

Article

Comparative Transcriptomic Analysis Reveals the Regulatory Role of PSK- δ in Trigonelline Biosynthesis in *Trigonella foenum-graecum*

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Abstract

Trigonelline is a bioactive pyridine alkaloid in *Trigonella foenum-graecum*, which is a legume species, and is known for its hypoglycemic and hypolipidemic activities. Phytosulfokine- δ (PSK- δ), a recently identified legume-specific phytosulfokine peptide, has been implicated in the regulation of nodulation and root development. However, whether PSK- δ participates in the regulation of secondary metabolite biosynthesis remains unclear. In this study, exogenous PSK- δ treatment significantly promoted trigonelline accumulation in *T. foenum-graecum* seedlings, leading to a 93% increase in trigonelline content after 5 days relative to the scrambled-pentapeptide-treated control, whereas a slight downward trend was observed at 15 days. Comparative transcriptomic analyses between PSK- δ -treated seedlings and the corresponding controls at 5 and 15 days identified 15 candidate genes associated with trigonelline biosynthesis and PSK signaling, whose expression patterns were consistent with trigonelline accumulation dynamics. Co-expression network analysis between these 15 candidate genes and 831 transcription factor-encoding genes identified candidate transcription factors potentially involved in the coordinated regulation of trigonelline biosynthesis and PSK signaling. These findings suggest that PSK- δ may serve as a potential peptide-based biostimulant for regulating alkaloid accumulation in medicinal legume cultivation and provide insights into peptide hormone-mediated regulation of plant metabolism.

Keywords: *Trigonella foenum-graecum*; trigonelline biosynthesis; PSK- δ ; signaling pathway; transcription factor



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1. Introduction

Trigonella foenum-graecum L. (fenugreek), an annual leguminous herb, contains abundant bioactive components such as trigonelline, 4-hydroxyisoleucine, saponins, and galactomannans with potential hypoglycemic, hypolipidemic, and anticancer properties [1]. Among these metabolites, trigonelline, a methylated derivative of nicotinic acid, has been widely recognized for its prominent hypoglycemic activity [2–4]. It is a naturally occurring hydrophilic pyridine alkaloid widely distributed in plant families such as *Fabaceae* (e.g., fenugreek and alfalfa), *Rubiaceae* (e.g., coffee), and *Solanaceae* (e.g., *Datura*) [5–7]. Trigonelline also exhibits diverse pharmacological activities beyond its hypoglycemic effect, including antitumor potential via modulation of nuclear factor erythroid 2-related

factor 2 (Nrf2)-related signaling [8] and its role as a novel natural precursor involved in nicotinamide adenine dinucleotide (NAD⁺) metabolism, with potential in delaying aging and improving muscle function [9,10]. Owing to these pharmacological properties, trigonelline has attracted considerable attention as a promising bioactive compound in drug development [11].

Previous studies have largely elucidated the biosynthetic pathway of trigonelline, which is closely linked to nicotinic acid biosynthesis and the NAD⁺ metabolic cycle in plants [12–14]. Nicotinic acid, in its physiological form nicotinate, serves as the direct precursor of trigonelline and is converted into trigonelline via N-methylnicotinic acid N-methyltransferase (NANMT) using S-adenosyl-L-methionine (SAM) as the methyl donor [12,15]. In plants, nicotinic acid is mainly synthesized through de novo pathways associated with NAD⁺ metabolism and recycled via the pyridine nucleotide cycle, thereby maintaining cellular NAD⁺ homeostasis [14]. Although the metabolic pathway of trigonelline biosynthesis has been largely characterized, its regulatory mechanisms remain poorly understood.

Phytosulfokines (PSKs) are a class of plant sulfated peptide hormones first identified in the late 1980s that regulate diverse biological processes, including cell proliferation, development, nodulation, and responses to environmental stresses [16–20]. PSK- δ , a recently identified legume-specific PSK peptide from *Medicago truncatula*, has been implicated in nodulation and root-related developmental processes [21]. PSK precursor proteins undergo tyrosine sulfation by tyrosylprotein sulfotransferase (TPST) and subsequent proteolytic processing by subtilisin-like serine proteases (SBTs) to generate mature PSK peptides, which are perceived by plasma membrane-localized PSK receptors (PSKRs) and activate downstream signaling cascades [17,22,23]. Notably, the canonical PSK peptide PSK- α has been reported to promote the accumulation of secondary metabolites such as paclitaxel and hyoscyamine in *Taxus cuspidata* [24,25], suggesting that PSK signaling may participate in the regulation of plant specialized metabolism. However, whether PSK- δ is involved in regulating trigonelline biosynthesis remains unknown.

Transcription factors (TFs) are DNA-binding proteins that regulate gene expression by interacting with cis-regulatory elements in target promoters and forming regulatory networks with other proteins. In plants, TFs play central roles in integrating environmental signals and coordinating hormone signaling, thereby regulating growth, development, stress responses, and secondary metabolism [26–28]. Among them, WRKY, MYB, and bHLH TF families have been widely implicated as key regulators of plant secondary metabolism. For example, MYB-bHLH regulatory modules coordinately regulate monoterpenoid indole alkaloid biosynthesis, while the jasmonate-responsive bHLH transcription factor CrBIS1 controls key genes involved in vinblastine biosynthesis [29,30]. The evidence suggests that transcriptional regulation may also contribute to PSK- δ -mediated modulation of trigonelline biosynthesis.

Therefore, this study investigated the role of PSK- δ in trigonelline biosynthesis in fenugreek seedlings. Metabolite and transcriptome analyses were performed to evaluate the effects of exogenous PSK- δ treatment on trigonelline accumulation and the expression of metabolism-associated genes, and to identify candidate transcription factors potentially involved in the coordinated regulation of trigonelline biosynthesis and PSK signaling. These observations suggest that peptide hormone signaling may provide a promising strategy for the sustainable regulation of secondary metabolite accumulation in medicinal plant cultivation. These results suggest a potential role of PSK signaling in modulating trigonelline accumulation.

2. Materials and Methods

2.1. Plant Materials and Chemicals

Seeds of *Trigonella foenum-graecum* were obtained from Anhui Province, China. Trigonelline standard was purchased from YuanYe Biotechnology Co., Ltd. (Shanghai, China). The phytosulfokine peptide PSK- δ and a scrambled pentapeptide containing the same amino acid composition as PSK- δ were synthesized by Hangzhou DanGang Biotechnology Co., Ltd. (Hangzhou, China). The amino acid sequences of PSK- δ and the scrambled pentapeptide control were Y_{SO3}IY_{SO3}TN and TYN_YI, respectively [21,31].

2.2. Plant Growth and PSK- δ Treatment

Uniform fenugreek seeds were surface-sterilized with 75% (*v/v*) ethanol for 1 min and 5% (*w/v*) sodium hypochlorite for 10 min, followed by thorough rinsing with sterile distilled water. Seeds were germinated on 0.65% (*w/v*) water agar in the dark at 25 $\pm$ 2 $^{\circ}$ C for 42–44 h. PSK- δ was dissolved in sterile double-distilled water and added to 1/2 Murashige and Skoog (MS) liquid medium to a final concentration of 0.1 μ M. An equal concentration of a scrambled pentapeptide was used as the control. Uniformly germinated seedlings were transferred to treatment or control media (25 seedlings per bottle) and supported with sterile glass beads. Cultures were maintained at 25 $\pm$ 2 $^{\circ}$ C under a 16 h light/8 h dark photoperiod. Seedlings were harvested at 5, 10, and 15 days after treatment, with three biological replicates for each group. Primary root length and shoot length were measured. Samples were either frozen in liquid nitrogen and stored at -80° C for transcriptome analysis or dried at 37 $^{\circ}$ C to constant weight for trigonelline quantification.

2.3. Extraction and HPLC Analysis of Trigonelline

Approximately 20 mg of dried fenugreek seedling powder (dried to constant weight) was weighed, and 20 μ L of ursolic acid solution (1 mg/mL) was added as an internal standard. Subsequently, 1 mL of methanol was added for ultrasonic extraction, which was repeated three times. The extracts were combined and evaporated to dryness, and the residue was stored at -20° C. Prior to analysis, the residue was re-dissolved in 100 μ L of methanol.

Trigonelline was quantified using high-performance liquid chromatography (HPLC) on a Shimadzu LC-20AT system. Separation was performed on a Shim-pack GIST C18 column (250 mm $\times$ 4.6 mm, 5 μ m; XDB-C18) at 26 $^{\circ}$ C. The mobile phase consisted of ultrapure water and acetonitrile (8:92, *v/v*) at a flow rate of 0.4 mL/min. Detection was carried out at 265 nm, with a total run time of 30 min.

2.4. RNA Extraction and RNA Sequencing

Total RNA was extracted from collected plant materials. RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while integrity was assessed by agarose gel electrophoresis and an Agilent 5300 system (Agilent Technologies, Santa Clara, CA, USA). The high-quality RNA samples (OD_{260/280} = 1.8–2.2, RQN > 6.5) were used for submitting to Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) for subsequent RNA sequencing. cDNA libraries were constructed using the NEBNext[®] Ultra[™] II RNA Library Prep Kit for Illumina[®] (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Poly(A)⁺ mRNA was enriched using oligo(dT) magnetic beads before library construction. The libraries were sequenced on a NovaSeq X Plus platform (Illumina, San Diego, CA, USA). Raw reads were filtered using fastp (version 0.23.4) with default parameters. Adapter sequences were automatically removed, and reads containing more than five ambiguous bases (N > 5), low-quality bases (Phred quality score < 15), or more than 40% low-quality

bases were discarded. After quality filtering, approximately 49.04 Gb of clean data were obtained from eight RNA-seq libraries, with Q30 values above 96.08%. Three biological replicates were prepared for RNA sequencing, with each biological replicate consisting of 25 seedlings. For the 5 d samples, three biological replicates were independently sequenced. For the 15 d samples, three biological replicates were collected, and equal amounts of RNA from these replicates were pooled to generate a sequencing library for each treatment group.

2.5. Transcriptome Analysis

Transcriptome assembly was carried out with Trinity. Clean reads were mapped to the assembled transcriptome, and gene expression levels were estimated for each gene using the FPKM (fragments per kilobase of transcript per million mapped reads) method [32]. Functional annotation of genes was performed using multiple databases, including NR, Swiss-Prot, Pfam, eggNOG, the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG). Differentially expressed genes (DEGs) were identified using the DESeq2 R package with false discovery rate (FDR) < 0.05 and $|\log_2(\text{fold change})| \geq 1$ [33,34]. KEGG pathway enrichment analysis was subsequently conducted to investigate the functional significance of the identified DEGs.

2.6. Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from plant samples using the EASYspin Plus Plant RNA Rapid Extraction Kit (Aidlab, Beijing, China), and 1 µg of purified RNA was used as the template for first-strand cDNA synthesis with the TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen Biotech, Beijing, China). Both RNA extraction and cDNA preparation were conducted according to the protocols provided by the manufacturers.

Gene expression analysis was performed by qRT-PCR using the Taq Pro Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) on a Bio-Rad CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The qRT-PCR cycling conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and annealing/extension at 58 °C for 25 s. Fluorescence data were collected at the end of each annealing/extension step. After amplification, a melting curve analysis was performed from 65 °C to 95 °C with a temperature increment of 0.5 °C (5 s at each step) to confirm the specificity of the amplified products. The obtained Cq values were used for subsequent relative expression analysis. Relative transcript abundance was calculated using the $2^{-\Delta\Delta C_q}$ method. Each assay included three independent biological replicates, with three technical replicates performed for each biological sample.

2.7. Gene Co-Expression Network Analysis

To identify transcription factors closely associated with trigonelline biosynthesis and the PSK signaling pathway, a total of 831 transcription factor-encoding genes from the transcriptome dataset were subjected to correlation analysis with key genes involved in trigonelline biosynthesis and signal transduction. Key enzyme genes in the trigonelline biosynthetic pathway and PSK signaling- and MAPK signaling-related genes were selected as target genes. Pearson's correlation coefficients (r) were calculated based on FPKM expression values across all samples. Transcription factor genes showing a strong positive correlation ($r > 0.8$, $p < 0.05$) or a negative correlation ($r < -0.8$, $p < 0.05$) with the target genes were retained as candidate co-expressed regulators. The co-expression networks between target genes and transcription factors were subsequently visualized using Cytoscape (version 3.10.4).

2.8. Phylogenetic Analysis

Protein sequences were obtained from the NCBI database. Phylogenetic tree was generated using the neighbor-joining method implemented in MEGA software (version 7.0.26), and node reliability was evaluated with 2000 bootstrap replicates.

2.9. Statistical Analysis

Statistical analyses and heatmap visualization were performed using GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA). Gene expression data were normalized using $\log_2(\text{FPKM} + 1)$ transformation or Z-score scaling prior to visualization.

3. Results

3.1. PSK- δ Treatment Enhances Trigonelline Biosynthesis in Fenugreek Seedlings

Previous studies have shown that PSK peptides, including PSK- α and PSK- ϵ , promote the elongation of primary roots in plants, such as *Medicago truncatula* and *Arabidopsis thaliana* [16,35–37]. To examine the effects of PSK- δ on root growth, fenugreek seedlings were treated with three concentrations of PSK- δ (0.1, 0.5, and 1 μM). Among them, 0.1 μM PSK- δ showed the greatest increase in primary root elongation after 5 days of treatment compared with the scrambled pentapeptide-treated control, whereas higher concentrations (0.5 and 1 μM) did not result in further enhancement (Figure 1A and Figure S1). Therefore, 0.1 μM was selected for subsequent experiments.

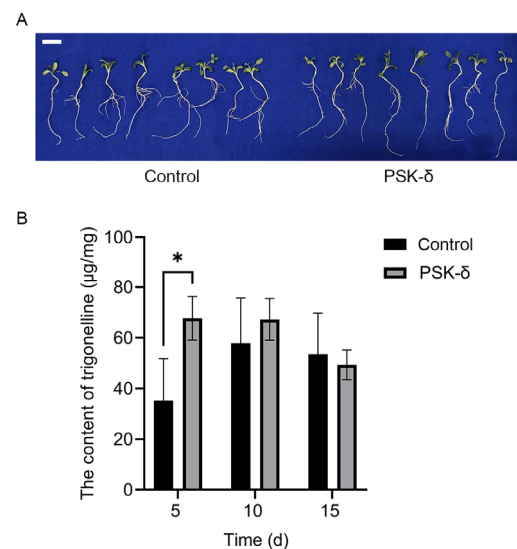


Figure 1. PSK- δ enhances primary root elongation and trigonelline accumulation in fenugreek. (A) Phenotype of fenugreek seedlings treated with 0.1 μM PSK- δ or 0.1 μM scrambled pentapeptide (control) for 5 days, showing differences in primary root length. Scale bar = 1 cm. (B) Trigonelline content in fenugreek seedlings with 0.1 μM PSK- δ or 0.1 μM scrambled pentapeptide (control) over 5, 10, and 15 days. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined by two-tailed Student's t -test, * $p < 0.05$ ($p = 0.036$).

Further analysis of trigonelline content in fenugreek seedlings treated with 0.1 μM PSK- δ for 5, 10, and 15 days showed that trigonelline levels ranged from 35.14 to 67.68 $\mu\text{g}/\text{mg}$. The highest trigonelline content was observed after 5 days of treatment (67.68 $\mu\text{g}/\text{mg}$), representing a significant 93% increase compared with the control. At 10 days, trigonelline content was numerically higher than that in the control (approximately 16%), but the difference did not reach statistical significance. By 15 days, trigonelline content showed a slight decline compared with the corresponding control (Figure 1B). Collectively, these

results indicate that PSK- δ treatment induced a time-dependent modulation of trigonelline accumulation in fenugreek seedlings.

3.2. Transcriptomic Analysis of Fenugreek Seedlings in Response to PSK- δ Treatment

To investigate the regulatory effects of PSK- δ on gene expression involved in trigonelline biosynthesis and PSK signaling pathways, comparative transcriptomic analysis was performed at 5 days and 15 days after treatment with PSK- δ and a scrambled pentapeptide control at a concentration of 0.1 μ M. These time points corresponded to dynamic changes in trigonelline accumulation, which increased at 5 days but showed a declining trend at 15 days (Figure 1B).

High-quality transcriptome data were obtained for all samples, with Q20 > 99.28%, Q30 > 96.08%, and GC content ranging from 41.74% to 42.99% (Table S1). A total of 42,526 and 36,345 expressed genes were identified at 5 days and 15 days, respectively, including 1802 and 755 novel genes. Most genes were shared between control and PSK- δ -treated groups at both time points (Figure 2A,B). At 5 days, 22,729 genes (94.42%) were commonly expressed, while 632 and 711 genes were specifically expressed in control and treatment groups, respectively. Similar patterns were observed at 15 days, with 22,032 shared genes (91.80%) and a small proportion of group-specific genes. Differential expression analysis identified 418 DEGs at 5 d (178 upregulated and 240 downregulated) and 448 DEGs at 15 d (156 upregulated and 292 downregulated) under the threshold of FDR < 0.05 and $|\log_2FC| \geq 1$ (Figure 2C,D).

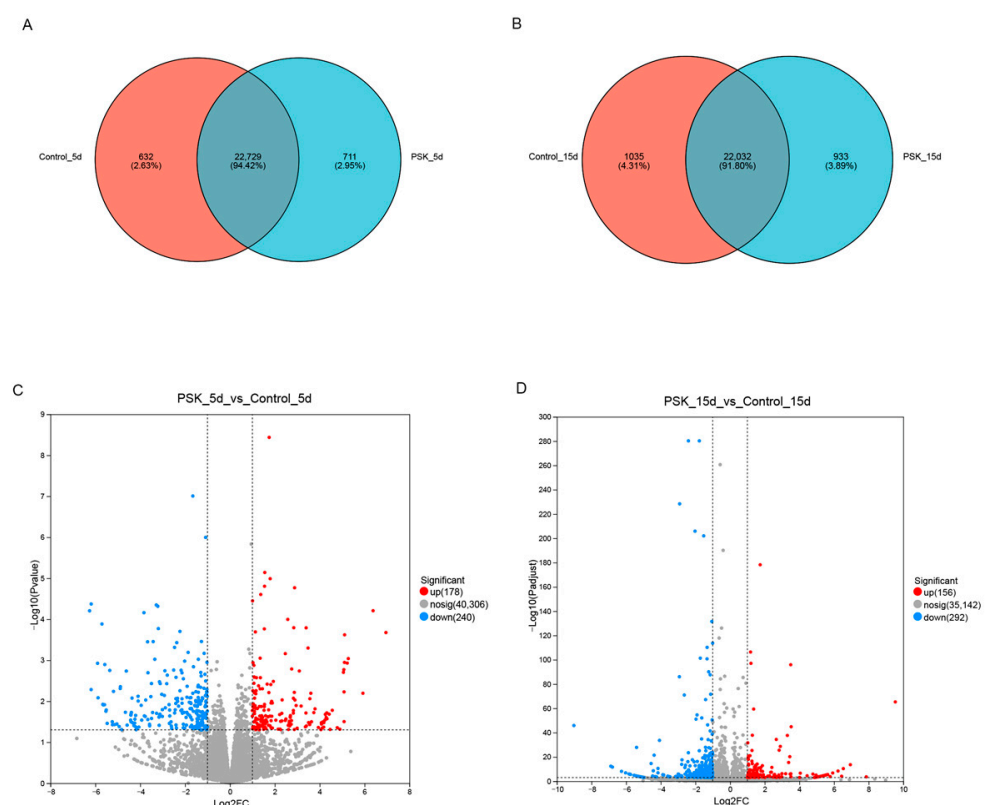


Figure 2. Venn diagram and volcano plot of gene expression in PSK- δ -treated groups at 5 and 15 days compared with their respective scrambled pentapeptide-treated controls. (A,B) Venn diagrams showing the shared and unique expressed genes between the Control and PSK-treated groups at 5 d and 15 d, respectively. (C,D) Volcano plots showing differentially expressed genes (DEGs) between the PSK-treated and corresponding Control groups at 5 d and 15 d, respectively. Red and blue dots indicate significantly upregulated and downregulated genes, respectively, whereas gray dots indicate genes with non-significant expression changes.

GO and KEGG enrichment analyses revealed that DEGs were predominantly associated with photosynthesis and secondary metabolic pathways following PSK- δ treatment (Figure 3A–D). At 5 days (PSK_5d vs. Control_5d), DEGs were mainly enriched in photosynthesis-related processes and terpene biosynthesis (Figure 3A). KEGG analysis further highlighted pathways including photosynthesis and sesquiterpenoid/triterpenoid biosynthesis, as well as plant hormone signaling and MAPK signaling pathways (Figure 3B). GO terms related to photosynthesis and terpene metabolism remained enriched (Figure 3C), while KEGG analysis additionally identified isoquinoline alkaloid biosynthesis (Figure 3D). These results indicate that PSK- δ treatment influences multiple metabolic and signaling pathways, including secondary metabolism, hormone signaling, and MAPK-associated processes.

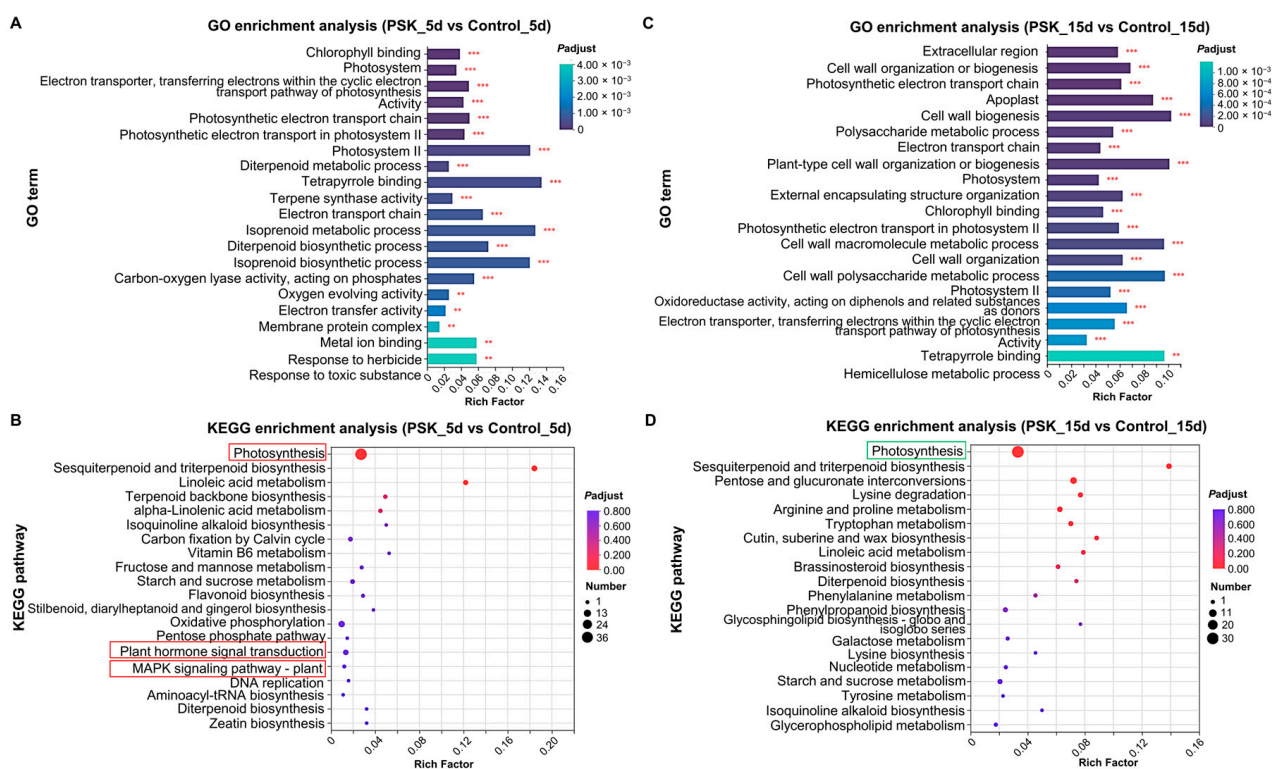


Figure 3. GO and KEGG enrichment analyses of differentially expressed genes in response to PSK- δ treatment. (A,B) GO enrichment of DEGs at 5 d and 15 d, respectively. Red boxes indicate highlighted enriched pathways. Significant terms are indicated by adjusted p -value ($Padjust$), with *** $Padjust < 0.001$ and ** $Padjust < 0.01$. (C,D) KEGG pathway enrichment of DEGs at 5 d and 15 d, respectively. The green box indicates the major enriched pathway. The color indicates the adjusted p -value ($Padjust$), and the dot size corresponds to the number of DEGs in each pathway.

To evaluate the concordance and temporal dynamics of DEG profiles after PSK- δ treatment at 5 d and 15 d, we performed a comparative Venn-diagram analysis (Figure S2). Among all identified DEGs, only 31 genes were shared between the two time points, including only four commonly up-regulated and seven commonly down-regulated DEGs, suggesting that PSK- δ induces largely distinct transcriptional responses at different time points. We further examined genes showing opposite expression patterns between 5 d and 15 d (Figure S2D,E). Only a small number of genes exhibited reversed regulatory patterns (16 genes were up-regulated at 5 d but down-regulated at 15 d, whereas 4 genes showed the opposite expression pattern), indicating that PSK- δ -responsive transcriptional changes were predominantly time-dependent.

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T. foenum-graecum clustered with their corresponding homologs from other plant species, supporting the reliability of their functional annotation.

3.4. Transcriptional Responses of PSK Signaling-Related Genes to PSK- δ Treatment

To investigate whether PSK- δ affects endogenous PSK signaling, the expression of the PSK signaling pathway-related genes was analyzed following PSK- δ treatment (Figure 5). At 5 d after PSK- δ treatment, four transcripts exhibited upward expression trends, whereas six showed downward expression trends (Figure 5B). At 15 d, five transcripts displayed upward expression trends and five exhibited downward expression trends (Figure 5C). Notably, the expression trends of *TPST*, *SBT1.1*, and *AHA2* were consistent with the pattern of trigonelline accumulation, suggesting the involvement of PSK maturation and signaling processes during PSK- δ -induced trigonelline biosynthesis.

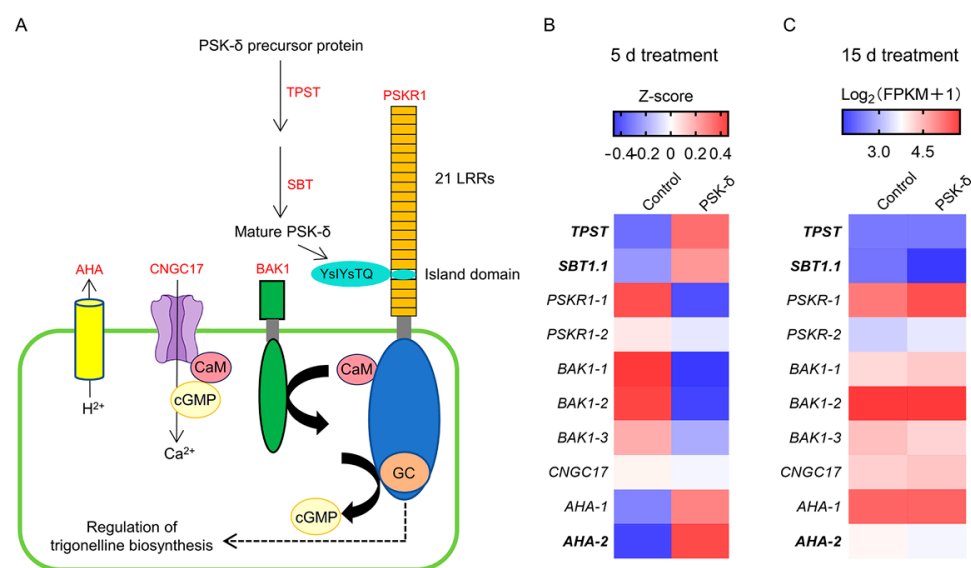


Figure 5. Transcriptional profiling of PSK signaling-related genes in response to PSK- δ . (A) Scheme of the PSK signaling pathway. Genes highlighted in red represent key components of the PSK signaling pathway, including *TPST*, *SBT*, *PSKR1*, *BAK1*, *CNGC17* and *AHA*, which encode tyrosylprotein sulfotransferase, subtilisin-like serine protease, phytosulfokine receptor 1, BRI1-associated receptor kinase 1, cyclic nucleotide-gated channel 17, and plasma membrane H^+ -ATPase, respectively. (B) Heatmap of gene expression profiles after 5 d of PSK- δ treatment, with values normalized as Z-scores. (C) Heatmap of gene expression profiles after 15 d of PSK- δ treatment, with expression values shown as $\log_2(\text{FPKM} + 1)$. In both heatmaps, blue and red indicate relatively low and high expression levels, respectively. Raw data are listed in Table S3.

3.5. Identification of the Key Transcription Factors Associated with PSK- δ -Mediated Trigonelline Biosynthesis and PSK Signaling

Transcription factors (TFs) are essential regulators of plant metabolic processes, particularly in response to phytohormone and peptide hormone signaling pathways such as PSK signaling [38,39]. Given that trigonelline accumulation was markedly enhanced after 5 days of PSK- δ treatment and TF-encoding genes were more comprehensively represented at this stage, the transcriptomic dataset from 5-day-treated fenugreek seedlings was selected for TF screening. A total of 831 TF-encoding genes were identified from the 5-day transcriptome library. These TFs belonged to 13 families, among which MYB (272 members) and NAC (188 members) were the most abundant (Figure 6A). Other major TF families, including WRKY, bHLH, and AP2/ERF, were also highly represented, suggesting their potential involvement in hormone-responsive signal transduction and secondary metabolism.

To identify candidate TFs associated with PSK- δ -induced trigonelline biosynthesis, co-expression analysis was performed using 15 key unigenes related to trigonelline biosynthesis and PSK signaling, including *QS*, *QPT*, *PARP-1*, *NPP-4*, *5'-NT-1/6*, *NRN-1/3*, *NaRN-1*, *NIC-3*, *NAPRT-4*, *NANMT*, *TPST*, *SBT1.1*, and *AHA-2*. A total of 163 TFs showed significant positive correlations with trigonelline biosynthetic genes, predominantly belonging to the MYB (59), bHLH (34), NAC (15), and WRKY (14) families. Similarly, 111 TFs were positively correlated with PSK signaling-related genes, mainly represented by MYB (47), bHLH (22) and GATA (12) family members. In contrast, 130 TFs were negatively correlated with trigonelline biosynthetic genes, primarily belonging to the MYB (30), WRKY (41), NAC (17), and bHLH (13) families. Likewise, 81 TFs showed negative correlations with PSK signaling pathway genes, including WRKY (35), MYB (14), NAC (10), bZIP (7) and GATA (6) members. Collectively, these results suggest the existence of a potential transcriptional regulatory network associated with PSK- δ -induced trigonelline biosynthesis.

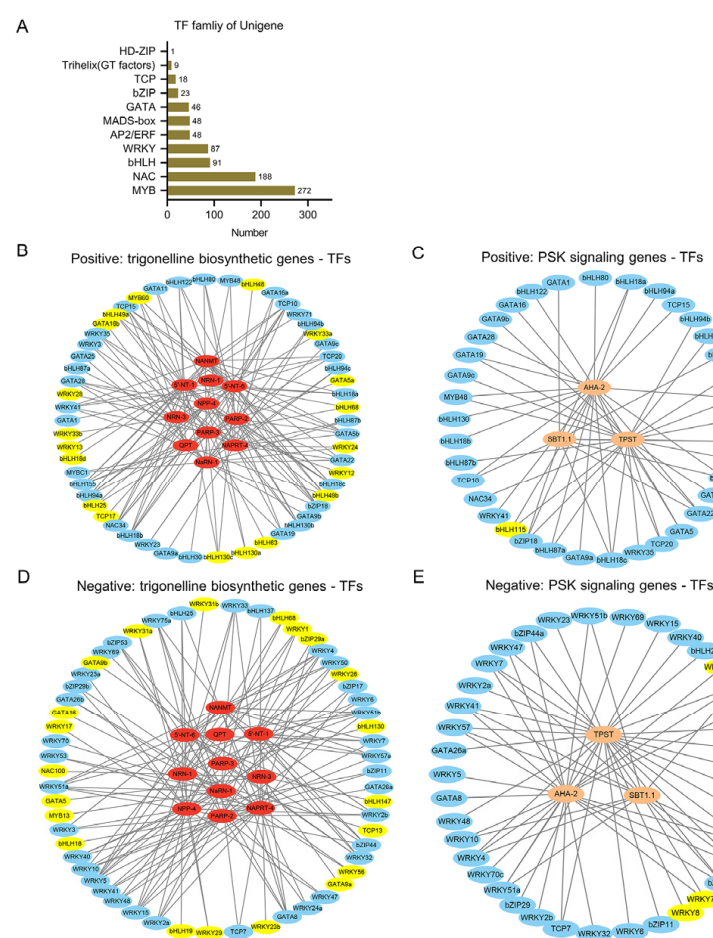


Figure 6. Positive and negative co-expression networks between transcription factors and trigonelline biosynthetic genes or PSK signaling-related genes. **(A)** Distribution of transcription factor (TF) families. **(B,D)** Co-expression networks of TF-encoding genes and trigonelline biosynthetic pathway genes. **(C,E)** Co-expression networks of TF-encoding genes and PSK signaling pathway genes. Edges indicate significant co-expression relationships ($|r| \geq 0.8$, $p < 0.05$), with line thickness proportional to the correlation coefficient (r). Inner nodes represent pathway-associated genes, whereas outer nodes represent transcription factors. Node size reflects the degree of connectivity. The inner red nodes represent pivotal genes involved in the trigonelline biosynthesis pathway, whereas the inner orange nodes correspond to key genes associated with the PSK signaling pathway. Yellow-filled nodes indicate TFs specifically co-expressed with pathway-associated genes at either 5 d or 15 d following PSK- δ treatment, while light blue nodes represent transcription factors specific to each regulatory pattern.

To further refine candidate regulators, TFs with well-annotated functions were selected for co-expression network construction with pathway-associated target genes. Based on Pearson correlation coefficients and functional annotation, 36 TFs were identified as shared positive regulators between the trigonelline biosynthetic and PSK signaling pathways (Figure 6B,C and Tables S7 and S8). In addition, 19 TFs were identified as positive regulators specific to the trigonelline biosynthetic pathway (Figure 6B), whereas only one TF was identified as a positive regulator specific to the PSK signaling pathway (Figure 6C). Similarly, 36 annotated TFs were identified in negatively correlated co-expression networks (Figure 6D,E and Tables S9 and S10). Additionally, 21 TFs were identified as negative regulators specific to the trigonelline biosynthetic pathway (Figure 6D), while three TFs were identified as negative regulators specific to the PSK signaling pathway (Figure 6E).

Given the enrichment of MAPK signaling in transcriptomic analysis (Figure 3B), considering that PSK signaling is mediated by receptor-like kinases that commonly activate downstream kinase cascades [40], additional co-expression analysis was performed between the total 831 TF-encoding genes and MAPK signaling pathway genes. First, MAPK signaling-related genes whose expression patterns were consistent with trigonelline accumulation trends after 5 and 15 days of treatment were selected as target genes (Table S11). These target genes were subsequently subjected to co-expression analysis with the 831 TF-encoding genes, resulting in the identification of 99 positively co-expressed TFs (Table S12) and 97 negatively co-expressed TFs (Table S13). Among the positively co-expressed TFs, 55 were also co-expressed with genes involved in trigonelline biosynthesis and PSK signaling pathway, mainly consisting of MYB (20 members), bHLH (10 members), and GATA (8 members). Among the negatively co-expressed TFs, 40 were simultaneously co-expressed with trigonelline biosynthesis genes and PSK signaling pathway genes, including WRKY (19 members), MYB (7 members), and NAC (5 members).

4. Discussion

Trigonelline is a pyridine alkaloid with diverse pharmacological activities, including antidiabetic, antioxidant, and metabolic regulatory effects [3,9,10]. Although previous studies on trigonelline biosynthesis in fenugreek have primarily focused on precursor supply and metabolic pathway characterization [41], the regulatory mechanisms governing its accumulation remain unknown. Phytosulfokine is a sulfated peptide hormone that plays important roles in plant growth, development, and stress adaptation through receptor-mediated signaling pathways [16–20]. However, evidence linking PSK signaling to specialized metabolite biosynthesis remains largely unclear. In the present study, exogenous PSK- δ treatment significantly promoted trigonelline accumulation in fenugreek seedlings (Figure 1B), revealing a previously uncharacterized association between PSK signaling and trigonelline metabolism. Given that trigonelline biosynthesis is highly responsive to hormonal and environmental regulation [42–44], these findings suggest that peptide hormone signaling may represent an additional regulatory layer controlling alkaloid accumulation in fenugreek.

Previous studies have shown that PSK peptides, including PSK- α , PSK- δ and PSK- ϵ , exert biological effects at low concentrations (0.1–1 μ M) in different plant species [16,21,31]. Therefore, a concentration range of 0.1–1 μ M PSK- δ was selected in this study based on previous reports to evaluate its effects on fenugreek seedlings. Although the differences in overall seedling morphology among treatments were relatively subtle, quantitative analysis revealed that 0.1 μ M PSK- δ resulted in the greatest increase in primary root length, whereas higher concentrations did not further enhance this response (Figure S1). Similar non-linear concentration responses have been observed for peptide signaling molecules, indicating that increasing peptide concentrations do not necessarily result in stronger

biological effects [16,21]. Thus, 0.1 μ M PSK- δ was selected as an effective concentration for subsequent physiological and transcriptomic analyses.

Interestingly, trigonelline accumulation displayed a distinct temporal pattern during PSK- δ treatment. Although PSK- δ significantly enhanced trigonelline content at 5 days, its stimulatory effect was not maintained at later stages (10 and 15 days), when trigonelline levels in control seedlings had already increased substantially. The elevated basal accumulation of trigonelline at later developmental stages may be associated with metabolic maturation and developmental progression of fenugreek seedlings, during which NAD-related precursor availability and secondary metabolism become progressively established [12–14]. In addition, endogenous physiological changes associated with prolonged cultivation may contribute to this gradual accumulation. The reduced response to PSK- δ at later stages suggests that PSK- δ -mediated regulation may be more prominent during early metabolic reprogramming. Alternatively, the increased basal trigonelline level at later developmental stages may limit further accumulation following exogenous PSK- δ application.

Comparative transcriptomic analyses further revealed coordinated transcriptional changes in genes associated with trigonelline biosynthesis following PSK- δ treatment. Most genes involved in trigonelline biosynthesis exhibited expression patterns consistent with trigonelline accumulation (Figure 4), suggesting that PSK- δ may transcriptionally influence multiple steps of the trigonelline biosynthetic pathway through transcriptional regulation of the broader NAD metabolic network rather than a single enzymatic reaction. Because trigonelline biosynthesis relies on the dynamic interconversion of NAD-related metabolites [12–14] (Figure 3A), simultaneous regulation of precursor-generating, recycling, and methylation-associated genes could increase metabolic flux toward trigonelline formation. Similar hierarchical transcriptional control of secondary metabolism has been widely reported in jasmonate-regulated pathways, where upstream signaling cascades activate multiple transcription factors and downstream biosynthetic genes in a coordinated manner [30,45,46]. Therefore, the transcriptional patterns observed here support the hypothesis that PSK- δ functions as an upstream signaling regulator that orchestrates multiple components of the trigonelline biosynthetic pathway.

In addition, several genes associated with PSK signaling, including *TPST*, *SBT1.1*, and *AHA2*, were transcriptionally responsive to PSK- δ treatment (Figure 5), indicating an active modulation of PSK processing and maturation, as well as downstream signal transduction. Previous studies have shown that PSK signaling involves receptor-mediated phosphorylation cascades and Ca^{2+} -related signaling events [22]. Consistent with this, genes associated with MAPK signaling pathways were also differentially expressed following PSK- δ treatment (Figure 3B and Table S11), suggesting activation of conserved kinase-mediated signaling networks downstream of PSK perception. Importantly, MAPK cascades function as conserved signaling modules in peptide-mediated responses and can regulate downstream transcription factors involved in specialized metabolism [47], suggesting that MAPK-associated regulatory processes may contribute to PSK- δ -induced trigonelline accumulation. However, as no direct biochemical validation of MAPK activation was performed in the present study, further investigations are required to confirm the involvement of MAPK signaling and clarify its relationship with candidate transcriptional regulators.

Interestingly, several receptor-associated genes, including *PSKR1* and *BAK1*, were transcriptionally downregulated following PSK- δ treatment despite enhanced trigonelline accumulation (Figures 1B and 5B). This observation suggests that sustained transcriptional activation of receptor genes may not be required for downstream metabolic responses and may instead reflect feedback regulation within the PSK signaling pathway. In contrast, the persistent induction of AHA-related genes (Figure 5B) suggests that proton trans-

port and membrane-associated metabolic regulation may contribute to PSK- δ -responsive physiological processes.

Several TF families, including MYB, WRKY, bHLH and NAC, emerged as potential regulators, consistent with their previously reported roles in plant secondary metabolism and hormone-responsive regulation. These transcription factor families are widely implicated in plant secondary metabolism and hormone-responsive transcriptional regulation, including controlling diverse alkaloid, phenylpropanoid, and terpenoid biosynthetic pathways [38,39], highlighting their potential roles as key regulators. Among these candidates, bHLH18/63, MYB48/60 and WRKY3/23 represent promising regulators for future functional characterization due to their strong co-expression relationships with pathway-associated genes (Tables S7 and S8). These TFs therefore constitute promising candidates for further functional characterization of trigonelline biosynthesis regulation in fenugreek. Together, these findings support the existence of a complex transcriptional regulatory network underlying PSK- δ -induced trigonelline accumulation. Future studies integrating genetic and molecular approaches will be needed to verify the regulatory functions of representative candidate genes and transcription factors, such as NANMT and selected MYB, WRKY, and bHLH members, and determine whether they directly regulate trigonelline biosynthetic genes.

Nevertheless, a limitation of this study is that RNA samples from the 15-day treatment group were pooled prior to library construction. Consequently, the late-stage transcriptomic dataset should be regarded as providing an overview of sustained transcriptional responses rather than a statistically replicated dataset. Therefore, the candidate genes and transcription factors identified in this study should be interpreted as putative regulators pending further validation. Future RNA-seq analyses based on independent biological replicates, together with functional characterization of key candidate genes, will be necessary to refine the proposed regulatory network.

Previous studies have demonstrated that auxin and salicylic acid participate in the regulation of trigonelline biosynthesis and accumulation in medicinal plants [48–51]. In addition, combined hormonal treatments involving methyl jasmonate and salicylic acid have been shown to markedly alter trigonelline accumulation and the expression of biosynthetic genes such as *NANMT* and *QPT* in fenugreek, highlighting the importance of hormone-mediated transcriptional regulation in trigonelline metabolism [52]. PSK signaling has also been reported to modulate auxin responses and interact with SA-associated signaling pathways [53–55]. Consistent with these observations, transcriptomic enrichment analyses revealed significant changes in plant hormone signaling pathways following PSK- δ treatment (Figure 3B). These findings suggest that PSK- δ may influence trigonelline accumulation through the modulation of multiple phytohormone-associated regulatory pathways. However, the molecular mechanisms underlying this potential hormonal crosstalk remain to be further elucidated.

Beyond the mechanistic insights into PSK- δ -mediated regulation of trigonelline biosynthesis, this study also highlights the potential agronomic application of sulfated PSK peptides as environmentally friendly biostimulants for medicinal legume cultivation. Exogenous elicitors such as methyl jasmonate and salicylic acid have been widely used to stimulate the biosynthesis of valuable secondary metabolites in medicinal plants [56]. Nevertheless, these elicitation strategies often involve trade-offs between metabolite accumulation and plant growth, and their efficacy can vary considerably depending on treatment conditions and plant species [56]. Previous studies have demonstrated that conventional elicitors, such as methyl jasmonate and salicylic acid, can enhance trigonelline accumulation in fenugreek. For example, combined MeJA and SA treatment (200 μ M each) increased seed trigonelline content by 2.6-fold in a high-accumulating fenugreek ecotype, accompanied by the activation of trigonelline biosynthetic and transport-related

genes [52]. In contrast, PSKs are naturally occurring endogenous peptide hormones that function at extremely low concentrations and are generally recognized as positive regulators of plant growth and development [17,21,23,36]. In the present study, exogenous PSK- δ treatment at 0.1 μ M simultaneously promoted primary root elongation and significantly enhanced trigonelline accumulation in fenugreek seedlings, with trigonelline content increasing by up to 93% compared with the control (Figures S1 and 1), suggesting that PSK- δ may positively coordinate growth-related processes and specialized metabolite production. This finding suggests that PSK- δ may serve as an alternative peptide-based elicitor to conventional chemical elicitors, such as methyl jasmonate and salicylic acid, for enhancing specialized metabolite production in medicinal plants. Although the current cost of chemically synthesized sulfated peptides remains relatively high, their nanomolar-level bioactivity substantially reduces application dosage requirements. In addition, recent progress in microbial expression systems and peptide engineering provides a promising basis for the future development of cost-effective production strategies for peptide-based biostimulants [57–59]. Therefore, PSK- δ -mediated elicitation may represent a potential peptide-based approach for enhancing the accumulation of high-value bioactive metabolites in medicinal plants, although further validation under field conditions is required. Future studies should focus on field-scale validation, optimization of application protocols, and development of cost-effective production systems to facilitate future evaluation of PSK-based biostimulants for medicinal plant cultivation.

5. Conclusions

This study provides evidence that PSK- δ is involved in regulating trigonelline accumulation, suggesting a potential role of peptide signaling in plant specialized metabolism. Metabolite analysis revealed that trigonelline content was significantly increased at 5 days after PSK- δ treatment. Transcriptomic analysis revealed that PSK- δ treatment induced the expression of key genes associated with trigonelline biosynthesis, PSK signaling and MAPK signaling. Comparative transcriptomic and co-expression network analyses identified multiple candidate transcription factors potentially involved in the coordinated regulation of PSK-responsive trigonelline accumulation, laying a foundation for further investigation of its regulatory mechanism.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16151509/s1>, Figure S1: Effects of different concentrations of PSK- δ on primary root and shoot growth in fenugreek seedlings. Figure S2: Comparison and concordance analysis of differentially expressed genes (DEGs) identified at 5 d and 15 d after PSK- δ treatment. Figure S3: Validation of RNA-seq data by qRT-PCR analysis of selected candidate genes in control and PSK- δ -treated fenugreek seedlings after 5 days of treatment. Figure S4: Phylogenetic analysis of the key enzymes involved in trigonelline biosynthesis. Table S1: Summary statistics of transcriptome sequencing and de novo assembly in *T. foenum-graecum* seedlings. Table S2: Expression levels and functional annotations of trigonelline biosynthesis genes in fenugreek seedlings following 5 and 15 days of PSK- δ treatment relative to controls. Table S3: Expression levels and functional annotations of PSK signaling genes in fenugreek seedlings following 5 and 15 days of PSK- δ treatment relative to controls. Table S4: Transcription levels of target genes in PSK- δ -treated fenugreek seedlings (5 and 15 d) selected for co-expression analysis based on their expression patterns consistent with trigonelline accumulation. Table S5: Protein sequence information for the phylogenetic tree shown in Figure S4. Table S6: Deduced amino acid sequences of the fenugreek-derived trigonelline biosynthetic enzymes used for phylogenetic analysis. Table S7: Candidate transcription factors positively co-expressed with trigonelline biosynthetic genes. Table S8: Candidate transcription factors positively co-expressed with genes involved in PSK signaling. Table S9: Candidate transcription factors negatively co-expressed with trigonelline biosynthetic genes. Table S10: Candidate transcription factors negatively co-expressed with genes involved in PSK signaling. Table S11:

Candidate MAPK signaling genes (target genes) highlighted in red showing expression patterns consistent with trigonelline accumulation at 5 d and 15 d treatments. Table S12: Candidate positive transcription factors identified by co-expression analysis between candidate MAPK signaling genes (target genes) and 183 transcription factor-encoding genes at 5 d after PSK- δ treatment. Table S13: Candidate negative transcription factors identified by co-expression analysis between candidate MAPK signaling genes (target genes) and 183 transcription factor-encoding genes at 5 d after PSK- δ treatment.

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Data Availability Statement: The RNA sequencing data of *Trigonella foenum-graecum* seedlings treated with PSK- δ and scrambled pentapeptide control have been deposited in the NCBI SRA database under accession number PRJNA1481979.

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Abbreviations

The following abbreviations are used in this manuscript:

5'-NT	5'-nucleotidase
AHA	Plasma Membrane H ⁺ -ATPase
AO	Aspartate Oxidase
BAK1	Brassinosteroid Insensitive 1 (BRI1)-associated Receptor Kinase 1
CNGC17	Cyclic Nucleotide-Gated Channel 17
MAPK	Mitogen-Activated Protein Kinase
NaAD	Nicotinic Acid Adenine Dinucleotide
NAD	Nicotinamide Adenine Dinucleotide
NADS	NAD synthase
NaMN	Nicotinic Acid Mononucleotide
NANMT	Nicotinate N-methyltransferase
NAPRT	Nicotinate Phosphoribosyltransferase
NaR	Nicotinic Acid Riboside
NaRN	Nicotinic Acid Riboside Nucleosidase
NIC	Nicotinamidase
NMN	Nicotinamide Mononucleotide
NMNAT	Nicotinamide Mononucleotide Adenylyltransferase
NPP	Nucleotide Pyrophosphatase
NR	Nicotinamide Riboside
NRN	Nicotinamide Riboside Nucleosidase
PARP	Poly (ADP-ribose) Polymerase
PSK	Phytosulfokine
PSKR	PSK Receptor
QPT	Quinolinate Phosphoribosyltransferase
QS	Quinolinate Synthase
SBT	Subtilisin-like Serine Protease
TPST	Tyrosylprotein Sulfotransferase

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