (D) Germination was faster in the *OE* lines after 20 h and 24 h of germination under light condition. n = 36. The data are presented as mean ± standard error (SE) of three independent replicates at $P < 0.05$ (*) and $P < 0.01$ (**).

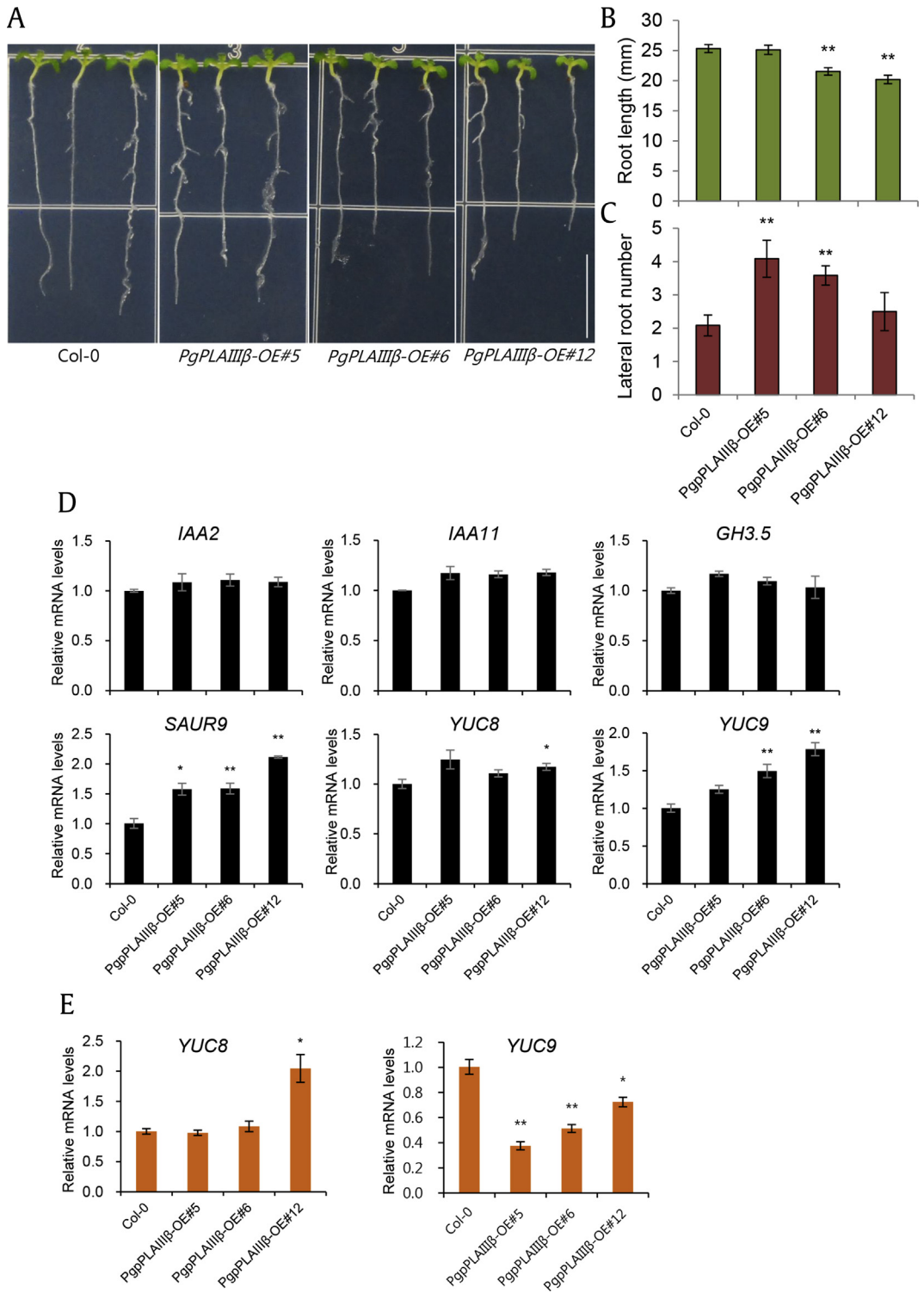

Fig. 6. Reduced primary root length of *PgpPLAIIIβ-OE* line is regulated by auxin-responsive genes. (A) Eight-day-old roots were shorter in *OE* lines than those in Col-0. Scale bar = 1 cm. (B) Root length and (C) the number of lateral roots in the *OE* lines. n = 11. (D) Transcript level of auxin-responsive genes was quantified by qRT-PCR using 8-day-old seedlings. (E) Transcript level of *YUCCA8* and *YUCCA9* was quantified in 7-week-old stems by qRT-PCR. The data are presented as mean ± SE of three independent replicates at $P < 0.05$ (*) and $P < 0.01$ (**).
qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SE, standard error.

and mechanical integrity, and it is a main component of secondary cell walls [28]. Auxin is associated with the regulation of most aspects of plant growth and development, including secondary growth of the stem [27]. Overexpression of *PgpPLAIIIβ* altered the expression of auxin response–related genes, especially the transcripts of *YUCCA9* that are involved in lignification, decreased in the stems (Fig. 6E). Therefore, we evaluated the amount of lignin (Fig. 7). Overexpression of *PgpPLAIIIβ* caused cell expansion (Fig. 7A) and reduced ultimate plant height. Phloroglucinol-HCl interacts with coniferaldehyde and sinapaldehyde end groups,

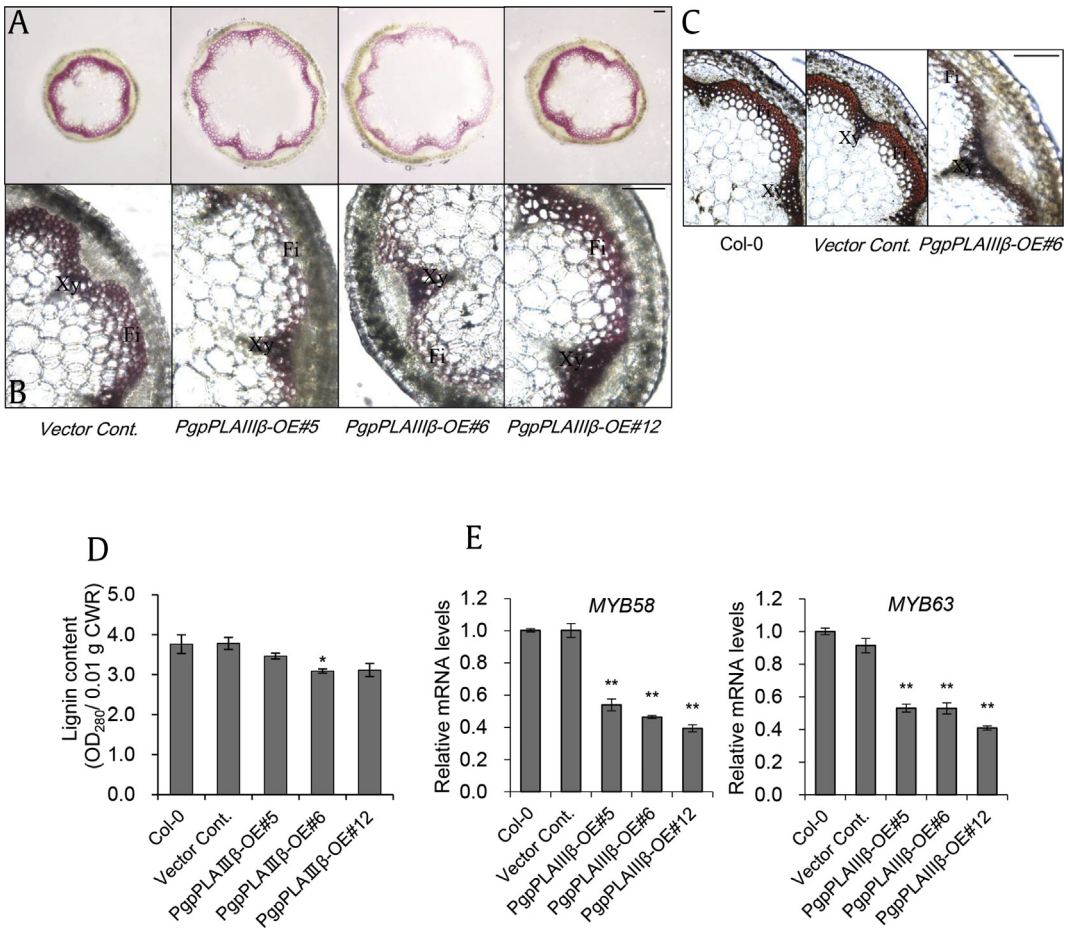

Fig. 7. Lignin content in the stems of *PgpPLAIIIβ-OE* lines decreased. (A and B) Histochemical staining of cross sections from second internode of 7-week-old stems from Col-0 and *PgpPLAIIIβ-OE* lines using phloroglucinol-HCl. Scale bars = 100 μm. (C) Mäule staining for S lignin staining. Scale bars = 100 μm. (D) Lignin content in 7-week-old stems determined using acetyl bromide. (E) Relative gene expression of transcription factors, *MYB58* and *MYB63*, involved in lignin biosynthesis decreased. The data are presented as mean ± SE of three independent replicates at $P < 0.05$ (*) and $P < 0.01$ (**).
SE, standard error.

and it is often used to stain lignifying cell walls [29]. All lines were stained red with phloroglucinol-HCl, but the staining was weaker in overexpressing lines, especially the interfascicular fibers (Fig. 7 A and B). Relatively weak expressing No. 12 *OE* line (Supplementary Fig. 2B) exhibited staining level similar to that of vector control (Fig. 7 A and B). This weaker staining can be explained by the increased expression of *YUCCA8*, offsetting the decreased expression of *YUCCA9* (Fig. 6E). The intensity of Mäule stain (red color), which specifically detects the syringyl (S) lignin units in xylem and interfascicular fibers, was also visibly decreased (Fig. 7C). The weakest staining was found in the highly expressing No. 6 *OE* line (Fig. 7A–C). This was consistent with the results obtained through direct lignin quantification using acetyl bromide, showing that the lignin content decreased significantly in highly expressing line (Fig. 7D). Two well-known transcription factors involved in lignin biosynthesis [30], *MYB58* and *MYB63*, were also concomitantly reduced (Fig. 7E).

Previously, it has been reported that the cellulose content increased in a knockout mutant of *pPLAIIIβ* and decreased in overexpressing lines, which showed a small and stunted phenotype [12], similar phenotype to *PgpPLAIIIβ*. Thus, we expected that the content of cellulose decreased and possibly lignin content increased to compensate the loss of cellulose or it was not altered. However, cellular cellulose was confirmed by Congo red staining and direct quantification (Supplementary Fig. 5), not to be changed. This indicates that ginseng *pPLAIIIβ* alters the functions of other homologous *pPLAIII* genes (Supplementary Fig. 4) and behaves differently for cell wall composition, when compared with *Arabidopsis pPLAIIIβ*. To the best of our knowledge, no functional study focused on lignin biosynthesis in ginseng and/or by *pPLAIII* genes was reported. Thus, this study is the first to report that the function of *PgpPLAIIIβ* reduced lignin content and reduced whole plant height by altering the polarity of cell elongation when overexpressed.

4. Conclusion

Studies on the pPLAIII group members, which lack a canonical catalytic serine motif, have been limited. Several *pPLAIII* genes have been studied in *Arabidopsis* [12,14] and rice [17,18], but the functional characterization of a ginseng-derived *pPLAIII* homolog has not been reported. The *pPLAIII* family from ginseng comprises 10 genes (*viz.* α, β, γ, δ, ε, ζ, η, θ, ι, κ, λ) (Fig. 1). *PgpPLAIIIβ* encodes a protein of 465 amino acids, and it is expressed in all organs of 2-year-old ginseng, with the highest expression in the leaves (Fig. 2). Heterologous overexpression of ginseng *pPLAIIIβ* in *Arabidopsis* severely altered cell elongation patterns and resulted in dwarf phenotype and loss of polarity in trichome cell elongation (Fig. 3). Reduced plant height and smaller leaf size are related with the reduced transcript levels of GA oxidases (Fig. 4). *PgpPLAIIIβ-OE* also resulted in distinct enlarged seed size and faster germination within 20 h of seed imbibition (Fig. 5). Shorter root length

correlated with increased expression of auxin-responsive genes at the seedling stage (Fig. 6). *PgpPLAIIIβ*-mediated suppression of *YUCCA9* transcripts (Fig. 6E) in fully grown plant stems was associated with the decrease in lignin content in *PgpPLAIIIβ-OE* (Fig. 7).

Enlarged seed size and enhanced initial germination are valuable agricultural traits, especially for pharmaceutically important medicinal plants such as ginseng [31]. Lignin is considered an antiquality component in forages owing to its negative effect on nutritional availability and digestibility of plant fiber [32]. Adventitious roots of ginseng are commercial resources for the production of ginsenosides [33]. Thus, the micropropagation of adventitious root cultures from ginseng by manipulating *PgpPLAIIIβ* can be a useful approach to enhance the digestibility of ginseng by reducing lignin content.

Conflicts of interest

The authors have no conflicts of interest to declare

Acknowledgment

This study was supported by a basic science research program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, & Future Planning (grant number: 2016R1A2B4012016).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2019.01.004.

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