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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A R T I C L E   I N F O

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

A B S T R A C T

Background: The patatin-related phospholipase AII family (pPLAIIls) 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.

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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 pPLAIIIs have been shown to be involved in the response of plants to auxin signaling, pathogens, and phosphate deficiency [7–10]. pPLAIIls 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 pPLAIIIβ overexpression [13,14]. Activation tagging of pPLAIIIβ [16] also decreases cell elongation and stunted growth, similar to that by pPLAIIIβ overexpression. In recessive rice mutant dep3, deficiency of OspPLAIIIβ resulted in dense and erect phenotype with shorter and wider epidermal cells [17]. Constitutive overexpression of rice pPLAIIIα was
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, pPLAIIIA 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 pPLAIIIs were also observed with the overexpression of ginseng pPLAIIIβ but with reduced lignin content instead of reduced cellulose content reported in other pPLAIIIs 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]ethane sulfonic acid (MES), and 0.8% phytagar; 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^{-ΔΔCt} method. Three independent experiments were performed for each primer set (Table S1). The gene-specific primers for PgpPLAIIIβ were 5′–GTA ATT TCT GAT ACC GGA G−3′ (forward) and 5′–TTT TCT CTT TTC ACC AGG−3′ (reverse). The primers for Arabidopsis β-actin were 5′–GTC TGT CTT TGT TCA GTC AGT CGT−3′ (forward) and 5′–ATG ACC TGC ATT GTC ACC CCG TAC T–3′ (reverse). The primers for ginseng β-actin (DC03005B05) were 5′–AGA GAT TCC GCT GCT 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 Sall and Spel sites as follows: 5′–AAT GAC ATT TGT CTT TCT GAT ACC CCA GAG A−3′ (forward) and 5′–AAT ACC TGC ATT GTC ACC CCG TAC T–3′ (reverse). 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 the stem sections were treated with saturated phloroglucinol in HCl and stained with a 1% aqueous alcoholic ferric chloride solution. The stems of 7-week-old seedlings were used for staining.

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 for 50 mm above the ground were treated sequentially with 70% EtOH 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. Hemicyclic 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% H2SO4, 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), AtPpPLAIIIs, 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]. Rapid amplification of cDNA ends PCR, full-length complementary DNA (cDNA) sequences of PgpPpPLAIIIIs (PLAIIIs from P. ginseng) were obtained. Using the Basic Local Alignment Search Tool (BLAST) tool, these PgpPpPLAIIIs 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 PpPLAIIIIs (PLAIII from P. ginseng) 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. PgpPpPLAIII protein was grouped close to AtPpPLAIII (pPLAIIIs from A. thaliana): 61.3%, 61.5%, 57% and 37% of amino acid identity with AtPpPLAIIz, AtPpPLAIIβ, AtPpPLAIIγ, and AtPpPLAIIδ, respectively (Fig. 1A). PgpPpPLAIII showed the closest identity with two pPLAIIIIs proteins, AtPpPLAIIz and AtPpPLAIIδ, with 1% difference. Thus, we decide to characterize PgpPpPLAIII gene, which can lead us to speculate the relevant roles of functional ortholog of two abundantly expressed genes [12]. PgpPpPLAIII is represented by a single gene encoding a protein of 465 amino acids with a predicted pl of 8.33 and molecular mass of 50.3 kDa using ProtParam [23].

The recently characterized pPLAIIIs contain noncanonical esterase box motif GXGXXG instead of catalytic serine-containing motif GXGXG [5,6], but they still exhibit lipase activity with broad substrate specificity [12–14]. All PgpPpPLAIIIs proteins, including PgpPpPLAIII, also contained the GXGXXG motif (Fig. 1B) instead of the GXGXG motif. The Ser (S) in the GXGXG 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 PgpPpPLAIII genes (α, β, γ, δ, ε, ζ, and η), except in the PgpPpPLAIII genes (γ, θ, ι, κ, and λ) (Fig. 1B). The phosphate- or anion-binding element was replaced into DGGXXN in the PgpPpPLAIII (α, β, and γ) protein, which was mostly conserved with DGGGXG [12–14].

3.2. Organ-specific expression pattern of PgpPpPLAIII

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 PgpPpPLAIII, all organs obtained from 2-year-old ginseng plants were analyzed by qRT-PCR (Fig. 2A). The expression of PgpPpPLAIII 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 pPLAIIIs has been performed only in Arabidopsis [12]. AtPpPLAIIIs has been reported to be expressed in all organs at various developmental stages with the highest in roots [12]. Thus, PgpPpPLAIII 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 pPLAIIIs might play important roles for annual regeneration from the rhizome.

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

Overexpression of patatin-related phospholipase pPLAIII in Arabidopsis resulted in stunted and dwarf phenotypes with altered cell elongation patterns [12]. To analyze whether ginseng PgpPpPLAIII also exhibits similar morphological characteristics, heterologous overexpression was carried out. Full-length cDNA encoding PgpPpPLAIII 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. PgpPpPLAIII was not expressed in Col-0 and vector control, indicating the specificity of primers used for qRT-PCR. The expression of PgpPpPLAIII 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 PgpPpPLAIII, the more stunted and dwarfed the leaf phenotype was (Fig. 3B, Supplementary Fig. 2A). For further characterization, line 6, in which the expression of PgpPpPLAIII 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 PgpPpPLAIII 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 rather than the stem cells, which is reminiscent to that of PgpPpPLAIII-OE, and this phenotype was explained by the
A

**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: pPLAIIIA (At2g39220), pPLAIIIB (At3g54950), At4g29800 (pPLAIIIG), and pPLAIIID (At3g63200) and OspPLAIIIs: OspPLAIIIA (LOC_Os03g14950), OspPLAIIIB (LOC_Os03g41880), OspPLAIIIC (LOC_Os03g57080), OspPLAIIID (LOC_Os06g46350), OspPLAIIIE (LOC_Os07g05110), and OspPLAIIIF (LOC_Os12g41720). The bar represents 0.1 substitution per amino acid position. (B) Alignment of PgpPLAIIIB protein with its closest homologs. The red-dotted box motifs represent the anion-binding box, esterase box, and catalytic dyad–containing motif.
reduction of bioactive gibberellin A3 (GA3) 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 PgpPLAIIIb-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 PgpPLAIIIb enlarges seed size and improved germination rate

Overexpression of rice pPLAIIa in rice [18] and Arabidopsis pPLAIII 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 pPLAIIIb 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,
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
\( \text{PgpPLAIII}_b \) increased not only seed width but also seed length (Fig. 5A and B).

To verify whether other endogenous gene expression of \( \text{pPLAIIIs} \)
altered by the overexpression of \( \text{PgpPLAIII}_b \) could cause the
enlarged seed, qRT-PCR was performed (Supplementary Fig. 4). It
explains that the endogenous \( \text{pPLAIII} \) isoforms are growth stage
differentially downregulated or upregulated, which suggests that
the cross-talks between \( \text{pPLAIII} \) isoforms and other growth-con-
trolling 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 imbib-
tion. 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 \( \text{PgpPLAIII}_b \) gene changes the seed size and also enhances
the initial germination rate.

3.5. \( \text{PgpPLAIII}_b \)-mediated reduced primary root length is via
increased auxin-responsive gene expression

To verify whether the stunted and dwarf phenotype observed in
\( \text{PgpPLAIII}_b \) 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). How-
ever, the number of lateral roots was higher in the overexpressing
lines than in the control (Fig. 6C). \( \text{Arabidopsis} \) seedlings grown on
auxin-containing media developed shorter primary roots and more
lateral roots than those of plants grown on media without exoge-
 nous auxin [25]. Thus, our data indicate the involvement of auxin-
responsive genes in the growth of roots.

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

Fig. 2. Organ-specific expression pattern of \( \text{PgpPLAIII}_b \). (A) Differential expression pattern of \( \text{PgpPLAIII}_b \) 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 \( \text{PgpPLAIII}_b \) in 4-year-old ginseng roots. The data are presented as mean \( \pm \) SD of three
independent replicates.

qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; SD, standard deviation.
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 YUCCA genes—YUCCA8 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 PgpPLAIIIb. 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 PgpPLAIIIb 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 $P < 0.01$ (**).
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.

![Image](image1.png)

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 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. 7A 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 PpPLAIIIb and decreased in overexpressing lines, which showed a small and stunted phenotype [12], similar phenotype to PgpPLAIIIb. 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 pPLAIIIb alters the functions of other homologous pPLAIII genes (Supplementary Fig. 4) and behaves differently for cell wall composition, when compared with Arabidopsis pPLAIIIb. 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 PgpPLAIIIb 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. a, b, γ, δ, ε, z, η, θ, i, k, l) (Fig. 1). PgpPLAIIIb 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 pPLAIIIb 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). PgpPLAIIIb-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 antiquity 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: 2016R1A2B410216).

Appendix A. Supplementary data

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

References

[1] Wang X. Plant phospholipases. Annu Rev Plant Physiol Plant Mol Biol 2001;52:211–31.
[2] Menijer HJ, Munnik T. Phospholipid-based signaling in plants. Annu Rev Plant Biol 2003;54:265–306.
[3] Ryu SB. Phospholipid-derived signaling mediated by phospholipase A in plants. Trends Plant Sci 2004;9:229–35.
[4] Matos AR, Pham-Thi AT. Lipid deacylating enzymes in plants: old activities, new genes. Plant Physiol Biochem 2009;47:491–503.
[5] Scherer GFE, Ryu SB, Wang X, Matos AR, Heitz T. Patatin-related phospholipase A, nomenclature, subfamilies and functions in plants. Trends Plant Sci 2010;15:691–700.
[6] Holk A, Rietz S, Zahn M, Quader H, Scherer GFE. Molecular identification of cytosolic, patatin-related phospholipases A from Arabidopsis with potential functions in plant signal transduction. Plant Physiol 2002;130:90–101.
[7] Rietz S, Holk A, Scherer GFE. Expression of patatin-related phospholipase A gene APPLA II in Arabidopsis thaliana is up-regulated by salicylic acid, wounding, ethylene, and iron and phosphate deficiency. Planta 2004;219:743–53.
[8] La Camera S, Geoffrey P, Samaha H, Ndiaye A, Rahim G, Legrand M, Heitz T. A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in Arabidopsis. Plant J 2005;44:810–25.
[9] Yang W, Devaiah SP, Fan X, Isaac G, Welti R, Wang X. APPLA III is an acyl hydrolase involved in basal jasmonic acid production and Arabidopsis resistance to Botrytis cinerea. J Biol Chem 2007;282:18116–28.
[10] Rietz S, Derrmendjeev G, Oppermann E, Tafesse FG, Effendi Y, Holk A, Parker JE, Teige M, Scherer GFE. Roles of Arabidopsis patatin-related phospholipases A in root development are related to auxin responses and phosphate deficiency. Mol Plant 2010;3:524–38.
[11] Andrews DL, Beames B, Summers MD, Park WD. Characterization of the lipid acyl hydrolase activity of the major potato (Solanum tuberosum) tuber protein, patatin, by cloning and abundant expression in a baculovirus vector. Biochem J 1988;252:199–206.
[12] Li M, Bahn SC, Guo L, Musgrave W, Berg H, Welti R, Wang X. Patatin-related phospholipase pPLAIIIβ-induced changes in lipid metabolism alter cellulose content and cell elongation in Arabidopsis. Plant Cell 2011;23:1107–23.
[13] Lin CC, Chu CF, Liu PH, Lin HH, Liang SC, Hsu WE, Lin JS, Wang HM, Chang LL, Chien CT, et al. Expression of an Oncidium gene encoding a patatin-like protein delays flowering in Arabidopsis by reducing gibberelin synthesis. Plant Cell Physiol 2011;52:421–35.
[14] Li M, Bahn SC, Fan C, Li J, Phan T, Ortiz M, Roth MR, Welti R, Jaworski J, Wang X. Patatin-related phospholipase pPLAIII α increases seed oil content with long-chain fatty acids in Arabidopsis. Plant Physiol 2013;162:39–51.
[15] Li M, Wei F, Tawfall A, Tang M, Saettele A, Wang X. Overexpression of patatin-related phospholipase AlII α altered plant growth and increased seed oil content in camellia. Plant Biotechnol J 2013;13:766–78.
[16] Huang S, Cervy RE, Bhat DS, Brown SM. Cloning of an Arabidopsis patatin-like gene, STURDY, by activation-T-DNA tagging. Plant Physiol 2001;125:573–84.
[17] Qiao Y, Piao R, Shi J, Lee SI, Jiang W, Kim BK, Lee JH, Han I, Ma W, Koh HJ. Fine mapping and candidate gene analysis of dense and erect panicle 3 (DEP3), which confers high grain yield in rice (Oryza sativa). Theor Appl Genet 2011;122:1439–49.
[18] Liu G, Zhang K, Ji J, Deng X, Hong Y, Wang X. Patatin-related phospholipase A, pPLAIII β, modulates the longitudinal growth of vegetative tissues and seeds in rice. J Exp Bot 2015;66:5945–55.
[19] Bechtold N, Pelletier G. In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol Biol 1998;82:259–66.
[20] Yuan L, Xu C, Kang Y, Gu T, Wang D, Zhao S, Xia G. The heterologous expression in Arabidopsis thaliana of sorghum transcription factor ShbHLH11 down-regulates lignin synthesis. J Exp Bot 2012;64:3021–32.
[21] Kumar M, Turner S. Protocol: a medium-throughput method for determination of cellulose content from single stem pieces of Arabidopsis thaliana. Plant Methods 2015;11:46.
[22] Kim MK, Lee BS, In JG, Sun H, Youn JH, Yang DC. Comparative analysis of expressed sequence tags (ESTs) of ginseng leaf. Plant Cell Rep 2006;25:609–606.
[23] Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 1999;82:59–66.
[24] Hentrich M, Böttcher C, Düchting P, Cheng Y, Zhao Y, Berkowitz O, Masle J, Chory J. A role for the Arabidopsis transcription factor AtMYB103 in the modulation of transcription factors. Plant Cell Physiol 2011;52:1856–67.
[25] Wilkins MR, Gasteiger E, Bairoch A, Sanchez JC, Williams KL, Appel RD, Hochstrasser DF. Protein identification and analysis tools in the ExPASy server. Methods Mol Biol 1999;82:59–66.
[26] Higginson T, Li SF, Parish RW. AMY1B03 regulates tapetum and trichome development in Arabidopsis thaliana. Plant J 2003;35:177–92.
[27] Hembree E, Estelle M. The aux1 auxin-resistant mutants of Arabidopsis thaliana define a gene important for root gravitropism and lateral root initiation. Plant J 1995;7:211–200.
[28] Zhao Y, Christensen SK, Fankhauser C, Kashman JR, Cohen JD, Weigel D, Chory J. A role for flavin monooxygenase-like enzyme in auxin biosynthesis. Science 2001;291:396–9.
[29] Henritsch M, Böttcher C, Duchtling P, Cheng Y, Zhao Y, Berkowitz O, Masle J, Medina J, Pollmann S. The jasmonic acid signaling pathway is linked to auxin homeostasis through the modulation of YUCCA8 and YUCCA9 gene expression. Plant J 2013;74:626–37.
[30] Zhong R, Lee C, McCarthy RL, Reeves CK, Jones EG, Ye ZH. Transcriptional activation of secondary wall biosynthesis by rice maize NAC and MYB transcription factors. Plant Cell Physiol 2011;52:1856–71.
[31] Ponomer F, Merino F, Barceló AR, O-4-linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. Protoplasma 2002;220:17–28.
[32] Zhou J, Lee C, Zhong R, Ye ZH. MYB58 and MYB63 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. Plant Cell 2009;21:248–66.
[33] Nguyen NQ, Lee OR. Overexpression of ginseng UGT72G1A1 causes organ fusion in the axillary leaf branch of Arabidopsis. J Ginseng Res 2017:41:419–27.
[34] Moore KD, Merino F, Barceló AR. O-4-linked coniferyl and sinapyl aldehydes. Journal of Research Management 2001;54:420–30.
[35] Murthy HN, Kim YS, Jeong CS, Kim SJ, Zhong J, Paek KY. Production of ginsenosides from adventitious root cultures of panax ginseng. Production of Biomass and Bioactive Compounds Using Bioreactor Technology 2014:625–51.