



# Expression of a novel PSK-encoding gene from soybean improves seed growth and yield in transgenic plants

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## Abstract

**Main conclusion** Expression of *GmPSKγ1*, a novel PSK-encoding gene from soybean, increases seed size and yield in transgenic plants by promoting cell expansion.

Phytosulfokine- $\alpha$  (PSK- $\alpha$ ), a sulfated pentapeptide hormone with the sequence YIYTQ, plays important roles in many aspects of plant growth and development. In this study, we identified a pair of putative precursor genes in soybean, *GmPSKγ1* and -2, encoding a PSK-like peptide: PSK- $\gamma$ . Similar to PSK- $\alpha$  in amino acid composition, the sequence of PSK- $\gamma$  is YVYTQ, and the tyrosines undergo sulfonylation. Treatment of Arabidopsis seedlings with synthetic sulfated PSK- $\gamma$  significantly enhanced root elongation, indicating that PSK- $\gamma$  might be a functional analog of PSK- $\alpha$ . Expression pattern analysis revealed that the two *GmPSKγ* genes, especially *GmPSKγ1*, are primarily expressed in developing soybean seeds. Heterologous expression of *GmPSKγ1* under the control of a seed-specific promoter markedly increased seed size and weight in Arabidopsis, and this promoting effect of PSK- $\gamma$  on seed growth was further confirmed in transgenic tobacco constitutively expressing *GmPSKγ1*. Cytological analysis of transgenic Arabidopsis seeds revealed that PSK- $\gamma$  promotes seed growth by inducing embryo cell expansion. In addition, expression analysis of downstream candidate genes suggested that PSK- $\gamma$  signaling might regulate cell wall loosening to promote cell expansion in Arabidopsis seeds. Overall, our results shed light on the mechanism by which PSK- $\gamma$  promotes seed growth, paving the way for the use of this new peptide for biotechnological improvement of crop seed/grain size and yield.

**Keywords** Arabidopsis · Cell expansion · Phytosulfokine · PSK- $\gamma$  · Seed size · Soybean

## Abbreviations

DAP Days after pollination  
PSK Phytosulfokine

Liangliang Yu and Yumin Liu contributed equally to this work.

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## Introduction

Seed growth is of great importance for agriculture productivity. In angiosperms, seed formation begins with a double fertilization event, generating a diploid embryo and a triploid endosperm (Chaudhury et al. 2001), and a protective seed coat originating from the maternal integument surrounds the embryo and endosperm (Figueiredo and Kohler 2014). Therefore, the coordinated growth and development of these three structures is required for the formation of normal-sized seed, which is one of the most important traits of seed yield (Li and Li 2016). Over the past decades, numerous signaling pathways and key regulatory factors have been identified that control seed size by regulating cell division and expansion in

the endosperm as well as growth of the seed coat (reviewed by Orozco-Arroyo et al. 2015; Li and Li 2016). Conversely, knowledge of the mechanism underlying embryo growth regulation during seed development is rather scarce.

In the model species *Arabidopsis thaliana*, seed development has been divided into three stages: the early embryogenesis stage (0–6 days after pollination, DAP), the maturation stage (7–16 DAP), and the late maturation stage (17–20 DAP) (Baud et al. 2002). During the second stage, embryo cells undergo a period of expansion, a process that contributes significantly to the embryo's volume and final seed size (Baud et al. 2002; Orozco-Arroyo et al. 2015). Nonetheless, the regulatory mechanism for embryonic cell growth is poorly understood and requires further investigation.

Phytosulfokine- $\alpha$  (PSK- $\alpha$ ) is a disulfated pentapeptide of the sequence Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Thr-Gln that has been identified as a new peptide hormone functioning in many aspects of plant growth and development (Sauter 2015). A truncated disulfated tetrapeptide with the sequence Tyr(SO<sub>3</sub>H)-Ile-Tyr(SO<sub>3</sub>H)-Thr (named PSK- $\beta$ ) was also found in the cell culture medium from which PSK- $\alpha$  was initially isolated (Matsubayashi and Sakagami 1996). However, PSK- $\beta$  was eventually classified as a degradation product of PSK- $\alpha$  (Yang et al. 1999), and only weak activity was detected (Matsubayashi et al. 1996). PSK- $\alpha$  is synthesized from prepropeptides of 80–120 amino acids that contain a putative signal peptide sequence at the N-terminus and the five-amino acid PSK- $\alpha$  motif near the C-terminus (Sauter 2015). PSK- $\alpha$  peptides mature through precursor processing by tyrosylprotein sulfotransferase (TPST) in the *trans*-Golgi (Komori et al. 2009) and subsequently by proteolytic enzyme activity in the apoplast (Srivastava et al. 2008). PSK- $\alpha$  precursors are encoded by small gene families widely distributed in the plant kingdom, including *Asparagus officinalis* (Matsubayashi and Sakagami 1996), *A. thaliana* (Matsubayashi et al. 2006), *Oryza sativa* (Yang et al. 1999; Lorbiecke and Sauter 2002), *Zea mays* (Lorbiecke et al. 2005), and *Lotus japonicas* (Wang et al. 2015a).

PSK- $\alpha$  was originally identified as a key effector inducing division in cell suspensions cultured at low density (Matsubayashi and Sakagami 1996; Matsubayashi et al. 1997). Recently, Heyman et al. (2013) reported that *AtPSK5*, transcriptionally activated by ERF115, regulates quiescent center (QC) cell division, therefore, maintaining stem cell niche longevity in root. Moreover, It has also been found that PSK- $\alpha$  plays important roles in cell expansion induction. Application of the PSK- $\alpha$  peptide or overexpression of the PSK- $\alpha$  precursor gene significantly enhanced root, hypocotyl, and leaf growth in *Arabidopsis* by promoting cell expansion (Kutschmar et al. 2009; Stührwohldt et al. 2011; Yu et al. 2016). It has also been reported that the cell growth induced by PSK- $\alpha$  might be attributed to enhanced

cell wall loosening (Yu et al. 2016) and/or protoplast expansion (Stührwohldt et al. 2011). In addition, PSK- $\alpha$  signaling has promoting effects on multiple developmental events, including somatic embryogenesis (Hanai et al. 2000; Igasaki et al. 2003), tracheary element differentiation (Matsubayashi et al. 1999), cotton fiber elongation (Han et al. 2014), *in vitro* regeneration competence in recalcitrant legumes (Ochatt et al. 2018), and legume nodulation (Wang et al. 2015a), and regulates immune responses against biotrophic and necrotrophic pathogens in an antagonistic manner (Igarashi et al. 2012; Mosher et al. 2013).

PSK- $\alpha$  is perceived by PSKRs, members of a family of membrane-localized leucine-rich repeat (LRR) receptor kinases (Matsubayashi et al. 2002, 2006). The intracellular kinase domain of a PSKR, which is modified by phosphorylation (Kaufmann et al. 2017) and activated by Ca<sup>2+</sup>/calmodulin binding (Hartmann et al. 2014), possesses overlapping serine/threonine kinase and guanylate cyclase activity (Kwezi et al. 2011; Hartmann et al. 2014). Recent structural data reveal that PSK- $\alpha$  binding stabilizes PSKR heterodimerization with SERK proteins, resulting in allosteric activation of PSKR (Wang et al. 2015b). Ladwig et al. (2015) reported that a plasma membrane-localized module including CNGC17, H<sup>+</sup>-ATPase, and BAK1 interacts with PSKR to transduce PSK- $\alpha$  signaling. Despite increasing knowledge regarding PSKRs, the signaling cascade involved, especially key factors downstream of the receptor, remains largely unknown.

In this study, we identified a novel type of PSK, PSK- $\gamma$ , from soybean. With a difference in only one amino acid, PSK- $\gamma$  exhibits a function similar to that of PSK- $\alpha$  in promoting plant growth. Heterologous expression of one precursor gene, *GmPSK $\gamma$ 1*, which is primarily expressed in soybean seeds, remarkably increased seed size and weight in both transgenic *Arabidopsis* and tobacco through embryo cell expansion induction. Further investigation indicates that PSK- $\gamma$  signaling might regulate cell wall loosening to promote cellular expansion in the seed. These results show that PSK- $\gamma$  is a new positive regulator in seed growth and can be exploited to improve crop seed/grain size and yield.

## Materials and methods

### Plant materials and growth conditions

*Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.] ecotype Columbia (Col-0; ABRC, Columbus, OH, USA) and tobacco (*Nicotiana tabacum* L.) cultivar K326 (Biorun Biotechnology, Wuhan, Hubei, China) were used in this study. Seeds of *Arabidopsis* and tobacco were surface sterilized for 15–20 min in 3% (v/v) sodium hypochlorite solution containing 0.2% Tween-20 and then rinsed

five times with sterile water. After stratification at 4 °C for 2 days, the seeds were germinated on Murashige and Skoog (MS) medium containing 1% (w/v) sucrose and 0.7% (w/v) agar. Seedlings were transplanted to a 3:1 (w/w) vermiculite:perlite mixture and cultured in environmental chambers under a light/dark cycle of 16/8 h. Temperatures were maintained at 23 °C and 26 °C for the growth of Arabidopsis and tobacco, respectively.

### Peptide treatment

The peptides used in this study, including PSK- $\gamma$  ( $Y_{S03}VY_{S03}TQ$ ), desulfated PSK- $\gamma$  (YVYTQ), and randomly arranged pentapeptide (TYQYV), were synthesized by Chinese Peptide Company (Hangzhou, China). Each peptide was dissolved in sterilized ddH<sub>2</sub>O at 1 mM, which was used as a stock solution. Stratified Arabidopsis seeds were sown on MS agar plates containing 1  $\mu$ M of each peptide. After 10 days of growth on the plates in the vertical position, seedlings were photographed to measure root length using Image J (National Institute of Health, Bethesda, MD, USA).

### Gene cloning, vector construction and transformation

The full-length and truncated *GmPSK $\gamma$ 1* CDS sequences were PCR amplified using cDNA reverse transcribed from RNA of soybean developing seeds (*Glycine max* Williams 82; seeds kindly provided by Professor Oliver Yu, Donald Danforth Plant Science Center, St. Louis, MO, USA); the primers used are listed in Suppl. Table S1. The obtained CDS fragments were cloned into the intermediate vector pRNAi downstream of the enhanced cauliflower mosaic virus (CaMV) 35S promoter using the restriction sites *Nco* I and *Bam*H I. The resulting *35Sp:GmPSK $\gamma$ 1:OC $\sigma$ ter* and *35Sp:GmPSK $\gamma$ 1tc:OC $\sigma$ ter* cassettes were subsequently ligated into the binary vector pCambia 2300 using the restriction sites *Kpn* I and *Sac* I. For construction of the seed-specific expression plasmid, the promoter region (~2000 bp) of *AtOLEO4* was PCR amplified from Arabidopsis (Col-0) genomic DNA using the primers listed in Suppl. Table S1. The plasmids were completed by replacing the 35S promoter with the *AtOLEO4* promoter in *35Sp:GmPSK $\gamma$ 1* and *35Sp:GmPSK $\gamma$ 1tc* (pCambia 2300) using *Kpn* I and *Nco* I. The seed-specific constructs were transformed into Arabidopsis by Agrobacterium-mediated transformation using the floral dip method that was performed by Pujie biotechnology company (Shanghai, China), and the *35Sp:GmPSK $\gamma$ 1* construct was transformed into tobacco by Agrobacterium-mediated transformation using the leaf disc method.

### Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was employed to determine the expression patterns of *GmPSK* genes and transcript levels of *GmPSK $\gamma$ 1* in transgenic plants. Samples were homogenated in liquid nitrogen to extract total RNA using an RNAPrep pure Plant kit (Tiangen, Beijing, China), and 1  $\mu$ g RNA of each sample was reverse transcribed using a PrimeScript RT reagent kit (Perfect RealTime) (TaKaRa, Dalian, China). Quantitative RT-PCR was carried out with cDNA and gene-specific primers (Suppl. Table S1); the Hieff qPCR SYBR Green Master Mix (Yeasen, Shanghai, China) and a CFX96 real-time PCR detection system (BioRad, Hercules, CA, USA) were used. The PCR procedure and data analysis were performed in accordance with methods established in a previous study (Yu et al. 2014).

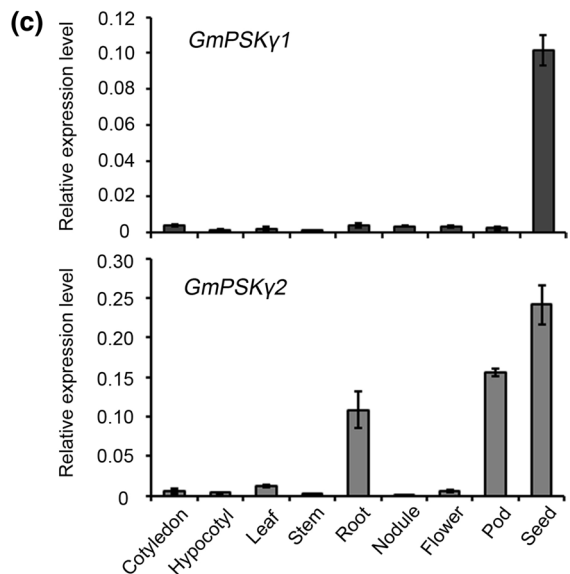
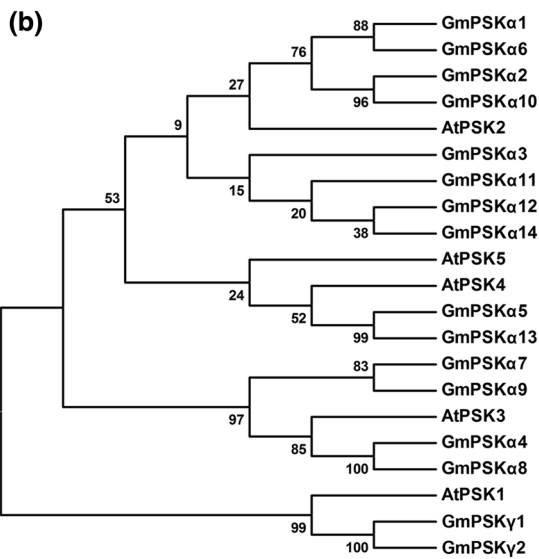
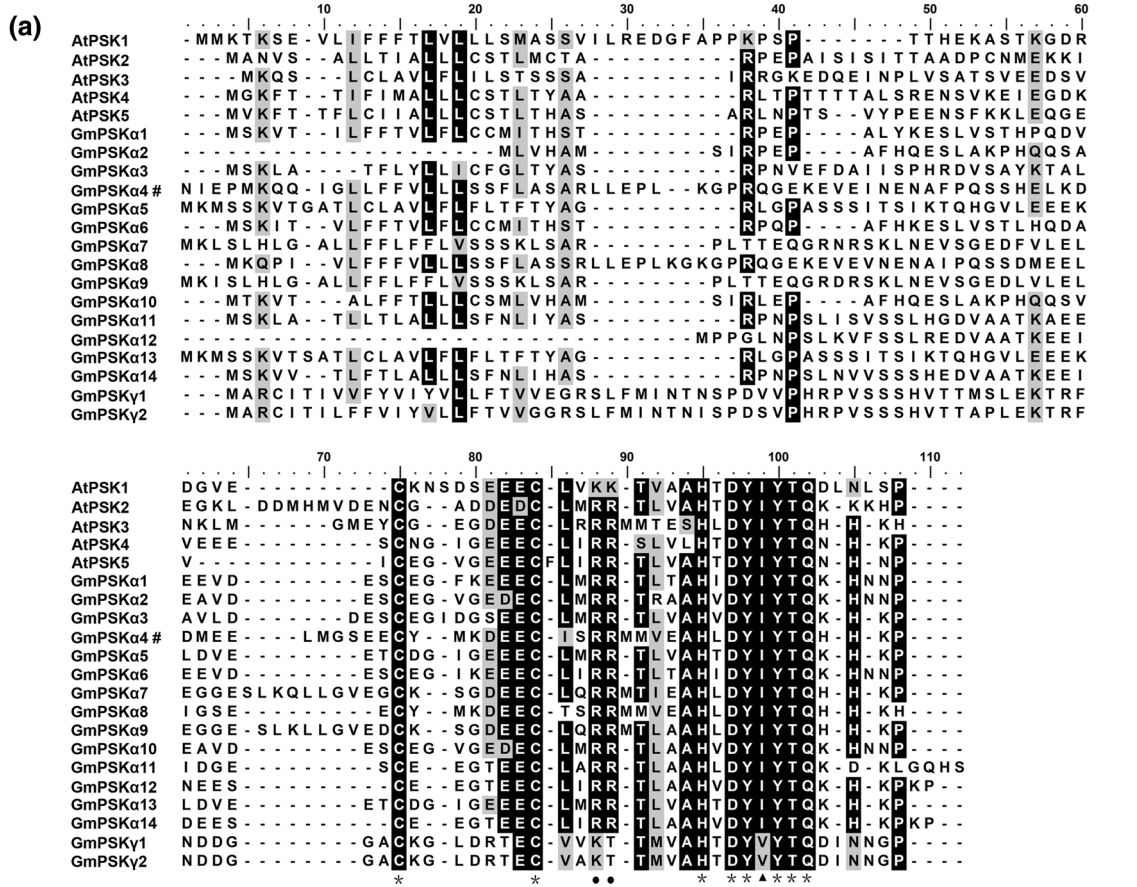
### Paraffin-embedded sections

Arabidopsis seeds at 10 DAP were dissected from siliques using needles and fixed in FAA (50% ethanol, 5% formaldehyde, 10% acetic acid, by vol.) solution overnight. The fixed seeds were dehydrated in a graded ethanol series and embedded in paraffin. Sections were prepared at 10  $\mu$ m thickness using a Leica RM2235 rotary microtome, stained with 0.05% toluidine blue, and examined and photographed under bright-field microscopy (Zeiss Axio Scope A1).

## Results

### Sequence, phylogeny, and expression pattern of GmPSK precursors

In a search of the genome database of *Glycine max* Williams 82 ([www.phytozome.net/search.php](http://www.phytozome.net/search.php)) using *AtPSK* sequences as queries, we identified 16 putative *GmPSK* genes encoding PSK precursors. Among them, 14 harbor a single PSK- $\alpha$  pentapeptide motif near the C-terminus; these were designated GmPSK $\alpha$ 1–14 (Fig. 1a). With the exception of GmPSK $\alpha$ 4 (170 aa in length), these GmPSK $\alpha$  precursor proteins have a typical length of ~80 aa and contain the conserved PSK signature motif C(x)<sub>6–12</sub>CxxRR(x)<sub>3–4</sub>AHxDYIYTQ (Sauter 2015) (Fig. 1a). The other two putative GmPSK precursors consist of 94 amino acids, a typical length of PSK precursors, but exhibit less conservation in the PSK signature motif. Obvious variations include substitution of the isoleucine in YIYTQ by valine and replacement of the double arginine by lysine and threonine (Fig. 1a). These two atypical PSK precursors are highly conserved in protein sequence, sharing 91% amino acid identity, and might have originated from a gene duplication event during evolution. Because



**Fig. 1** *GmPSKγ1* and *-2* are predicted to encode a novel type of phytosulfokine precursor protein. **a** Protein sequence alignment of AtPSKs and predicted GmPSKs. The full-length amino acid sequences of these proteins, with the exception of GmPSKα4 (indicated by #), were aligned using BioEdit software. GmPSKα4 is much longer than is a typical PSK precursor protein, and only the relatively conserved COOH-terminal portion was aligned. Stars indicate consensus amino acids; circles and triangle indicate the di-basic amino acids and iso-

leucine in the PSK signature motif, respectively. **b** Phylogenetic tree of AtPSKs and predicted GmPSKs. GmPSKγs are close to AtPSK1 in phylogeny. The tree was constructed using MEGA6 with the neighbor-joining method, and bootstrap values from 1000 replications were included. **c** Quantitative RT-PCR analyses of *GmPSKγ1* and *-2* expression in various soybean tissues. Values are the mean ± SD of three independent biological replicates normalized against the reference gene *GmEF1β*

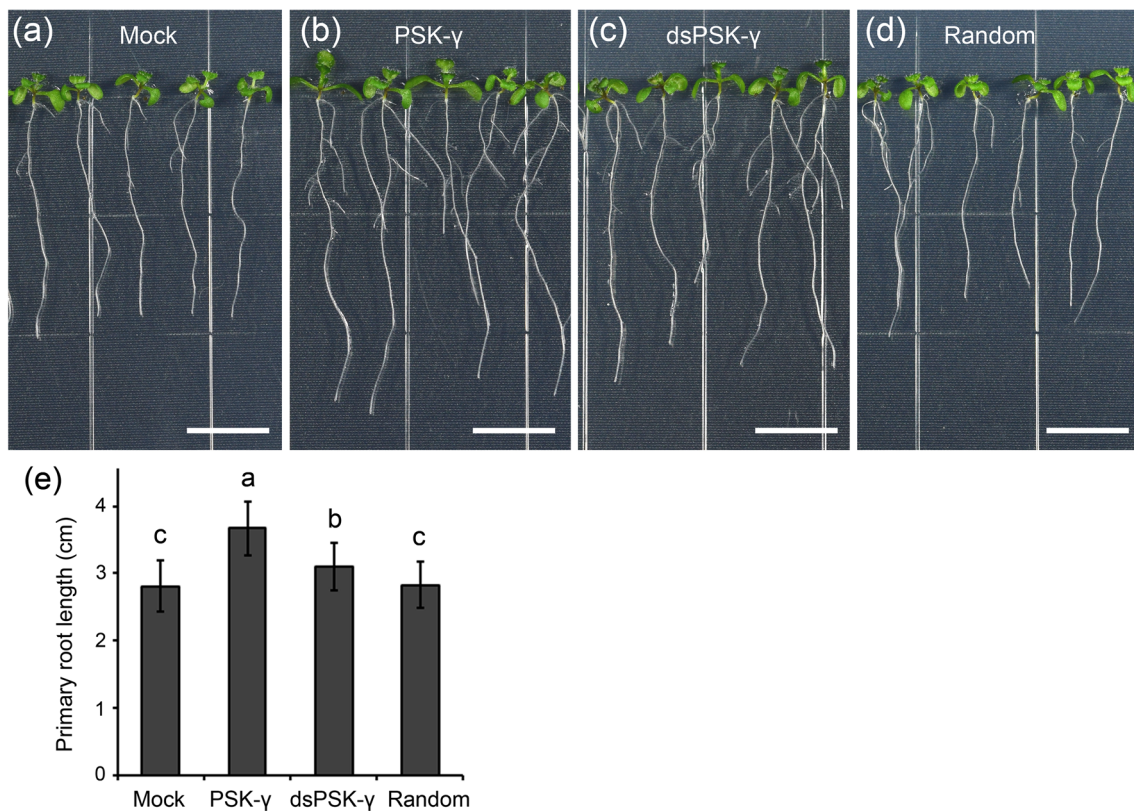


the name PSK- $\beta$  has been assigned to the tetrapeptide YIYT (Matsubayashi and Sakagami 1996), we named the predicted YVYTQ pentapeptide with tyrosine-sulfonylation PSK- $\gamma$  and accordingly, its precursor proteins in soybean GmPSK $\gamma$ 1 and -2 (Fig. 1a).

Phylogenetic relationship analysis revealed that although there are additional members, the GmPSK family comprises orthologs of the reported Arabidopsis PSK family. Indeed, both GmPSK $\gamma$  proteins are likely orthologs of AtPSK1 (Fig. 1b). Quantitative RT-PCR was employed to examine the expression patterns of *GmPSK* genes in various tissues of *G. max*. The results showed that the 14 *GmPSK $\alpha$*  genes were expressed in a tissue-specific or ubiquitous pattern (Suppl. Fig. S1); in contrast, *GmPSK $\gamma$ 1* was dominantly expressed in developing seeds, and *GmPSK $\gamma$ 2* was expressed in seeds, pods (without seeds), and roots, with the highest expression level detected in seeds (Fig. 1c). These results indicate that *GmPSK $\gamma$*  genes might encode a novel type of phytosulfokine that mainly functions in seed development.

## The PSK- $\gamma$ peptide promotes Arabidopsis root growth

To test the physiological activities of PSK- $\gamma$ , we treated wild-type Arabidopsis seedlings with 1  $\mu$ M sulfated PSK- $\gamma$  peptide and then observed root growth, which is reported as a sensitive system for evaluating the activity of PSK- $\alpha$  (Kutschmar et al. 2009). At 10 days of age, PSK- $\gamma$  treatment caused an approx. 31% increase in primary root length compared to seedlings treated with mock or 1  $\mu$ M randomly arranged pentapeptide having the same amino acid composition as PSK- $\gamma$  (Fig. 2a, b, d, e). To assess the effect of the sulfonylation modification on the activity of PSK- $\gamma$ , we also treated Arabidopsis seedlings with 1  $\mu$ M desulfated PSK- $\gamma$  (dsPSK- $\gamma$ ), and the results showed that dsPSK- $\gamma$  treatment caused an 11% increase in primary root length compared to controls (Fig. 2a, c, d, e), indicating that dsPSK- $\gamma$  retains part of PSK- $\gamma$  activity. These findings demonstrate that the PSK- $\gamma$  peptide has a promoting effect on root growth, which has also been reported for PSK- $\alpha$  (Kutschmar et al. 2009).



**Fig. 2** Treatment with the PSK- $\gamma$  peptide promotes root growth in Arabidopsis. Ten-day-old Arabidopsis (Col-0) seedlings grown on vertical MS agar plates containing mock (a), 1  $\mu$ M PSK- $\gamma$  (b), 1  $\mu$ M desulfated PSK- $\gamma$  (dsPSK- $\gamma$ , c), and 1  $\mu$ M randomly arranged pentapeptide with the same amino acid composition as dsPSK- $\gamma$  (random,

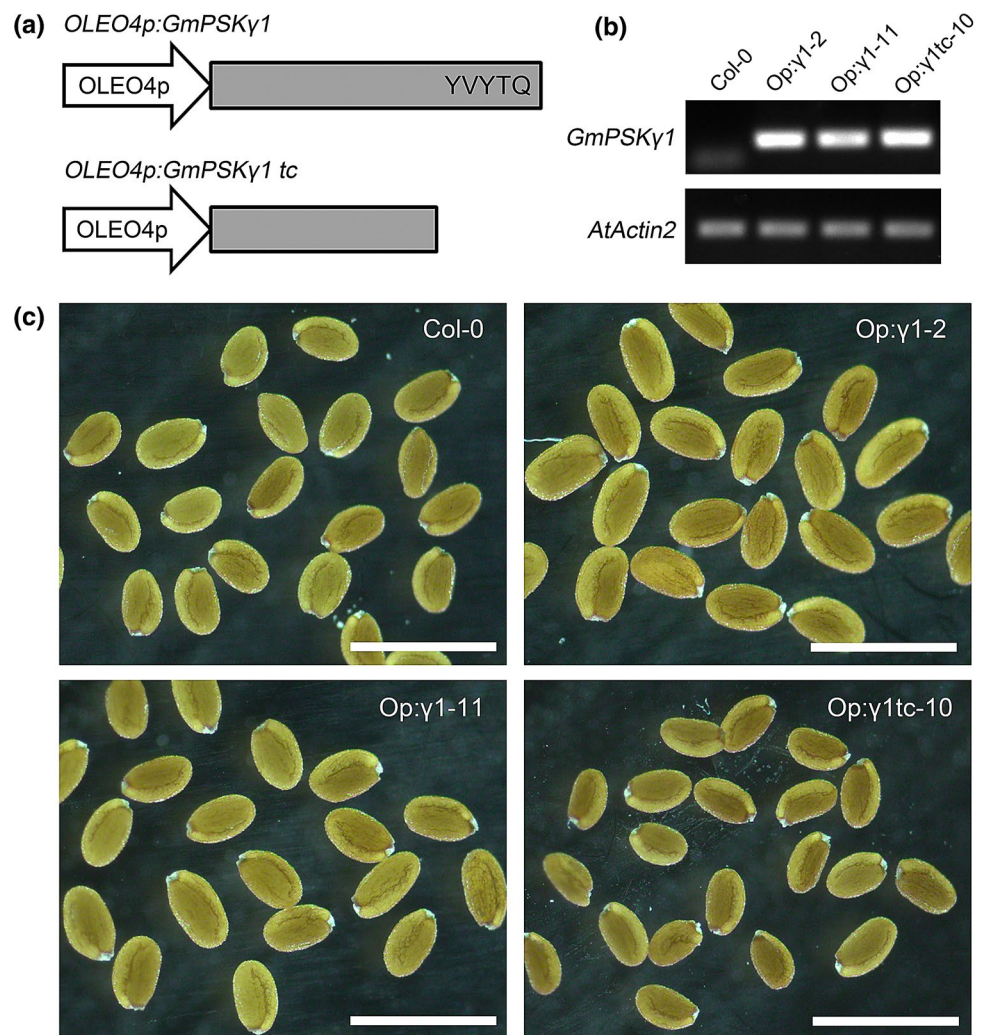
d). Bars=1 cm. e Lengths of the Arabidopsis primary roots shown in a–d. Values are the mean  $\pm$  SD from three independent biological replicates with a total of ~40 seedlings per treatment. Different letters indicate significantly different values,  $P < 0.01$ , ANOVA test

## Expression of *GmPSK $\gamma$ 1* promotes seed growth in Arabidopsis

Based on the findings that *GmPSK $\gamma$ 1* and -2, especially *GmPSK $\gamma$ 1*, are primarily expressed in soybean seeds, it is conceivable that PSK- $\gamma$  plays a role in seed growth and development. To test this assumption, a seed-specific expression plasmid was constructed and transformed into Arabidopsis due to its high transformation efficiency compared to soybean; in this case, *GmPSK $\gamma$ 1* was expressed under control of the promoter of *AtOLEO4* (*At3g27670*) (*Op: $\gamma$ 1*, Fig. 3a), a seed/embryo-specific gene reported by Baud et al. (2009) and verified by qRT-PCR expression data and microarray information from PlaNet (Suppl. Fig. S2a and b). A seed-specific expression plasmid of a truncated form of *GmPSK $\gamma$ 1* devoid of the peptide-encoding sequence was also constructed (*Op: $\gamma$ 1tc*, Fig. 3a) and transformed as a negative control. More than 20 independent

transgenic lines were generated for each construct. *Op: $\gamma$ 1* lines 2 and 11, and *Op: $\gamma$ 1tc* line 10 showed relatively high transcript abundance in developing seeds (Fig. 3b) and were chosen for further analyses. Phenotypical observation of dry mature seeds revealed that *GmPSK $\gamma$ 1* expression in Arabidopsis notably increased seed size (Fig. 3c). Specifically, the seed length of the *GmPSK $\gamma$ 1*-expressing lines increased by 15–20% compared to that of wild type and the truncated control, and the seed width increased by 15–17% (Table 1). Accordingly, the weight of the dry seeds increased by 20–31% in *GmPSK $\gamma$ 1*-expressing lines compared to controls (Table 1), while seed number per silique, another key factor determining yield, was not influenced by *GmPSK $\gamma$ 1* expression (Table 1). As expected, seed-specific expression of *GmPSK $\gamma$ 1* did not influence the growth of vegetative organs, such as leaves and roots (Suppl. Fig. S3), demonstrating that *GmPSK $\gamma$ 1* expression was the direct factor enhancing seed growth.

**Fig. 3** Seed-specific expression of *GmPSK $\gamma$ 1* promotes seed growth in Arabidopsis. **a** Schematic representation of *GmPSK $\gamma$ 1* expression constructs. The *GmPSK $\gamma$ 1* CDS, in both full-length and truncated forms, was constructed under control of the seed-specific *AtOLEO4* promoter. **b** Semi-quantitative RT-PCR analyses of *GmPSK $\gamma$ 1* transcript abundances in developing seeds of the indicated transgenic Arabidopsis lines. Expression of *AtActin2* was used as an internal control. **c** Comparison of dry mature seeds of wild-type and the indicated transgenic Arabidopsis lines. Bars = 1 mm





**Table 1** Seed-specific expression of *GmPSKγ1* increases seed size and weight in Arabidopsis

	Seed length (mm)	Seed width (mm)	Seed weight (mg)	Seed number per silique
Col-0	0.446 ± 0.022	0.267 ± 0.013	10.1 ± 0.9	50.6 ± 5.7
Op:γ1-2	0.535 ± 0.030**	0.313 ± 0.018**	13.2 ± 0.6**	52.2 ± 4.0
Op:γ1-11	0.512 ± 0.026**	0.308 ± 0.015**	12.0 ± 0.3**	52.1 ± 4.4
Op:γ1tc-10	0.442 ± 0.018	0.259 ± 0.019	9.9 ± 0.6	51.1 ± 3.0

Mature dry seeds of each genotype were photographed and measured for seed length and width using Image J software. Values represent the mean ± SD from three biological replicates, with a total of 38–46 seeds. Five hundred seeds were weighed as the seed weight. Values represent the mean ± SD from three biological replicates

\*\* $P < 0.01$ , Student's  $t$  test

### Expression of *GmPSKγ1* promotes embryo cell growth in Arabidopsis seeds

In a mature Arabidopsis seed, the embryo occupies the entire space between the integuments and is thus one of the major determinants of seed size. Embryos of seeds at 10 DAP were dissected and observed under a stereomicroscope, and the embryos of *GmPSKγ1*-expressing seeds appeared to be markedly larger than were those of wild-type and truncated control seeds (Fig. 4a, b). To reveal the cellular basis underlying the enlarged embryo, seeds were cross sectioned at 10 DAP, a time during which embryo cells undergo expansion and differentiation (Baud et al. 2002). The results showed that expression of *GmPSKγ1* significantly promoted cell expansion in embryos (Fig. 4c). In contrast to controls, the transverse areas of radical cells, cotyledonal spongy cells, and palisade cells in *GmPSKγ1*-expressing seeds increased by approximately 42, 52, and 29%, respectively (Fig. 4d–f). Therefore, seed-specific expression of *GmPSKγ1* promotes embryo cell growth, leading to seed size and weight enhancement.

### Constitutive expression of *GmPSKγ1* promotes growth of seeds and vegetative organs in tobacco

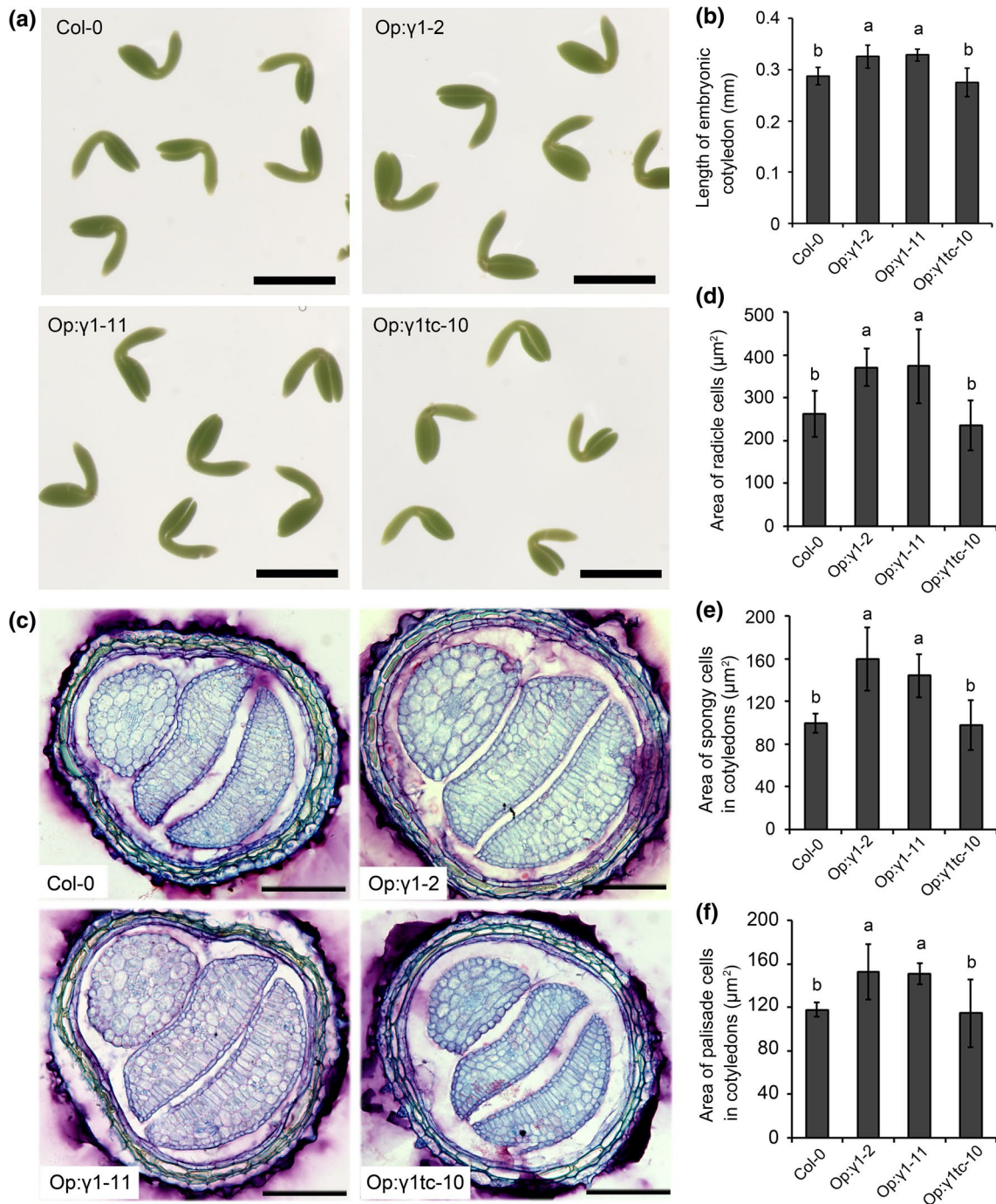
To investigate whether the effect of *GmPSKγ1* on seed growth is conserved in the plant kingdom, we constructed a constitutive expression plasmid comprising *GmPSKγ1* driven by the enhanced CaMV 35S promoter (Fig. 5a) and transformed it into *Nicotiana tabacum*. Twenty-three independent transgenic lines were generated, among which lines 7, 11, and 22 exhibited high transcript levels of *GmPSKγ1* in developing seeds (Fig. 5b). Phenotypic observation of dry mature seeds revealed that *GmPSKγ1* expression in tobacco remarkably increased seed size (Fig. 5c): the projected areas of *GmPSKγ1*-expressing seeds were 12–30% larger than those of wild-type seeds (Fig. 5d). Moreover, the weight of the dry seeds increased by 7–29% (Fig. 5e). Therefore, the effect of PSK-γ on seed growth is conserved between

Arabidopsis and tobacco, indicating that PSK-γ function might be conserved in plant species, at least in dicots.

Due to the constitutive 35S promoter, *GmPSKγ1* was also highly expressed in vegetative organs such as leaves (Suppl. Fig. S4), significantly promoting tobacco leaf growth (Fig. 5f and g) and increasing plant weight (Fig. 5h). Additionally, *GmPSKγ1*-expressing tobacco seedlings developed longer roots than did wild-type seedlings (Fig. 5i and j). These data demonstrate that similar to PSK-α, PSK-γ can also promote plant vegetative growth.

### *GmPSKγ1* expression in Arabidopsis seeds regulates expression of genes associated with cell growth

To investigate the mechanism by which *GmPSKγ1* promotes seed growth, we performed RNA sequencing (RNA-Seq) to identify differentially expressed genes (DEGs) in 10 DAP Arabidopsis seeds between *Op:γ1* and *Op:γ1tc* transgenic lines. The RNA-Seq raw data were submitted to the National Center for Biotechnology Information Gene Expression Omnibus under the series entry GSE121567. However, due to lack of biological replicates, these data can only serve as a guidance to understand the regulation mechanism of PSKγ1; therefore, qRT-PCR with biological replicates was performed to verify the expression of the candidate DEGs. The results showed that a number of genes with putative functions in plant cell growth were differentially expressed in *GmPSKγ1*-expressing seeds (10 DAP) relative to controls. Particularly, two cell expansion-promoting genes, *Cel2* (*At1G02800*, encoding an endo-1,4-β-glucanase) and *SAUR74* (*At3G12955*, encoding a SAUR-like auxin-responsive protein), were significantly upregulated (Fig. 6a). In contrast, several cell expansion-inhibiting genes were downregulated, including peroxidase-encoding genes *POD11* (*At1G68850*), *-49* (*At4G36430*), and *-64* (*At5G42180*) and ethylene-responsive factor family genes *ERF13* (*At1G77640*), *-53* (*At2G20880*), and *-71* (*At2G47520*) (Fig. 6b).



**Fig. 4** Seed-specific expression of *GmPSKγ1* promotes embryonic cell growth in Arabidopsis seeds. **a** Comparison of embryos dissected from the developing seeds (10 days after pollination, DAP) of wild-type and the indicated transgenic Arabidopsis lines. Bars=0.5 mm. **b** Measurement of the length of embryonic cotyledons at 10 DAP. **c** Transverse sections of developing seeds at 10 DAP. The paraffin-

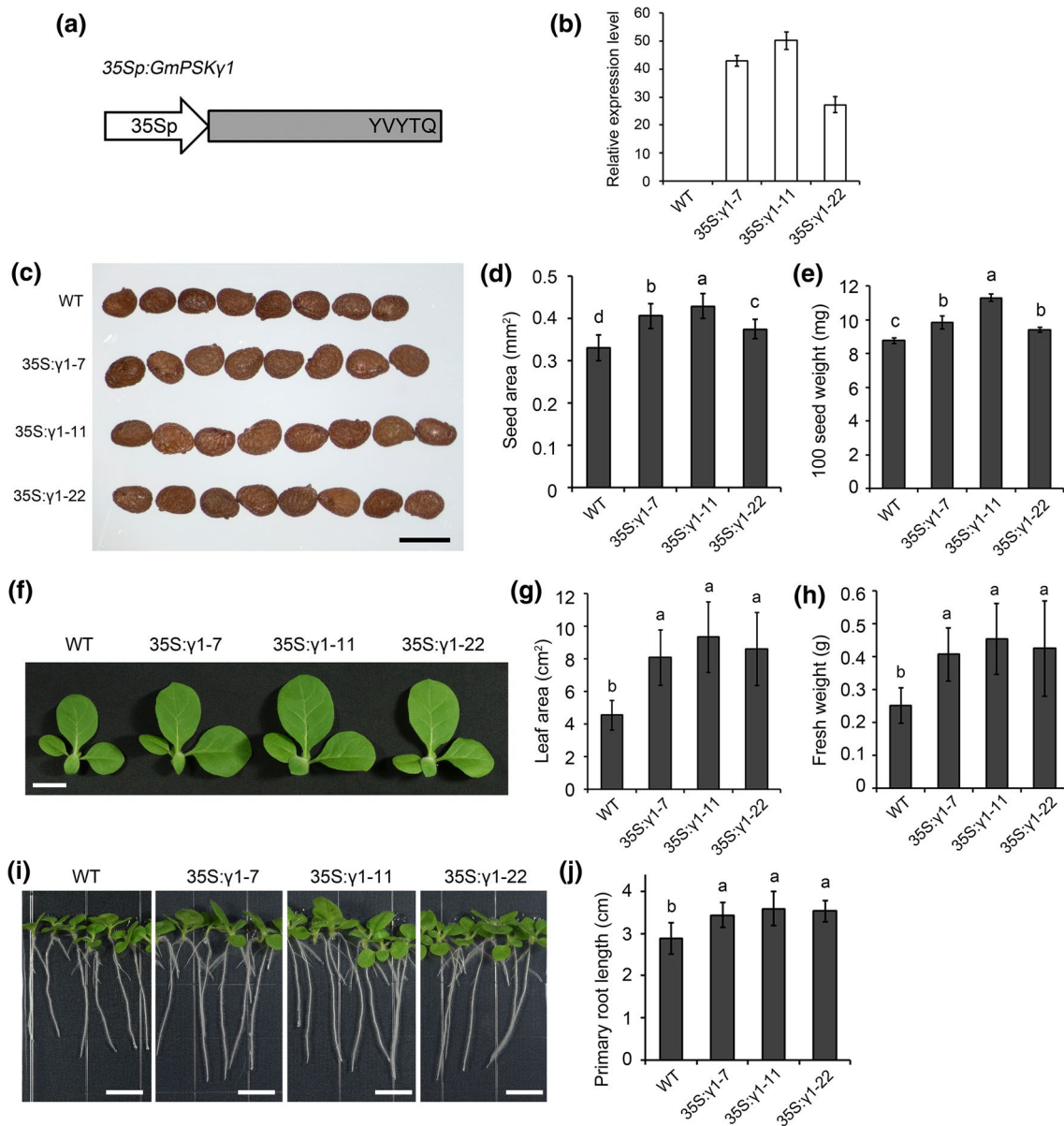
embedded sections were stained with toluidine blue. Bars=100 μm. Measurement of transverse areas of radical cells (**d**) and spongy (**e**) and palisade (**f**) cells in cotyledons at 10 DAP. Values are the mean ± SD from three independent biological replicates, with a total of 212–244 cells measured for each cell type. Different letters indicate significantly different values,  $P < 0.01$ , ANOVA test

## Discussion

### PSK-γ is a novel type of phytosulfokine from soybean

Over the past 2 decades, PSK-α precursors have been extensively identified and studied in multiple plant species. Although weak sequence conservation is observed among these precursor proteins, the PSK-α pentapeptide motif is





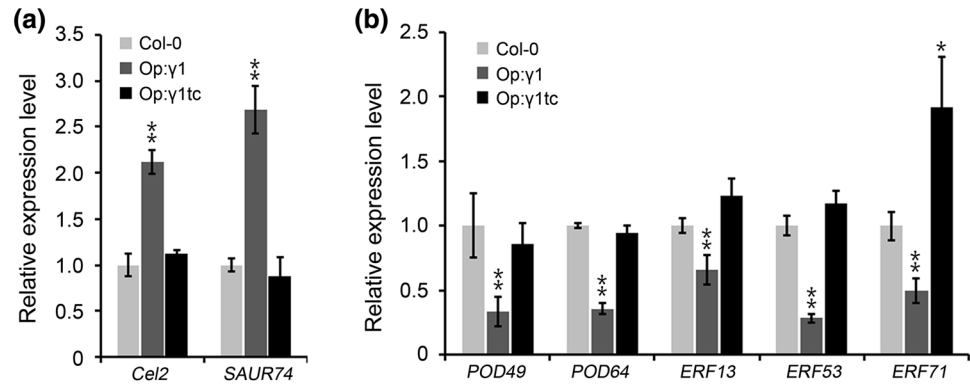
**Fig. 5** Constitutive expression of *GmPSKγ1* promotes growth of seeds and vegetative organs in tobacco. **a** Schematic representation of the *GmPSKγ1* constitutive expression construct. Full-length *GmPSKγ1* CDS was constructed under control of the enhanced CaMV 35S promoter. **b** Quantitative RT-PCR analysis of *GmPSKγ1* transcript levels in developing seeds of *GmPSKγ1*-overexpressing tobacco lines. Values are the mean ± SD of three independent biological replicates normalized against the reference gene *NtUBC2*. **c** Comparison of dry mature seeds of wild-type and the indicated transgenic tobacco lines. Bar: 1 mm. **d** Measurement of the projected areas

of dry mature seeds shown in (c). *n*=144–152. **e** 100 seed weight of the mature dry seeds shown in (c). *n*=3 biological replicates. **f** One-month-old wild-type and *GmPSKγ1*-overexpressing tobacco plants. Bar=2 cm. Leaf (the largest one) areas (**g**) and fresh weight of above-ground parts (**h**) of the 1-month-old tobacco plants shown in (f). *n*=3 biological replicates. **i** Seventeen-day-old wild-type and *GmPSKγ1*-overexpressing tobacco seedlings grown on vertical MS-agar plates. Bars=1 cm. **j** Primary root length of the tobacco seedlings shown in (i), *n*=3 biological replicates. Different letters indicate significantly different values, *P*<0.01, ANOVA test

highly conserved (Lorbiecke and Sauter 2002). However, it is still unknown whether any novel PSK peptide or PSK-α isoform exists in the plant kingdom. In this study, we identified a pair of soybean genes that encode a new type of PSK: PSK-γ. Both *GmPSKγ* precursors have a typical amino acid length and a PSK signature motif, which is, however, less

conserved compared to PSK-α. The isoleucine in PSK-α is substituted by a valine in PSK-γ. Regardless, given that isoleucine and valine are similar in chemical characteristics, it is conceivable that the two types of PSK peptides have similar biological functions. As expected, treatment of *Arabidopsis* seedlings with synthetic sulfated PSK-γ

**Fig. 6** Quantitative RT-PCR analysis of expression of upregulated (a) and downregulated (b) genes associated with cell growth in 10 DAP Arabidopsis seeds. *AtActin2* was used as an internal control. Values represent the mean  $\pm$  SD from three lines of each genotype with two biological replicates for each line. \* $P < 0.05$ , \*\* $P < 0.01$ , Student's *t* test



markedly promoted root elongation, a conserved function also reported for PSK- $\alpha$  (Kutschmar et al. 2009). Moreover, similar to PSK- $\alpha$ , sulfonation of the tyrosines in PSK- $\gamma$  is important for its full activity. Another sequence variation between PSK- $\gamma$  and PSK- $\alpha$  is that the di-basic amino acids upstream from the PSK- $\alpha$  pentapeptide are replaced by one basic amino acid (lysine) and a threonine in PSK- $\gamma$ . Although di-basic residues are speculated to be required for proteolytic processing of precursors (Srivastava et al. 2008), a single basic residue in the case of OsPSK, a well-characterized rice PSK- $\alpha$ , is sufficient for maturation (Yang et al. 1999), indicating that maturation of PSK- $\gamma$  from its precursors might occur with a less-conserved PSK signature motif. Based on their phylogenetic relationship, GmPSK $\gamma$ s are orthologs of AtPSK1. These data led us to hypothesize that PSK- $\gamma$  evolved from a PSK- $\alpha$  ancestor present in the soybean genome and retained conserved activities.

### PSK- $\gamma$ promotes seed growth by enhancing embryo cell expansion

Although the seed development process has been extensively studied in Arabidopsis, little is known about the embryo enlargement process during seed maturation. In this study, we found that GmPSK $\gamma$  genes, especially GmPSK $\gamma$ 1, are primarily expressed in developing soybean seeds, indicating potential roles in seed development. By expressing GmPSK $\gamma$ 1 under the control of a seed/embryo-specific promoter, we found that both the seed size and weight of transgenic Arabidopsis lines to be notably increased. This promoting effect of PSK- $\gamma$  on seed size and weight was further confirmed by constitutive expression of GmPSK $\gamma$ 1 in tobacco. Moreover, detailed analysis of cytological alterations in transgenic Arabidopsis seeds at 10 DAP (middle maturation stage) revealed that PSK- $\gamma$  significantly promoted cell expansion in embryo tissues, including cotyledons and radicles, resulting in an enlarged embryo. This observation is reminiscent of the function of PSK- $\alpha$  in promoting cell elongation and expansion in various tissues (Kutschmar et al. 2009; Stührwohldt et al. 2011; Yu et al. 2016). On

the basis of these findings, it is reasonable to presume that although slightly different in amino acid composition, PSK- $\gamma$  and PSK- $\alpha$  share similar biochemical features and conserved functions in promoting plant cell growth.

### PSK- $\gamma$ promotes embryo cell expansion possibly by loosening the cell wall

The growth of plant cells requires two basic cellular actions: cell wall loosening mediated by cell wall-remodeling enzymes and protoplast expansion driven by intracellular turgor pressure. Our previous data showed that overexpression of AtPSK4 in Arabidopsis promotes cell expansion by affecting cell wall development (Yu et al. 2016), and Stührwohldt et al. (2011) also reported that PSK- $\alpha$  peptide application induced protoplast expansion. These findings indicate that PSK- $\alpha$  regulates cell growth with dual functions. To investigate the molecular mechanism of PSK- $\gamma$  regulation of embryo cell expansion, qRT-PCR analysis was performed to identify DEGs in developing seeds between GmPSK $\gamma$ 1-expressing and control Arabidopsis lines. It was shown that a number of genes associated with cell expansion to be differentially regulated, among which, the endo-1,4- $\beta$ -glucanase-encoding gene *Cel2* was significantly upregulated. Endo-1,4- $\beta$ -glucanases are important factors responsible for promoting cell growth by loosening the cell wall structure (Cosgrove 2005; Yu et al. 2013); therefore, increase in *Cel2*'s expression potentially promotes cell expansion. Moreover, two peroxidase family genes, *POD49* and *-64*, were transcriptionally repressed in GmPSK $\gamma$ 1-expressing seeds. It is known that peroxidases act as inhibitors of cell expansion by enhancing cross-linking and deposition of cell wall materials (Brownleader et al. 2000; Raggi et al. 2015). Thus, repression of these genes by PSK- $\gamma$  signaling facilitates cell expansion. Although more evidence is required, our data indicate that PSK- $\gamma$  might regulate embryo cell expansion by loosening cell wall.

In conclusion, our study demonstrates that PSK- $\gamma$ , a novel PSK from soybean, can promote seed growth and weight by inducing embryo cell expansion. The findings highlight the

potential use of PSK- $\gamma$  signaling in molecular breeding to generate crops with larger seed/grain size and higher yield.

**Author contribution statement** LY and LL conceived and designed the research. LY and YL performed the experiments. LY, YL, SZ, JY and EW analyzed the data. LY and LL wrote the manuscript. All authors read and approved the manuscript.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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