miPEP858/miR858-MYB3-PSK4 module regulates growth and development in *Arabidopsis*

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ABSTRACT

Small molecules, peptides and miRNAs, are the crucial regulators of plant growth. Here, we show the importance of cross-talk between miPEP858a/miR858a and Phytosulfokine (PSK4) in regulating plant growth and development in *Arabidopsis*. Genome-wide expression analysis suggested modulated expression of PSK4 in miR858 mutant and overexpression, miR858OX, plants. The silencing of PSK4 in miR858OX plants compromised the growth, whereas overexpression of PSK4 in miR858 mutant rescued the developmental defects. The exogenous application of synthetic PSK4 further complemented the plant development in mutant plants. Exogenous treatment of synthetic miPEP858a in PSK4 mutant led to clathrin-mediated internalization of the peptide however did not enhance growth as in the case of wild-type plants. We also demonstrate that the MYB3 is an important molecular component participating in miPEP858a/miR858a-PSK4 module. Finally, our work highlights the signalling between miR858/miPEP858-MYB3-PSK4 in modulating the expression of key elements involved in auxin responses leading to the regulation of growth.

Keywords: miPEPs, MYB3, phytohormones, PSK4, signalling peptides, transcriptome.

INTRODUCTION

In multicellular organisms like plants, growth and development are precisely coordinated and tightly controlled via cell-to-cell interactions. Plant growth and development is a well-coordinated process that is controlled by numerous factors, including hormones, transcription factors, miRNAs, small peptides, microProteins and other signaling molecules (Tavormina et al., 2015; Catalanotto et al., 2016; Roberts et al., 2016; Bhati et al., 2021; Takahashi et al., 2018). Until recently, classical phytohormones held a dominant role in our understanding of the regulatory mechanisms that lead to plant development and diverse cellular responses (Olsson et al., 2019; Sauter 2015). However, in just over a decade, numerous small peptides and RNAs have been recognized as important signalling molecules in plants that regulate cell-cell communication and assist in the co-ordination and integration of cellular functions (Czyzewicz et al., 2013). Plant peptides possess an extensive range of biological functions that include growth, defence and stress tolerance in response to various biotic and abiotic stresses (Butenko et al., 2009; Grillet et al., 2018; Pearce et al., 1991; Butenko et al., 2003; Reichardt et al., 2020). Despite the tremendous roles the peptides play, the detailed signalling mechanisms and the cross-talks of the small peptides with other plant regulatory molecules (miRNAs and transcription factors) and the downstream pathways activated by these peptides are largely unknown.

MicroRNAs (miRNAs) are small, non-coding, endogenous RNAs that acts as the key regulators of a variety of plant and animal developmental processes (Ambros 2001; Chen 2009). miRNAs are processed products of primary miRNAs (pri-miRNAs). A few recent studies have suggested that primary miRNA (pri-miRNA), initially whose regulatory function was largely unknown, harbours short open reading frames (ORFs) with potential to encode regulatory peptides, termed as miRNA-encoded peptides (miPEPs). miPEPs are reported to positively regulate the accumulation of their associated miRNAs (Lauressergues et al., 2015; Couzigou et al., 2015; Sharma et al., 2020). In *Arabidopsis thaliana*, miR858a plays a crucial role in the regulation of the phenylpropanoid pathway and plant growth (Sharma et al., 2016). In our recent study, we have demonstrated that primary transcript of miR858a encodes a small regulatory peptide, called miPEP858a. This peptide regulates the expression of miR858a at the transcriptional level and thereby affects the expression of target genes involved in the phenylpropanoid pathway as well as plant growth and development (Sharma et al., 2020).

The MYB transcription factors represent one of the major families of transcriptional regulators in plants. In recent years, the R2R3-MYB and R3-MYB transcriptional repressors have arose as crucial players in the regulation of the phenylpropanoid pathway and plant growth (Zhou et al., 2017; Ma et al., 2019; Dubos et al., 2010). In *Arabidopsis*, miR858a is known to control the flavonoid biosynthesis by downregulating the expression of various MYB transcription factors including MYB11, MYB12 and MYB111 which are involved in the regulation of flavonol biosynthesis (Sharma et al., 2016; Piya et al., 2017). Apart from modulations observed in flavonol, anthocyanin and lignin content, a robust alteration in growth patterns is observed in miR858a overexpression and its mutant plants (Sharma et al., 2016; Piya et al., 2017). Although significant progress has been made towards our understanding of the regulation of the phenylpropanoid pathway by miR858a, it remains completely unknown to what downstream factors are responsible for the significant modulations observed in growth. In this study, by performing the RNA-seq analysis, we investigated the underlying molecular components involved in miR858a-dependent growth and development in *Arabidopsis thaliana*.

In this study, we propose that Phytosulphokine4 (PSK4), a disulphated pentapeptide, controls plant growth in a miR858-dependent manner. In *Arabidopsis*, five PSK (PSK1-5) genes exist that harbours the canonical YIYTQ sequence (Sauter 2015; Matsubayashi & Sakagami 1996). The PSK precursor genes show differential gene expression throughout the plant life cycle (Yang et al., 2001). PSK precursors undergo tyrosine sulfation by a tyrosylprotein sulfotransferase (TPST) followed by proteolytic cleavage in the apoplast by *AtSBT1.1* to generate biologically functional, mature peptide (5 amino-acid in length) (Sauter 2015; Srivastava et al., 2008). The mature PSK4 binds to its corresponding plasma membrane-localized receptor-like kinases (RLKs) to initiate the downstream signalling processes (Ma et al., 2016; Wang et al., 2015). *Arabidopsis* contains two PSK receptor genes, *AtPSKR1* and *AtPSKR2*, that belongs to the leucine-rich repeat receptor-like kinase (LRR-RLK) family of the receptor. However, *AtPSKR1* is majorly involved in the perception of PSK4 and it expresses in all tissues with varying abundance (Sauter 2015). The detailed signalling mechanisms and pathways activated by PSK are unknown. Therefore, deciphering the detailed function and its cross-talk with other signalling molecules is essential to understand their role in intercellular communication fundamental to plant biology.

Here, we report that PSK4 is an important molecular component involved in miR858a-dependent plant growth. The differential gene expression analysis revealed a significant modulation in the levels of precursor genes encoding for PSK4 in contrasting lines of miR858a. Further, the knock-out lines of PSK4 in miR858OX plants compromised the growth whereas over-expression of in *miR858^{CR}* plants complemented the plant growth and development. The exogenous application of miPEP858a on transgenic lines expressing reporter gene under the control of PSK4 promoter revealed an important co-relation between miPEP858/miR858 in regulating the expression of PSK4. Elevated levels of expansins triggered an increase in the root elongation zone. In this study, we demonstrate PSK4 mediated regulation of plant growth and development in miR858OX and its mutants is through auxin. In this study, we also studied about the internalization of miPEP858s in *Arabidopsis thaliana*. We have also identified MYB3 as an essential component required in the PSK4 and miR858 signalling cascade. This study demonstrates an additional layer of regulation of miR858/miPEP858- MYB3-PSK4 module in finely balancing expression of genes to ensure optimal growth and development in *Arabidopsis thaliana*.

RESULTS

Identification of molecular components involved in miR858 dependent growth

In an earlier study by Sharma et al., 2016, we demonstrated that miR858a regulates the phenylpropanoid pathway as well as plant growth (Sharma et al., 2016). Apart from modulation in flavonoid contents, miR858a overexpressing plants displayed enhanced growth compared to WT, whereas *MIM858* (transgenic lines expressing an artificial miRNA target mimic) exhibited compromised and delayed growth (Sharma et al., 2016). Recently, we showed that the primary transcript of miR858a encodes a regulatory peptide known as miPEP858, which increases the transcript of miR858a and regulates flavonoid biosynthesis along with plant growth and development (Sharma et al., 2020). miPEP858OX plants also exhibited increased growth, while miPEP858a edited lines displayed delayed and compromised growth compared to WT plants. Therefore, to gain a deeper insight into miR858 function and identify the molecular component(s) involved in miR858-dependent plant growth and development, we carried out genome-wide transcriptome analysis of miR858OX and *MIMIC858* plants (Supplemental Figure S1, Supplemental Table S1). The analysis of rosette and stem tissues of these plants suggested that 341 and 382 genes uniquely expressed in miR858OX and *MIMIC858* plants, respectively, along with 175 common differentially modulated genes (Figure 1A). Differentially modulated genes belonged to members of various transcription factor families and genes associated with stress response, hormones, transporters, as well as small peptides (Supplemental Table S2). Interestingly, we identified that precursor encoding for a disulfated pentapeptide called Phytosulfokine4 (AtPSK4) was significantly up-regulated in miR858OX lines, while its expression decreased significantly in *MIM858* lines.

PSK4 belongs to post-translationally modified peptides (Mastubayashi 2014). The Phytosulfokine family in *A. thaliana* consists of five members (PSK1-5), encoded by different precursor genes with differential expression (Yang et al., 2001). To validate the transcriptome data and define the role of PSK4 in regulating miR858a/miPEP858a-dependent growth, we analyzed the expression of PSK4 along with the other members of the Phytosulfokine family (AtPSK1-5) in miR858OX and *MIMIC858* lines along with *miR858^{CR}*, *miPEP858^{CR}* and miPEP858OX lines developed in our previous study (Sharma et al., 2020). Reassuringly, precursor encoding for PSK4 was also significantly reduced in *miR858a^{CR}* and *miPEP858^{CR}*

lines, while levels of PSK4 enhanced in miPEP858OX lines. The analysis revealed that the expression of PSK4 was significantly enhanced in miR858OX and miPEP858OX plants. On the contrary, the expression of PSK4 was significantly down-regulated in mutants (*MIMC858*, *miR858^{CR}* and *miPEP858^{CR}* plants) (Figure 1B and 1C). Further, the qRT-PCR analysis suggested that only the expression of PSK4 (no other PSK family members) was significantly modulated in miPEP858 overexpressing lines (Supplemental Figure S2). Altogether, the above results strongly infer that as the levels of miR858a increases (miR858OX and OX miPEP858a), there is a significant increase in the expression of PSK4. While, in mutants (*MIM858*, *miR858a^{CR}* and *miPEP858a^{CR}*) where the levels of miR858 decreases, there is a significant reduction in the expression of PSK4. These results suggest that PSK4 may play a significant role during growth and development in miR858a/miPEP858a dependent manner. Interestingly, many earlier studies have implicated the functional role of PSK4 in the regulation of growth and other physiological processes (Igarashi et al., 2012; Kutschmar et al., 2009; Geng et al., 2020; Yu et al., 2016; Matsubayashi et al., 2006) however other components involved in these processes were not investigated in detail. It is thus reasonable to hypothesize that PSK4 might control the growth in miR858a mutants and overexpression lines.

miPEP858a controls the expression of PSK4 via miR858

Earlier studies have shown that the exogenous application of miPEP enhances the expression of its corresponding miRNA (Laressergues et al., 2015; Couzigou et al., 2015; Sharma et al., 2020). To determine whether the application of synthetic miPEP858 also increases the transcript of PSK4 (as it increases the accumulation of miR858), WT seedlings were grown on media supplemented with different concentrations of miPEP858. Interestingly, the application of miPEP858 increased the transcript of PSK4 along with the transcript of miR858a as observed in miR858OX lines (Figure 2A and 2B). Also, the expression of PSK4 which was initially reduced in *miPEP858^{CR}* plants also enhanced significantly in *miPEP858^{CR}* and in WT plants grown on media supplemented with miPEP858a (Figure 2E). These experiments reveal that miPEP858a enhances the expression of PSK4.

The internalization of miPEP858 inside the plant cells was analyzed by incubating the WT seedling in the liquid media containing FAM-labeled miPEP858 (FAM-miR858a). When WT seedlings were visualized under a confocal microscope, green florescence was observed inside

the cells (Figure 2D). Also, when the WT roots were stained with propidium iodide (PI), which stains the cell wall, the presence of green fluorescence was clearly observed inside the cell (Figure 2C). Altogether, these results clearly indicate that miPEP858a penetrate inside the plant cell. Recently it has been shown that miPEP165a is internalized inside the plant via clathrin-mediated endocytosis (Ormancey et al., 2020). Therefore, we hypothesized that if miPEP858a also follows a similar route, the exogenous application of miPEP858a in the media should not affect the phenotypes as well as the expression of miR858 in the clathrin mutants. To validate this, we grew seeds of WT along with clathrin mutants (*chc1.1*, *1.2*, *2.1* and *2.2*) on media supplemented with and without miPEP858a. The root length analysis demonstrated that the exogenous application of miPEP did not cause any change in the root growth on *chc* mutant, whereas WT seedlings showed a significant increase in root length (Supplementary Figure S3A-S3C). We have also analyzed the expression of pre-miR858a in seedling of WT and *chc* mutants grown on media supplemented with or without miPEP858a and expression analysis suggested that application of miPEP858a did not affect the expression of pre-miR858a in *chc* mutants while as expected, WT seedlings showed a significant increase in the expression of pri-miR858 (Supplemental Figure S3D). To further verify the effect of miPEP858a, we have performed exogenous peptide assays on mature plants. The application of miPEP858a did not show any significant modulation in phenotypes of mature plants, similar to the results obtained with seedlings (Supplemental Figure S3E). Together, these results suggested that clathrin mutants do not show any significant modulation in the phenotype as well as expression upon application of miPEP858a, demonstrating that clathrin-mediated endocytosis might be required for miPEP858a functions. Therefore, to confirm that clathrin-mediated endocytosis is essential for miPEP858 endocytosis, we have incubated the seedlings of clathrin mutant (*chc1.2*) with FAM-labeled miPEP858a and fluorescence analysis clearly showed that clathrin mutant was unable to endocytose FAM-miPEP858a. As a result, no fluorescence was detected in the cells of clathrin mutant (Figure 2D and Supplemental Figure S4). Overall, these results suggested that clathrin-mediated endocytosis is required for the internalization of miPEP858a in *Arabidopsis thaliana*. Finally, based on this study we propose a model suggesting that clathrin-mediated endocytic pathway is essential for miPEP858a uptake in *Arabidopsis thaliana* to regulate the transcription of miPEP858 (Supplemental Figure S5).

To obtain a deeper insight into the co-relation between miR858/miPEP858 and PSK4, a promoter-reporter (*ProPSK4::GUS*) expressing transgenic lines of *Arabidopsis thaliana* was developed. As an exogenous application of miPEPs enhances the accumulation of mature miRNAs, we hypothesized that the application of miPEP858a might affect PSK4 expression, by enhancing the expression of miR858a. Interestingly, the exogenous application of synthetic miPEP858 to *ProPSK4* lines resulted in higher GUS activity, as shown by GUS histochemical assay and expression analysis (Figure 2E and 2F). Our results strongly reveal that miR858/miPEP858 have the potential to enhance the transcription of PSK4.

PSK4 participates in regulating miPEPa/miR858a-associated phenotypes

To understand the involvement of the miPEP858-miR858-PSK4 module in regulating plant growth and development, we generated transgenic plants overexpressing *AtPSK4* under the control of the CaMV35S promoter and its knock out mutants using the CRISPR/Cas9 genome editing approach in Col-0 background. PSK4 knock-out lines carried Indels in the PSK4 peptide-coding region that is expected to cause a premature translational termination in the PSK4 motif (YIYTQ), rendering the peptide to be non-functional (Supplemental Figure S6). Further, the expression analysis study also showed that the transcript level of PSK4 was several folds up-regulated in PSK4 over-expression lines while its expression was significantly down-regulated in PSK4 mutants (*PSK4^{CR}*) (Figure 3D and 3E).

To be sure that changes in plant growth are specifically due to PSK4 and is not the synergistic effect of other PSK family members, we also tested the expression of other members of the PSK family in PSK4 OX and PSK4 mutants. No significant change in the transcripts of other members was observed, signifying the specific differential expression of PSK4 in these lines (Supplemental Figure 7A and 7B). We analyzed the effect of PSK4 over-expression and mutation on root growth. PSK4 OX seedlings displayed increased primary root length than the WT seedlings, whereas a significant reduction in the primary root length was observed in *PSK4^{CR}* seedlings (Figure 3A-3C). Also, PSK4 OX plants showed a significant enhancement in plant growth (early bolting, rosette diameter and plant height). In contrast, delayed and

compromised growth was observed in PSK4 edited lines compared to that of WT plants (Figure 3I, 3J and Supplemental Figure S8).

As previously reported, expansins affect the root length growth in PSK over-expression lines in *Arabidopsis thaliana* and expansin activity is also regulated during the root elongation (Yu et al., 2016). Thus, we analyzed the expression of growth-related expansins, i.e., *EXPA2*, *EXPA11* and *EXPA15* in PSK4 OX and *PSK4^{CR}* plants. Our results revealed a significant upregulation in the expression of these genes in over-expression lines while their expression was significantly reduced in PSK4 edited lines (Figure 3F). To further determine the involvement of PSK4 in *Arabidopsis* root system architecture, we analyzed the alteration in root cell elongation zone underlying the faster root growth in PSK OX lines and slower root growth in *PSK4* mutants. Our results reveal a significant increase in the root elongation zone in PSK4 OX lines (Figure 3G, 3H and Supplemental Figure S9). These results demonstrated that the PSK4 is involved in root morphogenesis. The reduced primary root growth in the PSK4 knock-out plants is likely caused by defective cell division and elongation processes.

Exogenous PSK4, not miPEP858a, complements function in *PSK4^{CR}* plants

Further, to study the effect of synthetic PSK4 peptide on plant growth, WT seedlings were grown on media supplemented with sulphated PSK (PSK), desulphated PSK (dPSK) and scrambled PSK (ScPSK) peptide. Supplementation with sulphated PSK showed a potential increase in the root length and seedlings weight in WT plants similar to PSK4 OX plants (Figure 4A-4C). In addition, our analysis also suggested that the application of synthetic PSK and, to a lesser extent, dPSK can also complement the root length phenotype and gene function in *PSK4^{CR}* plants. Interestingly, the application of synthetic PSKs could restore the functions in *miR858^{CR}* and *miPEP^{CR}* plants (Figure 4D-4H).

To show the specificity and involvement of PSK4 in the miPEP858 signaling module, we carried out the complementation analysis of *PSK4^{CR}* lines with synthetic miPEP858a. Interestingly, the application of miPEP858 and a non-specific miPEP (NSP) did not cause any significant change in the root length of *PSK4^{CR}* lines (Figure 4I and 4J). In *Arabidopsis*, it has been shown that the tyrosylprotein sulfotransferase (TPST), sulfates the PSK precursor leading to the formation of bioactive PSK (Sauter 2015; Matsubayashi et al., 2014). In the *tpst-1* mutant

with no endogenous active PSK, the root growth is significantly lowered, that was restored by exogenous application of synthetic PSK. The unsulphated PSK (dPSK) also showed weak root growth promotion but to a lesser extent and no significant root growth was observed after treatment with scrambled peptide (ScPSK) (Figure 4K, 4M). *Arabidopsis* encodes two PSK receptor genes, *AtPSKR1* and *AtPSKR2*, of these *AtPSKR1* is expressed in all organs with variable abundance and contributes majorly to the PSK signalling. Seedlings that lack *AtPSKR1* have a shorter root length than the WT plants (Kutschmar et al., 2009). Exogenous application of PSK could not restore the PSK4 functions in *pskr1* mutant (Figure 4L and 4M). As exogenous miPEP858a significantly enhanced the expression of PSK4 in WT seedlings, we further analyzed the effect of the application of synthetic miPEP858 to *tpst* and *pskr1* mutants and no significant change in the root length was observed (Figure 4N-4P). These results imply that PSK and its receptor tightly regulate the PSK signalling and PSK4 is involved in miPEP858-dependent modulation in growth parameters in *Arabidopsis*.

PSK4 modulates the miR858a/miPEP858a-associated growth phenotypes

To analyse the potential role of PSK4 in miR858-dependent growth, we characterized its loss of function mutant phenotype in miR858 over-expressing plants. In miR858 mutants, the expression of PSK4 is significantly reduced and plants exhibit compromised and delayed growth. Therefore, we hypothesized that if PSK4 is responsible for miR858-mediated growth, over-expression of PSK4 in *miR858^{CR}* background should complement the growth phenotypes as well as the expression of PSK4. As anticipated, over-expression of PSK4 in *miR858^{CR}* background caused a significant increase in the primary root growth (Figure 5A, 5B). The expression of PSK4 also enhanced significantly in these lines, which is comparable to WT (Figure 5C). Later, at maturity, *miR858^{CR}* plants over-expressing PSK4 gained all the growth phenotypes that were initially compromised in *miR858^{CR}* mutants. The plants displayed enhanced vegetative growth, reflected by an increased number of rosette leaves and diameter along with the overall plant growth (bolting time and plant height) as similar to WT plants (Figure 5G and Supplemental Figure S11). Together, this indicated that the developmental defects in *miR858^{CR}* mutant are possibly caused by deficiency of PSK4.

Since over-expression of PSK4 promoted growth in *miR858a^{CR}* plants, we developed PSK4 mutant in miR858OX lines to study the effect on the plant growth. Therefore, to

functionally characterize the role of the PSK4 in association with miR858, we knocked out PSK4 in miR858 over-expression lines (Supplemental Figure S10). Interestingly, the primary root length was highly reduced in these plants compared with that of miR858OX lines, which is reduced to WT plants (Figure 5D and 5E). The expression of PSK4 was also significantly reduced in these lines (Figure 5F). At the later stages, *PSK4^{CR}* in miR858OX plants displayed developmental defects such as delay in bolting time and reduced growth (Figure 5H and Supplemental Figure S11). These results highlight that PSK4 comprises an essential molecule in regulating growth of the Arabidopsis.

To investigate the effect on levels of expansins, we further analyzed the expression of growth-related expansins in *PSK4^{CR}* in miR858OX and PSK4OX in *miR858^{CR}* plants. Interestingly, the analysis suggested that the expression of expansins was significantly reduced in *PSK4^{CR}* in miR858OX plants while enhanced significantly in PSK4OX in *miR858^{CR}* plants (Figure 5I). Taken together, our results suggest that PSK4 mediated modulations in expansins play an important role in growth in *miR858^{CR}* and miR858OX plants.

miPEP858-miR858-PSK4-mediated growth requires downstream auxin biosynthesis

Auxin is one of the most important signalling molecules that regulate plant growth and development (Brumos et al., 2018). An earlier report has shown the role the PSK4 in auxin-dependent immunity in response to the necrotrophic pathogens in tomatoes (Zhang et al., 2018). Therefore, we studied the involvement of auxin in miPEP858-miR858-PSK4-mediated growth and development. To determine the relationship between auxin and PSK4, the expression of *YUC* genes (*YUC1* and *YUC4*) involved in auxin biosynthesis was investigated in WT seedlings grown on media supplemented with different concentrations of synthetic PSK. Interestingly, supplementation with PSK significantly enhanced the expression of YUCs in WT seedlings (Figure 6A). To verify the involvement of auxin pathways in PSK signalling, we further analyzed the expression of these genes in PSK4 OX and *PSK4^{CR}* plants. Remarkably, PSK4 mutants showed significantly reduced expression of YUC1 and YUC4, whereas an enhanced expression of these genes was observed in PSK4 OX plants (Figure 6B).

Previous studies report that elongation of root length is influenced by the expression of expansins (Lee et al., 2003; Trivedi & Nath 2004). To gain additional insights into the

correlation between the expression of the PSK4 and expansins in regulating growth, we analyzed the expression of expansins in the seedlings grown on media supplemented with different concentrations of indole-3-acetic acid (IAA, auxin). Strikingly, the expression of expansin genes (*EXPA2*, *EXPA11* and *EXPA15*) enhanced significantly compared to that of control (Figure 6C). Further, to examine whether PSK4 regulates plant growth and development through auxin, we analysed the growth of WT, *PSK4^{CR}* and PSK4 OX after germination on a half-strength MS medium with or without supplementation with 2,3,5-triiodobenzoic acid (TIBA; 25 μ M), an auxin transport inhibitor. Seedlings grown on TIBA-supplemented media exhibited restricted growth suggesting the involvement of an auxin-dependent mechanism in PSK4 function (Figure 6D and 6F-6H). Also, the expression of *EXPA2*, *EXPA11* and *EXPA15* reduced significantly in WT seedlings when grown on media supplemented with TIBA compared to that of control (Figure 6E). To conform PSK4 regulate plant growth via auxin, we measured in vivo levels of auxin in WT, *PSK4^{CR}* as well as PSK4 OX seedling. The analysis suggested that the auxin level significantly reduced in *PSK4^{CR}* lines while PSK4 OX lines have higher accumulation (Supplemental Figure S12). Mutation in *PSK4^{CR}* lines caused reduction in the level of auxin. We hypothesized that exogenous application of PSK to *PSK4^{CR}* lines might complement its function. As expected, supplementation with synthetic PSK enhanced the level of auxin in *PSK4^{CR}* lines (Supplemental Figure S12). Thus, PSK4-induced IAA accumulation and modulations in the expression of expansins function together in controlling growth in miR858 over expression and miR858 mutants.

MYB3 participates in miR858-PSK4 module to regulate plant growth

To study the molecular mechanism associated with miPEP858/miR858a-PSK4-dependent plant growth, we carried out *in silico* promoter analysis of PSK4 and miR858a/miPEP858a. The promoter analysis revealed the presence of several *cis*-acting elements implicated in transcriptional regulation of light (AE-box, G-box, LTR and MRE), hormones (ABRE and TATC-box) and abiotic stress (MBS). Interestingly, we found the presence of binding sites of a newly characterized transcription factor, MYB3, which belongs to Subgroup 4 of R2R3-MYB transcription factors on the promoters of PSK4 and miR858 (Figure 7A and Supplemental Figure S13A). MYB3 is a novel identified repressor in phenylpropanoid biosynthesis. Therefore, we hypothesized that MYB3 might be one of the crucial factors involved in miPEP/miR858-PSK4-

mediated plant growth. To unravel this, we have generated the edited lines of MYB3 using CRISPR/Cas9 and the expression of MYB3 was reduced significantly in *MYB3^{CR}* lines (Figure 7B). When we analysed the expression of PSK4 and miR858 in MYB3 edited lines, we found that the expression of both PSK4 and miR858 were significantly downregulated in MYB3 edited lines (Figure 7B and Supplemental Figure S13B). To further investigate the role of MYB3, we developed the over-expression lines of MYB3 under the control of the CaMV35S promoter. The expression of MYB3 was several fold higher in MYB3 OX lines. Also, the expression of both PSK4 and miR858 was significantly enhanced in overexpression lines as compared to WT (Figure 7C and Supplemental Figure S13B).

At the seedling stage, MYB3 OX lines exhibited a higher primary root length, whereas *MYB3^{CR}* plants displayed a significantly shorter primary root length compared to WT plants (Figure 7E and 7F). The mature MYB3 OX transgenic plants (30-day-old) grew rapidly and showed vigorous growth like early bolting, plant height and rosette diameter compared to WT plants. Concomitantly, *MYB3^{CR}* plants displayed delayed growth. A remarkable difference was also observed in the plant height in MYB3 OX and *MYB3^{CR}* lines compared to WT plants (Supplemental Figure S14). These results suggested that the modulation in expression of MYB3 affects the plant growth.

To verify the biological function of MYB3, we have grown the MYB3 edited seedlings on the media supplemented with exogenous PSK and miPEP858. These results demonstrated no significant change in root length of MYB3 edited seedlings after the application of exogenous PSK and miPEP858 (Figure 7E and 7F). These results also suggest that the application of synthetic miPEP858 could not complement the PSK4 and miR858a expression in MYB3 mutants, hence indicated that MYB3 is essential for the expression of PSK4 and miR858 (Figure 7D and Supplemental Figure S13C). To show that MYB3 is required for the function of PSK4 and gain an additional understanding of regulation of the PSK4 by MYB3, we have developed the promoter-reporter lines of PSK4 in the *MYB3^{CR}* background. The histochemical GUS staining showed the activity of GUS in the positive control (empty vector; EV) and ProPSK4::GUS in WT background. Interestingly, partially absent or significantly reduced GUS activity was observed in ProPSK4::GUS in the *MYB3^{CR}* background (Figure 7G). All together

388 these results demonstrated that the MYB3 transcription factor is necessary for
389 miPEP858/miR858-PSK4-dependent plant growth and development.

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DISCUSSION

The identification of functional peptides in plants represents one of the most fascinating research areas and has led to advancement in understanding how these few amino acids can effectively control growth and other vital processes of plant life (Amano et al., 2007; Haruta et al., 2014; Schardon et al., 2016). With the advancement in the field of genomics, numerous predicted peptide-encoding genes and their probable receptors have been investigated (Ma et al., 2016). In recent studies by various groups worldwide, several miPEPs have been identified and has been demonstrated to play crucial roles in the plant. Despite, the tremendous roles played by the peptides, their detailed signalling mechanisms and the cross-talks of these small peptides with other plant regulatory molecules (phytohormones, miRNAs and transcription factors) and the downstream pathways activated by these peptides are largely unknown.

In *A. thaliana*, the miR858 family comprises two family members i.e., miR858a and miR858b and plays a crucial role in the regulation of the phenylpropanoid pathway and plant growth (Sharma et al., 2016; Piya et al., 2017). In an earlier study, mutants of miR858a/b/ab and pre-miR858a (*miR858a^{CR}*, *miR858b^{CR}*, *miR858ab^{CR}*, *pre-miR858a^{CR}*) were developed using CRISPR/Cas9 approach¹⁸. In addition, miPEP858a, a regulatory peptide encoded by the primary transcript of miR858 was functionally characterized by developing miPEP858a OX and *miPEP858a^{CR}* plants. miPEP858 regulates the expression of miR858a at the transcriptional level and thereby affects the expression of target genes involved in the phenylpropanoid pathway as well as plant growth and development. Together, the miR858 family and miPEP858a controls anthocyanin, flavonoids and lignin biosynthesis by regulating the phenylpropanoid pathway.

This study provides downstream molecular components responsible for the significant modulations observed in the vigorous change in growth patterns observed in miR858/miPEP858 overexpression and its mutant plants. In this study, through RNA-seq analysis, with a major focus on identifying genes related to plant growth, we identified differential expression of precursor genes encoding for a disulfated pentapeptide, PSK4, in WT miR858OX and *MIM858* lines. Earlier reports have suggested that PSK4 is majorly known to play an important role in plant growth. As miPEP858 affects the expression of miR858 and affects growth, to confirm the role of PSK4 in regulating miR858/miPEP858-dependent growth, the expression of PSK4 along with the other members of the Phytosulfokine family (AtPSK1-5) was examined in *miPEP858^{CR}*

and miPEP858OX lines along with miR858OX, *MIMIC858*, and *miR858^{CR}* lines. The analysis suggested that only the expression of PSK4 (no other PSK family members) was significantly altered in miPEP858OX lines. Our study also revealed a significant increase in the transcript levels of PSK4 in WT and *miPEP^{CR}* seedlings supplemented with synthetic miPEP858a compared to control. Exogenous treatment of ProPSK4::GUS seedlings with synthetic miPEP858a significantly enhanced the GUS gene activity suggesting that miR858a/miPEP858a positively affect the expression of PSK4 in *Arabidopsis*. To validate the function of PSK4 in regulating miPEP858/miR858 dependent plant growth, we generated mutant plants defective in PSK4 in WT and miR858OX background through CRISPR–Cas9 approach. Various studies suggest that the overexpression of peptide encoding genes affects plant growth and development. To provide better insight into the role of PSK4 in governing miR858 mediated growth, we also generated PSK4 overexpression lines in WT and *miR858^{CR}* background. Interestingly, knocking out PSK4 in miR858OX plants compromised the plant growth while overexpression of PSK4 in *miR858^{CR}* complemented the defects in growth parameters observed in *miR858^{CR}* plants.

Our work highlights that expansins perform an important function in regulating PSK4-mediated growth based on the observations reported here. Auxin is one of the classical growth-regulating hormones in plants that modulate plant growth; however, very few studies have investigated the roles of small peptide-mediated phytohormone modulation in plant growth (Brumos et al., 2018). Our results, together with previously published data, support the view that the auxin biosynthesis and associated signalling play an important role in plant development and is required for PSK4-mediated growth of *miR858^{CR}* and miR858OX plants. As shown, the application of exogenous PSK enhanced the abundance of genes associated with auxin signalling. In addition, the application of exogenous IAA enhanced the transcript levels of growth-related expansins suggesting that the activation of several downstream pathways may underlie the effects of PSK4 in regulating growth in miR858 dependent manner. In plants, clathrin-mediated endocytosis (CME) refers to one of the key internalization routes (Kitakura et al., 2011; Dhonukshe et al., 2007). This study has also studied the internalization of miPEP858 in *Arabidopsis* seedlings by using FAM-labeled peptide. The results showed that the internalization of miPEP858a is clathrin-mediated. Moreover, our study also demonstrates that exogenous application of miPEP858 to clathrin mutant (*chc1.2*) does not affect any plant phenotype and the expression of miR858 and target genes, suggesting that clathrin-mediated internalization is essential for the

uptake of miPEP858a. In this study, we have also identified MYB3 as an essential molecular component that positively regulates the expression of the PSK4 and miR858. Plant growth was significantly modulated in MYB3 edited and over-expression lines. The expression analysis results suggested that the expression of PSK4 was significantly enhanced in MYB3 OX lines, while it was reduced in *MYB3^{CR}* lines. Further analysis suggested that exogenous application of miPEP858 did not cause any significant modulation in the expression of PSK4 in the *MYB3* mutant. Additionally, we also found that the exogenous supplementation of PSK and miPEP858 does not complement the root length in *MYB3* mutant plants. In agreement with these results, our study shows that the MYB3 might be crucial for the function of PSK4 in miR858-dependent plant growth. Further proof in support of understanding of the regulation of PSK4 by MYB3 is the demonstration that the GUS activity was significantly reduced in ProPSK4::GUS in the *MYB3^{CR}* background.

On the basis of our findings, we propose a model depicting the participation of PSK4 as a signal molecule in miR858a/miPEP858a-dependent growth and development in *Arabidopsis* (Figure 8). Manipulation of miPEP858/miR858-PSK4 mediated growth represents an attractive approach that could potentially be used to enhance growth and productivity in many diverse plant species. How PSK4 regulates expansins and auxin-mediated growth in miR858 over-expression and mutants needs to be investigated in detail. Whether miPEP-miR858-PSK4-mediated regulation of growth is restricted to *Arabidopsis thaliana* or is more broadly distributed in other plants remains an open question. Small plant peptides possess numerous unique features that could be explored and utilized for their usefulness and would deliver opportunities to resolve agricultural-related problems. Together, this study provides an essential insight into the molecular control of growth regulated by cross-talk between miRNA and peptides.

MATERIALS AND METHODS

Plant material and growth condition. *Arabidopsis thaliana* (Col-0) was used as the WT plant, and the CRISPR–Cas9 approach was used for the editing of the *AtPSK4* gene, homozygous and Cas9-free edited plants (T4 generation) were used for study. For genome-wide analysis, miR858aOX and *MIMIC858* lines were used (19). *tpst-1* (AT1G08030; SALK_009847) and *pskr1* (AT2G02220; SALK_033212), *chc1* (AT3G11130 SALK_112213, *chc1.2*,

SALK_103252), *chc2* (AT3G08530 SALK_042321, *chc2.1* SALK_028826) mutants were purchased from ABRC. For germination, the seeds were surface sterilized and placed on half-strength MS medium (Hi-Media) containing 1.5% sucrose, pH 5.72–5.8. After stratification for two days at 4°C in the dark, the plates were transferred to a growth chamber (Conviron) set at a 16-h-light/8-h-dark photoperiod cycle, 180 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 22°C temperature and 50–60% relative humidity. Ten-day-old seedlings were used for the root length measurements. The miPEP858a, over-expressing (miPEP858aOX), *miR858CR*, *miPEP858CR* lines were developed earlier by our group, and used in this study for analysis (Sharma et al., 2020). To study the effect of TIBA on the growth of *Arabidopsis* seedlings, a half-strength MS medium was supplemented with TIBA (25 μM) dissolved in ethanol. The control plates were supplemented with an equal amount of ethanol. Three-day-old light-grown *Arabidopsis* seedlings were transferred from control plates to plates containing TIBA, and root growth was measured.

RNA-Seq analysis. For RNA sequencing, total RNA was extracted from 30-days-old rosette leaves and stem of Col-0, *MIMIC858* and *miR858OX* plants using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) and treated with RNase free DNaseI (Ambion, USA) according to manufacturer's instructions. Transcriptome datasets of these samples were developed through SciGenom Labs Private Limited (Cochin, Kerala, INDIA). Based on quality of sequence reads (Base quality score distribution, sequence quality score distribution, average base content per read, GC distribution in the reads, PCR amplification issue, check for over-represented sequences) sequence read were trimmed where necessary; to retain only high-quality sequence for further analysis. In addition, the low-quality sequence reads were excluded from the analysis. From the Trimmed paired-end reads, unwanted sequences were removed, especially non-polyA tailed RNAs. The Adapters were trimmed using cutadapt and quality trimming was performed using Sickle. The contamination removal step had been performed using the help of bowtie2 (version 2.2.2.6), in-house Perl scripts and picard tools (version 1.100). The pre-processed reads were aligned to the reference *Arabidopsis* genome and their gene model downloaded from Ensembl plants (Release 31). The alignment was performed using Tophat program (version 2.1.0) with default parameters. Only uniquely mapped reads were used for further analysis. The overall alignment summary is shown in Table S2. After aligning the reads with reference gene model, the aligned reads were used for estimating expression of the genes and transcripts; using

cufflinks program (version=2.2.1). Differential expression analysis was performed using cuffdiff program of cufflink package with default settings (Total up and down regulated genes (p-value $\leq$ 0.01) found using cuffdiff analysis).

Expression analysis. For gene expression, qRT-PCR were performed using total RNA from different samples. In brief, total RNA was treated with TURBO DNase (Ambion), and 1 μ g RNA was reverse transcribed using the Revert AidH Minus First Strand cDNA Synthesis Kit (Fermentas) as per the manufacturer's instructions. The cDNA was diluted 20 times with nuclease-free water, and 2 μ l was used as a template for quantitative PCR performed using Fast SYBR Green Mix (Applied Biosystems) in a Fast 7500 Thermal Cycler instrument (Applied Biosystems). The expression was normalized using Tubulin and analysed through the comparative $\Delta\Delta$ CT method (Livak & Schmittgen 2001).

Plasmid construct. For the editing of the PSK4 gene using the CRISPR–Cas9 system in *A. thaliana*, gRNA for *AtPSK4* (AT3G49780) gene was designed using the CRISPR Arizona software (<http://www.genome.arizona.edu/crispr>). gRNA was cloned into the binary vector pHSE401 using the *Bsa*I restriction site (Xing et al., 2014). This vector contains Cas9 endonuclease-encoding gene under the dual CaMV35S promoter as well as genes encoding neomycin phosphotransferase and hygromycin phosphotransferase as plant selection markers. All the constructs were sequenced from both the orientations using plasmid-specific forward and vector reverse primers. For the over-expression of PSK4, the ORF was amplified using cDNA of Col-0. The amplicon was cloned into pTZ57R/T and then transferred into plant expression vector pBI121 under the control of the CaMV35S promoter. For the histochemical assay, promoter lines were developed with GUS reporter gene, using around 1.8 kb upstream sequence from the coding region. The amplicons were cloned into pCAMBIA1303 plant binary vector using the *Hind*III and *Nco*I restriction sites.

Generation of transgenic *Arabidopsis* plants and analysis of mutations. The pHSE401 vector having gRNA for PSK4 and pBI121 (PSK4 ORF under the CaMV35S promoter) and pCAMBIA1303 having PSK4 promoter, vectors were transformed into *Agrobacterium* (strain GV3101) using the freeze–thaw method. *Arabidopsis thaliana* (Col-0) plants were used for

transformation by using the floral dip method (Clough & Bent 1998). The collected T0 seeds were screened on half-strength MS plates containing hygromycin (20 mg/ml) for pHSE401 and pCAMBIA1303 and on kanamycin (50 mg/ml) for pBI121. Positive plants were transferred to soilrite for the maturation. Genomic DNA from the leaves was isolated using the GenElute Plant Genomic DNA Miniprep Kit (Sigma). Regions around the target sites were amplified by PCR, and mutations were detected using the Takara Guide-it Mutation Detection Kit (Takara). PCR products from positive plants were cloned in the cloning vector pTZ57R/T and sequenced using M13F and M13R primers. For PSK4 OX, the cloned sequences were amplified using CaMV35S-F and NosT-R primers and sequenced using gene-specific primers to confirm the construct. For each construct, we generated multiple independent lines.

Synthetic peptide assay. The synthetic peptides [PSK (sulphonated, de-sulphonated and scrambled) and miPEP858a] (purity >98% and 95% respectively) were synthesized through Link Biotech (<http://www.linkbiotech.com>). The peptides were dissolved in water (stock concentration, 10 mM for PSK and 5 mM for miPEP858a). The seedlings were treated with concentrations of 0.25 μ M for miPEP858a, 0.5 μ M to 1 μ M for PSK and peptide diluted in the agar medium or in water.

Peptide sequences:

PSK (Sulphonated): Y(SO₃H)IY(SO₃H)TQ

dPSK (de-Sulphonated): YIYTQ

ScPSK (Scrambled): QTIYY

miPEP858a: MGGIESLLFTIVRDIGRYGTVCVVYNIKCVYTTRTKASTRTSHP

NSP: MNIRFSQIAVQDFAKQGSTNISGEFWCSTSQKAYRNTIPKSI

Histochemical GUS staining. The GUS staining was performed using a previously described method (Jefferson 1989). Seedlings of the *ProPSK4::GUS* transgenic lines were immersed in a solution containing 100 mM sodium phosphate buffer (pH 7.2), 10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide and 1 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl- β -D-glucuronide at 37°C for 4 h. The chlorophyll was removed by incubation and multiple washes using 70% ethanol. The seedlings were observed under a Leica microscope (LAS version 4.12.0, Leica Microsystems) for the GUS staining.

In vivo quantification of auxin by GC-MS. The endogenous IAA levels were quantified as earlier described method with slight modification (Pan et al., 2010). Briefly, about 100 mg of seedling were collected and immediately frozen in liq. N₂. Crushed in 1 ml (80% methanol), after 1 min vortex derivatization were performed with 40 µl pyridine and 90 µl BSTFA. The samples were mixed on thermo shaker at 800 rpm (40 min at 4⁰C) followed by centrifuged 12,000 rpm (15 min at 4⁰C). Supernatant were used for analysis through GC-MS (PerkinElmer Mass Spectrometer Clarus SQ 8 C). The calibration curve was made using Synthetic IAA (Sigma).

Statistical analysis. Data are plotted as means ±SD with error bars as standard deviation. The statistical tests and *n* numbers, including sample sizes or biological replications, are described in the figure legends. All the statistical analyses were performed using two-tailed Student's *t*-tests using GraphPad Prism version 8.4.3 software (* *P* < 0.1; ** *P* < 0.01; *** *P* < 0.001). All the experiments were repeated at least three times independently, with similar results.

Supplemental Data

Supplemental Figure S1. Representation of overview of the samples and the sets used in transcriptome analysis study.

Supplemental Figure S2. Quantification of AtPSK1-5 in miPEPOX lines.

Supplemental Figure S3. Role of clathrin in miPEP858a-dependent growth in *Arabidopsis*.

Supplemental Figure S4. Uptake of miPEP858a is clathrin-mediated.

Supplemental Figure S5. Proposed model for the entry of miPEP858a in *Arabidopsis* via clathrin-mediated endocytosis (CME).

Supplemental Figure S6. Analysis of CRISPR/Cas9 edited PSK4 mutants in WT plants.

Supplemental Figure S7. Quantification of AtPSK4 in PSK4 OX and *PSK4^{CR}* lines.

Supplemental Figure S8. Alteration of morphology in PSK4 OX and *PSK4^{CR}* lines.

Supplemental Figure S9. Full-length microscopic image of the PSK4 OX root.

Supplemental Figure S10. Analysis of CRISPR/Cas9 edited PSK4 mutants in miR858OX plants.

Supplemental Figure S11. Alteration of morphology in OX PSK4 in *miR858a^{CR}* and *PSK4^{CR}* in miR858OX lines.

Supplemental Figure S12. PSK affect auxin levels.

Supplemental Figure S13. MYB3 modulates the expression of miR858a.

Supplemental Figure S14. Phenotypes of mature *MYB3^{CR}* and MYB3 OX plants.

Supplemental Table 1. Read alignment summary.

Supplementary Table 2. Differentially expressing genes as analysed from transcriptome datasets

Supplemental Table 3. Oligonucleotides used for development of constructs and expression analysis

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Author contributions: P.K.B., A.S. and P.K.T. conceived and designed the study. P.K.B., A.S. and H.G. participated in the execution of all the experiments. P.K.B., A.S. and P.K.T. interpreted and discussed the data. A.S., P.K.B., H.G. and P.K.T. wrote the manuscript.

Competing interests: The authors declare that they have no competing interests.

FIGURE LEGENDS

Figure 1. Comparative transcriptome analysis and validation. (A) Venn diagram analysis showing differentially expressed genes in 30 days old rosette and stem of WT VS miR858OX and WT VS *MIMIC858* plants with p value cutoff of 0.01. (B) Quantification of AtPSK4 in five-day-old seedlings of WT, miR858OX and *MIMIC858* plants grown on half-strength MS medium (the small open circles represent the individual values). (C) Quantification of AtPSK1-5 in five-day-old seedlings of WT, *miR858a^{CR}* and *miPEP858a^{CR}* and OX *miPEP858a* grown on half-strength MS medium (the small open circles represent the individual values). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ S.D. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001.

Figure 2. miPEP858 regulates the expression of PSK4. (A) Quantification of pre-miR858a in five-day-old WT seedlings grown on half-strength MS medium supplemented with water (control) and different concentrations of miPEP858a. (B) Quantification of AtPSK4 in five-day-

old WT seedlings grown on half-strength MS medium supplemented with water (control) and different concentrations of miPEP858a. (C) Confocal images of the uptake of fluorescein (FAM)-labelled miPEP858a (5-FAM miPEP) and Propidium iodide (PI) in WT roots after incubation for 12 h. (D) Green fluorescence, bright-field and merged microscopic images of WT and *chc1.2* root cells in the presence of 50 μ M 5-FAM-miPEP858a after incubation for 12 h. Scale bars, 50 μ m. Confocal images are representative of three independent experiments. (E) Quantification of AtPSK4 in five-day-old seedlings of WT and the CRISPR–Cas9-edited *miPEP^{CR}* plants grown on half-strength MS medium supplemented with water (control) and 0.25 μ M miPEP858a. (F) Histochemical staining showing GUS activity in five-day-old seedlings of ProPSK4::GUS grown on half-strength MS medium supplemented with water (Control), miPEP858a (0.25 μ M). The experiment was repeated three times independently (n=20 seedlings) with similar results. Scale bar, 2,000 μ m. (G) Relative expression of GUS in ProPSK4::GUS in five-day-old seedlings of Pro:PSK4::GUS grown on half-strength MS medium supplemented with water (Control) and miPEP858a (0.25 μ M). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001.

Figure 3. PSK4-mediated modulation in plant growth and development. (A) Representative image of ten-day-old WT and PSK4OX seedlings. (B) Representative image of ten-day-old WT and *PSK4^{CR}* seedlings grown on half-strength MS medium. Scale bar, 1 cm. (C) Root lengths of ten-day-old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. *n* = 30 independent seedlings (small open circles). The experiment was repeated three times independently, with similar results. (D) Quantification of *AtPSK4* gene expression in five-day-old WT and PSK4 OX seedlings. (E) Quantification of *AtPSK4* gene in five-day-old WT and *PSK4^{CR}* seedlings. The small open circles represent the individual values in D and E. These experiments were repeated three times independently, with similar results. (F) Quantification of *EXPA2*, *EXPA11* and *EXPA15* in five-day-old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. (G) Representative image of 3-day-old root elongation zone of WT, *PSK4^{CR}* and PSK4 OX seedlings. (H) Length of root elongation zone of three-day old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. *n* = 10 independent seedlings (small open circles). (I and J) Representative image of 30-day-old WT, PSK4 OX and *PSK4^{CR}* plants. The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001, ns represents no significant difference.

Figure 4. Effect of PSK4 on *PSK4^{CR}*, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings. (A) Representative image of ten-day-old WT seedlings grown on half-strength MS medium supplemented with water (control) or different concentrations of PSK4. Scale bar, 1 cm. (B) Root lengths of ten-day-old WT seedlings grown on half-strength MS medium supplemented with water (control) or different concentrations of PSK4. *n* = 30 independent seedlings (small

open circles). (C) Seedling weight of ten-day-old WT grown on half-strength MS medium supplemented with water (control) or different concentrations of PSK4. $n = 10$. (D) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium. (E) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M PSK. (F) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M dPSK. (G) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M ScPSK. Scale bar, 1 cm. (H) Root length of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. $n = 30$ independent seedlings (small open circles). (I), Representative image of ten-day-old *PSK4^{CR}* seedlings grown on half-strength MS medium supplemented with NSP and miPEP858a (0.25 μ M). Scale bar, 1 cm. (J) Root length of ten-day-old *PSK4^{CR}* seedlings grown on half-strength MS medium supplemented with NSP and miPEP858a (0.25 μ M). $n = 30$ independent seedlings (small open circles). (K) Representative image of ten-day-old WT and *tpst* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. Scale bar, 1 cm. (L), Representative image of ten-day-old WT and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. Scale bar, 1 cm. (M) Root lengths of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. $n = 25$ independent seedlings (small open circles). (N) Representative image of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control). Scale bar, 1 cm. (O) Representative image of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with miPEP858a (0.25 μ M). Scale bar, 1 cm. (P) Root lengths of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or miPEP858a (0.25 μ M). $n = 15$ independent seedlings (small open circles). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$, ns represents no significant difference.

Figure 5. miR858-mediated growth and development in *Arabidopsis* involve PSK4 signaling. (A) Representative image of ten-day-old WT, *miR858a^{CR}* and PSK4 OX in *miR858a^{CR}* seedlings grown on half-strength MS medium. Scale bar, 1 cm. (B) Root lengths of ten-day-old WT, *miR858a^{CR}* and PSK4 OX in *miR858a^{CR}* seedlings grown on half-strength MS medium. $n = 30$ independent seedlings (small open circles). (C) Quantification of AtPSK4 gene in five-day-old seedlings of WT, *miR858a^{CR}* and OX PSK4 in *miR858a^{CR}* grown on half-strength MS medium (the small open circles represent the individual values). (D) Representative image of ten-day-old WT, *miR858OX* and *PSK4^{CR}* in *miR858OX* seedlings grown on half-strength MS medium. Scale bar, 1 cm. (E) Root length of ten-day-old WT, *miR858OX* and *PSK4^{CR}* in *miR858OX* seedlings grown on half-strength MS medium. $n = 30$ independent seedlings (small

open circles). (F) Quantification of AtPSK4 gene in five-day-old seedlings of WT, OXmiR858 and *PSK4^{CR}* in miR858OX grown on half-strength MS medium (the small open circles represent the individual values). (G) Representative image of 30-day-old WT *miR858a^{CR}* and PSK4 OX in *miR858a^{CR}* plants. (H) Representative image of 30-day-old WT miR858OX and *PSK4^{CR}* in miR858OX plants. (I) Quantification of EXPA2, EXPA11 and EXPA15 gene in five-day-old WT, OXmiR858 and *PSK4^{CR}* in miR858OX grown on half-strength MS medium (the small open circles represent the individual values). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001, ns represents no significant difference.

Figure 6. PSK4-mediated growth involves downstream expansins and auxin biosynthesis pathways. (A) Quantification of YUC1 and YUC4 gene in five-day-old WT seedlings grown on half-strength MS medium supplemented with different concentrations of PSK (the small open circles represent the individual values). (B) Quantification of YUC1 and YUC4 gene in five-day-old seedlings of *PSK4^{CR}* and PSK4 OX (the small open circles represent the individual values). (C) Quantification of EXPA2, EXPA11 and EXPA15 gene in five-day-old WT seedlings grown on half-strength MS medium supplemented with different concentrations of IAA (the small open circles represent the individual values). (D) Representative image of 10-day old WT and CRISPR/Cas9 edited *PSK4^{CR}*, PSK4 OX, *PSK4^{CR}* in miR858OX, PSK4 OX in *miR858^{CR}*, *miR858^{CR}* and miR858OX seedlings grown on half-strength Murashige and Skoog (MS) medium supplemented with ethanol (control) and 25 μ M TIBA (scale bars, 1 cm). (E) Quantification of EXPA2, EXPA11 and EXPA15 gene in five-day-old seedlings of *PSK4^{CR}* and PSK4 OX (the small open circles represent the individual values). (F) Root length of ten-day-old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength Murashige and Skoog (MS) medium supplemented with ethanol (control) and 25 μ M TIBA *n* = 30 independent seedlings (small open circles). (G) Root length of ten-day-old WT, miR858OX and *PSK4^{CR}* in miR858OX, *miR858^{CR}*, PSK4 OX in *miR858^{CR}* seedlings grown on half-strength Murashige and Skoog (MS) medium supplemented with ethanol (control) and 25 μ M TIBA. *n* = 30 independent seedlings (small open circles). (H) Root length of ten-day-old WT, miR858 OX and *miR858^{CR}* seedlings grown on half-strength Murashige and Skoog (MS) medium supplemented with ethanol (control) and 25 μ M TIBA. *n* = 30 independent seedlings (small open circles). The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001, ns represents no significant difference.

Figure 7. MYB3 is involved in PSK4-mediated plant growth and development. (A) Schematic representation of the PSK4 promoter up-stream of PSK4 precursor. (B) Quantification of *MYB3* and *PSK4* gene expression in five-day-old WT and *MYB3^{CR}* seedlings. (C) Quantification of *MYB3* and *PSK4* gene expression in five-day-old WT and MYB3 OX seedlings grown on half-strength Murashige and Skoog (MS) medium (the small open circles represent the individual values in B and C). (D) Quantification of PSK4 in five-day-old WT and *MYB3^{CR}*

seedlings (L-1 and L-2) grown on half-strength MS medium supplemented with water (control) and 0.25 μ M miPEP858a. (the small open circles represent the individual values). (E) Representative image of ten-day-old WT, *MYB3^{CR}* and MYB3 OX seedlings grown on half-strength MS medium and *MYB3^{CR}* seedlings supplemented with 1 μ M PSK and 0.25 μ M miPEP858a. (F) Root length of ten-day-old WT, *MYB3^{CR}* and MYB3 OX seedlings grown on half-strength MS medium and *MYB3^{CR}* seedlings supplemented with 1 μ M PSK and 0.25 μ M miPEP858a. $n = 30$ independent seedlings (small open circles). (G) Histochemical staining showing the GUS activity in rosette leaf (empty vector (EV) and ProPSK4::GUS in *MYB3^{CR}* background. The experiment was repeated three times independently ($n=20$ seedlings) with similar results. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$, ns represents no significant difference.

Figure. 8. Proposed model for the regulation of plant growth via miPEP/miR858-MYB3-PSK4 module. PSK4 participates as an important signal molecule in miR858a/miPEP858a-dependent growth and development in *Arabidopsis*. MYB3 is an essential component required for the expression of PSK4. Modulated levels of PSK4 also affect downstream auxin signalling and expansins, which together regulate plant growth and development.

Figure 1. Comparative transcriptome analysis and validation. (A) Venn diagram analysis showing differentially expressed genes in 30 days old rosette and stem of WT VS miR858OX and WT VS MIMIC858 plants with p value cutoff of 0.01. (B) Quantification of AtPSK4 in five-day-old seedlings of WT, miR858OX and MIMIC858 plants grown on half-strength MS medium (the small open circles represent the individual values). (C) Quantification of AtPSK1-5 in five-day-old seedlings of WT, miR858a^{CR} and miPEP858a^{CR} and OX miPEP858a grown on half-strength MS medium (the small open circles represent the individual values). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ S.D. The error bars represent standard deviations. The asterisks indicate significant differences: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$.

Figure 2. miPEP858 regulates the expression of PSK4. (A) Quantification of pre-miR858a in five-day-old WT seedlings grown on half-strength MS medium supplemented with water (control) and different concentrations of miPEP858a. (B) Quantification of AtPSK4 in five-day-

old WT seedlings grown on half-strength MS medium supplemented with water (control) and different concentrations of miPEP858a. **(C)** Confocal images of the uptake of fluorescein (FAM)-labelled miPEP858a (5-FAM miPEP) and Propidium iodide (PI) in WT roots after incubation for 12 h. **(D)** Green fluorescence, bright-field and merged microscopic images of WT and *chc1.2* root cells in the presence of 50 μ M 5-FAM-miPEP858a after incubation for 12 h. Scale bars, 50 μ m. Confocal images are representative of three independent experiments. **(E)** Quantification of AtPSK4 in five-day-old seedlings of WT and the CRISPR–Cas9-edited *miPEP^{CR}* plants grown on half-strength MS medium supplemented with water (control) and 0.25 μ M miPEP858a. **(F)** Histochemical staining showing GUS activity in five-day-old seedlings of ProPSK4::GUS grown on half-strength MS medium supplemented with water (Control), miPEP858a (0.25 μ M). The experiment was repeated three times independently (n=20 seedlings) with similar results. Scale bar, 2,000 μ m. **(G)** Relative expression of GUS in ProPSK4::GUS in five-day-old seedlings of Pro:PSK4::GUS grown on half-strength MS medium supplemented with water (Control) and miPEP858a (0.25 μ M). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: **P* < 0.1; ***P* < 0.01; ****P* < 0.001.

Figure 3. PSK4-mediated modulation in plant growth and development. (A) Representative image of ten-day-old WT and PSK4OX seedlings. (B) Representative image of ten-day-old WT and *PSK4^{CR}* seedlings grown on half-strength MS medium. Scale bar, 1 cm. (C) Root lengths of ten-day-old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. $n = 30$

independent seedlings (small open circles). The experiment was repeated three times independently, with similar results. **(D)** Quantification of *AtPSK4* gene expression in five-day-old WT and PSK4 OX seedlings. **(E)** Quantification of *AtPSK4* gene in five-day-old WT and *PSK4^{CR}* seedlings. The small open circles represent the individual values in **D** and **E**. These experiments were repeated three times independently, with similar results. **(F)** Quantification of *EXPA2*, *EXPA11* and *EXPA15* in five-day-old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. **(G)** Representative image of 3-day-old root elongation zone of WT, *PSK4^{CR}* and PSK4 OX seedlings. **(H)** Length of root elongation zone of three-day old WT, PSK4 OX and *PSK4^{CR}* seedlings grown on half-strength MS medium. $n = 10$ independent seedlings (small open circles). **(I and J)** Representative image of 30-day-old WT, PSK4 OX and *PSK4^{CR}* plants. The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: $*P < 0.1$; $**P < 0.01$; $***P < 0.001$, ns represents no significant difference.

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914 **Figure 4. Effect of PSK4 on *PSK4^{CR}*, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings. (A)**
 915 **Representative image of ten-day-old WT seedlings grown on half-strength MS medium**
 916 **supplemented with water (control) or different concentrations of PSK4. Scale bar, 1 cm. (B)**

Root lengths of ten-day-old WT seedlings grown on half-strength MS medium supplemented with water (control) or different concentrations of PSK4. $n = 30$ independent seedlings (small open circles). (C) Seedling weight of ten-day-old WT grown on half-strength MS medium supplemented with water (control) or different concentrations of PSK4. $n = 10$. (D) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium. (E) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M PSK. (F) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M dPSK. (G) Representative image of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with 1 μ M ScPSK. Scale bar, 1 cm. (H) Root length of ten-day-old WT, *miR858a^{CR}* and *miPEP858a^{CR}* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. $n = 30$ independent seedlings (small open circles). (I), Representative image of ten-day-old *PSK4^{CR}* seedlings grown on half-strength MS medium supplemented with NSP and *miPEP858a* (0.25 μ M). Scale bar, 1 cm. (J) Root length of ten-day-old *PSK4^{CR}* seedlings grown on half-strength MS medium supplemented with NSP and *miPEP858a* (0.25 μ M). $n = 30$ independent seedlings (small open circles). (K) Representative image of ten-day-old WT and *tpst* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. Scale bar, 1 cm. (L), Representative image of ten-day-old WT and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. Scale bar, 1 cm. (M) Root lengths of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or 1 μ M PSK, dPSK and ScPSK. $n = 25$ independent seedlings (small open circles). (N) Representative image of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control). Scale bar, 1 cm. (O) Representative image of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with *miPEP858a* (0.25 μ M). Scale bar, 1 cm. (P) Root lengths of ten-day-old WT, *tpst* and *pskr1* seedlings grown on half-strength MS medium supplemented with water (control) or *miPEP858a* (0.25 μ M). $n = 15$ independent seedlings (small open circles). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$, ns represents no significant difference.

Figure 5. miR858-mediated growth and development in *Arabidopsis* involve PSK4 signaling. (A) Representative image of ten-day-old WT, *miR858^{CR}* and PSK4 OX in *miR858^{CR}* seedlings grown on half-strength MS medium. Scale bar, 1 cm. (B) Root lengths of ten-day-old WT, *miR858^{CR}* and PSK4 OX in *miR858^{CR}* seedlings grown on half-strength MS medium. $n = 30$ independent seedlings (small open circles). (C) Quantification of AtPSK4 gene in five-day-old seedlings of WT, *miR858^{CR}* and OX PSK4 in *miR858^{CR}* grown on half-strength MS medium (the small open circles represent the individual values). (D) Representative image of

ten-day-old WT, miR858OX and *PSK4^{CR}* in miR858OX seedlings grown on half-strength MS medium. Scale bar, 1 cm. **(E)** Root length of ten-day-old WT, miR858OX and *PSK4^{CR}* in miR858OX seedlings grown on half-strength MS medium. $n = 30$ independent seedlings (small open circles). **(F)** Quantification of AtPSK4 gene in five-day-old seedlings of WT, OXmiR858 and *PSK4^{CR}* in miR858OX grown on half-strength MS medium (the small open circles represent the individual values). **(G)** Representative image of 30-day-old WT *miR858a^{CR}* and PSK4 OX in *miR858a^{CR}* plants. **(H)** Representative image of 30-day-old WT miR858OX and *PSK4^{CR}* in miR858OX plants. **(I)** Quantification of EXPA2, EXPA11 and EXPA15 gene in five-day-old WT, OXmiR858 and *PSK4^{CR}* in miR858OX grown on half-strength MS medium (the small open circles represent the individual values). The statistical analysis was performed using two-tailed Student's *t*-tests. The data are plotted as means $\pm$ s.d. The error bars represent standard deviations. The asterisks indicate significant differences: * $P < 0.1$; ** $P < 0.01$; *** $P < 0.001$, ns represents no significant difference.

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