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The Evolution and Developmental Expression Profile of the *PIN-FORMED* Family in *Setaria viridis*

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Abstract

Auxin is one of the major driving forces of plant development and requires careful regulation of transporter proteins to establish polar auxin transport. The *PIN-FORMED* (*PIN*) family plays a pivotal role in plant development by establishing auxin gradients that govern vascular patterning and organogenesis. However, the *PIN* family remains severely underexplored in *Setaria viridis*, a well-established model for C_4 monocots. In this study, we identified and characterized 13 *PIN* genes in the *S. viridis* genome. Phylogenetic and collinearity analyses revealed duplication events in the *SvPIN1*, *SvPIN5* and *SvPIN10* subfamilies. Structural analysis uncovered unique features, including potential pseudogenization of *SvPIN5a*. Expression profiling across five developmental stages unveiled the potential developmental roles of *SvPINs*, with *SvPIN1* and *SvPIN10* paralogues predominantly expressed in shoots and panicles, *SvPIN2* and *SvPIN9* in roots, while *SvPIN5b* showed leaf-enriched expression, suggesting potential involvement in Kranz anatomy development. Hormonal treatments in callus cultures revealed auxin-mediated upregulation of *SvPIN1b*, *SvPIN2*, *SvPIN5d*, *SvPIN8* and *SvPIN10a*. Our findings provide significant insights into the role of *PIN* genes in *S. viridis* and other C_4 monocots, establishing a foundation for future functional studies and offering potential targets for crop improvement through auxin transport manipulation.

Keywords: Auxin transport; PIN-FORMED; Green foxtail; C4 grasses; Gene expression; Plant development

Key Message

This study characterizes the *PIN-FORMED* auxin transporter family in the C_4 model *Setaria viridis*, revealing gene duplication, tissue-specific expression, and hormonal regulation that may influence C_4 traits.

Introduction

Auxin was the first ever discovered phytohormone (Darwin 1883) and was first isolated in the form of indole-3-acetic acid (IAA) (Went 1926). Throughout the decades of plant developmental science, it became clear that auxin is involved with a myriad of transcriptional and non-transcriptional signaling processes, influencing cell growth, organ initiation and patterning (Leyser 2018; Bogaert et al. 2022). One particularly important process regulated by auxin is vascular development. The evolution of vascular systems was pivotal for the colonization of terrestrial environments by land plants, enabling not only the rapid transport of molecules and signals, but also providing structural support (Ruonala et al. 2017). Vascular differentiation relies on the establishment of local auxin maxima and gradients, mediated by plasma membrane (PM)-localized transporter proteins in provascular cells (Petrášek and Friml 2009; Vosolsobě et al. 2020). The polar distribution of auxin transporters in the PM is essential for the unique directional flow of auxin, or polar auxin transport (PAT), which drives vascular bundle formation and leaf initiation (Scarpella et al. 2010). Beyond vascular development, PAT regulates embryogenesis, organogenesis, apical dominance and organ tropism (Ung et al. 2023).

Among the most well-studied auxin transporters is the *PIN-FORMED* (*PIN*) family. Since their discovery (Okada et al. 1991), PIN proteins have been recognized as central players in plant development. As efflux carriers, canonical PINs (PIN1, -2, -3/4/7, -10 and -11) are localized in the plasma membrane and export auxin to

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neighboring cells, establishing PAT and facilitating environmental and developmental responses (Adamowski and Friml 2015; Ung et al. 2023). Some PINs localize to the endoplasmic reticulum (ER) membrane, and thus are classified as non-canonical (PIN5, -6, -8 and -9), where they regulate intracellular auxin homeostasis by sequestrating auxin within the ER (Mravec et al. 2009; Ung et al. 2023).

PIN proteins are present in streptophyte algae, though their functions remain poorly understood and appear to differ significantly from those in land plants. Land plant *PINs* are thought to have originated from a single algal ancestor. (Viaene et al. 2013; Bennett 2015). *PIN* diversification coincides with that of land plants and key evolutionary innovations throughout their history. In bryophytes (non-vascular plants), PINs regulate critical developmental processes, including sporophyte development and spermatogenesis (Viaene et al. 2014; Lüth et al. 2023). During the rise of flowering plants, the *PIN-FORMED* family underwent major duplications, which contributed to floral development and the diversification of angiosperms (Zhang et al. 2020). Furthermore, *PIN* diversification has been linked to developmental innovations in monocots, particularly in grass architecture (Balzan et al. 2014; Wakeman and Bennett 2023), contributing to the major success of this group, which encompasses some of the world's most important crops.

In addition to their role in plant development, especially in flowering and embryogenesis, *PIN* genes have been associated with abiotic stress responses. In sorghum, for instance, certain *PIN* family members colocalize with stay-green quantitative trait loci (QTLs) and have been associated with drought tolerance and increased yield (Borrell et al. 2022; Wong et al. 2023). Recent studies also implicate PIN proteins in the thermonastic movement (nondirectional responses to changes in temperature) of leaves mediated by PIN3 (Park et al. 2019), and changes in PIN2 polarization in response to oxidative stress (Zwiewka et al. 2019). Despite these advances, much remains unknown about auxin biology and the potential applications of *PIN* genes in crop improvement.

Setaria viridis is closely related to economically significant crops such as maize, sorghum, and sugarcane, sharing the same NADP-ME C_4 photosynthetic pathway (Brutnell et al. 2010). This makes *S. viridis* an ideal model for genetic studies focused on C_4 species. While *PIN-FORMED* genes have been extensively characterized in *Arabidopsis* and other C_3 plants, their roles in C_4 species remain largely unexplored. In this study, we systematically identified and characterized 13 *PIN-FORMED* genes in the *Setaria viridis* genome. Our integrative approach combined phylogenetic reconstruction, gene structure and promoter analyses, and developmental and hormonal expression profiling. We uncovered lineage-specific features such as potential pseudogenization of *SvPIN5a*, as well as tissue- and stage-specific roles for *SvPIN1*, *SvPIN2*, *SvPIN9*, and *SvPIN10* subfamilies.

Materials and Methods

Plant Materials

Seeds of the A10.1 accession (PI 669942 accession in the USDA Germplasm Resources Information Network) were germinated on filter paper following the gibberellic acid protocol described by Sebastian et al. (2014). Eight days after imbibition (DAI), seedlings were transplanted into a 3:1 (v/v) soil-vermiculite substrate and grown until flowering at 42 DAI. Plants were harvested for gene expression analysis at five key developmental stages, following the *S. viridis* BBCH scale proposed by Junqueira et al. (2020). The stages are marked by the full

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expansion of the first (1.10), third (1.30) and seventh (1.70) leaves, partial panicle emergence (5.50), and appearance of the first visible diaspores (7.10) (**Supplementary Fig. S1**).

For callus induction, dehusked seeds from the A10.1 accession were placed on callus induction medium (CIM) as described by Martins et al. (2019). Morphogenic calli were cultured for 15 days on media containing varying concentrations of the phytoregulators 2,4-dichlorophenoxyacetic acid (2,4-D) and Kinetin (K). Three experimental groups, each consisting of 15 calli, were grown on: standard CIM (2 mg/L 2,4-D + 0.5 mg/L K), Auxin-rich medium (3 mg/L 2,4-D + 0.25 mg/L K) and Cytokinin-rich medium (1 mg/L 2,4-D + 0.75 mg/L K). Three pools of five calli per treatment were harvested for molecular analysis.

Orthologue Identification and Phylogenetic Analyses

Genomic data from 16 species (*Amborella trichopoda* v1.0, *Ananas comosus* v3, *Arabidopsis thaliana* TAIR10, *Brachypodium distachyon* v3.2, *Glycine max* Wm82.a4.v1, *Oryza sativa* v7.0, *Panicum virgatum* v5.1, *Physcomitrium patens* v3.3, *Populus trichocarpa* v4.1, *Selaginella moellendorfii* v1.0, *Setaria italica* v2.2, *Setaria viridis* v4.1, *Solanum lycopersicum* ITAG4.0, *Sorghum bicolor* v3.1.1, *Vitis vinifera* v2.1 and *Zea mays* RefGen_V4) representing major angiosperm lineages, lycophytes and mosses were retrieved from the Phytozome v13 database (Goodstein et al. 2012). *PIN-FORMED* orthologues were identified using HMMER v3.3.2 (http://hmmer.org; Finn et al., 2011) with the PIN protein HMM profile (PF03547) as a query. Adjusted HMM profiles were generated for each taxon, and results were manually curated via phylogenetic analysis, BLASTP (Altschul et al. 1990), and conserved domain analysis using InterProScan (Jones et al. 2014).

Amino acid sequences were aligned using MUSCLE in MEGA X (Kumar et al. 2018). A maximum-likelihood phylogenetic tree was constructed using IQ-TREE v2.0.7 (Minh et al. 2020), with branch support assessed using 2000 bootstrap replicates (Hoang et al. 2018) and 2000 single-branch test iterations (Guindon et al. 2010). The JTT+R7 amino acid substitution model was selected according to the Bayesian information criterion by the ModelFinder algorithm (Kalyaanamoorthy et al. 2017). The *Klebsormidium flaccidum PIN* orthologue (GenBank AJA79000.2), identified via BLASTP, served as an outgroup.

Chromosomal Localization and Duplication Analysis

Chromosomal positions of *S. viridis PIN* genes were extracted from the Phytozome v13 annotation file and visualized using the chromoMap R package (Anand 2018). Gene duplication events were identified with MCScanX (Wang et al. 2012), and collinearity maps were generated using the AccuSyn online platform (Núñez Siri et al. 2020).

Gene Structure, Conserved Motifs and Transmembrane Domain Analyses

Gene structure (intron/exon composition) were derived from the Phytozome v13 annotation and visualized with GSDS 2.0 (Hu et al. 2015). Conserved motifs in the amino acid sequence of *S. viridis* PIN proteins were identified using MEME v5.5.7 (Bailey and Elkan 1994). Transmembrane domain structure was predicted using DeepTMHMM v1.0.44 (Hallgren et al. 2022) and visualized using MembraneFold v0.0.71 (Gutierrez et al. 2022).

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Quantitative Gene Expression Analysis

Total RNA was extracted from frozen plant and callus tissue using the ReliaPrep RNA Tissue Miniprep System (Promega™), followed by cDNA synthesis with TaqMan Reverse Transcription Reagents (Thermo Scientific™). Primer design was performed using Primer3Plus (Untergasser et al. 2007). Reference *genes folyl-polyglutamate synthase* (*Si035045*) and *phosphoglucomutase* (*Si034613*) were used for normalization during expression analysis (Lambret-Frotté et al. 2015). Quantitative PCR was conducted on a 7500 Fast Real-Time PCR System (Applied Biosystems™) according to the Platinum Taq DNA Polymerase (Invitrogen™) protocol. Raw fluorescence data was processed with Real-Time PCR Miner (Zhao and Fernald 2005). Relative expression across developmental stages was analyzed using qBase+ v3.3 (Hellemans et al. 2008), while differential expression in callus was assessed with REST-384 (Pfaffl 2002).

Cis-Acting Regulatory Elements (CARE) Analysis

The 2000 base pair upstream region of each *S. viridis PIN* locus was analyzed for CAREs using the New PLACE database (Higo et al. 1999). Identified CAREs were classified into discrete categories based on the database annotation. CARE enrichment analysis was performed in RStudio using a Fisher exact test with Benjamini-Hochberg false discovery rate (FDR) correction. Results were plotted using the *ggplot2* (Wickham 2016) R package.

Results

Phylogenetic Analysis Reveals 13 PIN-FORMED Genes in Setaria viridis

PIN-FORMED proteins underly plant evolution since the first land plants began colonizing the aerial environment. Understanding the evolutionary history of *PINs* is a crucial step in the characterization of the family. Therefore, extensive phylogenetic analysis identified 13 *PIN-FORMED* genes in the *S. viridis* genome (**Table 1**), distributed across seven subfamilies (**Fig. 1A; Supplementary Fig. S2**). The *PIN1* clade comprises four paralogues (*SvPIN1a*, *SvPIN1b*, *SvPIN1c* and *SvPIN1d*), divided into two subfamilies: *PIN1* (*SvPIN1a* and *SvPIN1b*) and *SISTER OF PIN1* (*SoPIN1*; *SvPIN1c* and *SvPIN1d*). These genes are scattered across chromosomes 4, 1, 7 and 8, respectively (**Fig. 1B**). Collinearity analysis revealed that segmental duplications gave rise to the *SvPIN1a/1b* and *SvPIN1c/1d* pairs (**Fig. 1C**). Only one *PIN2* subfamily member (*SvPIN2*) was identified, located on chromosome 4 (**Fig. 1B**), and was classified as a dispersed duplication event by the collinearity algorithm. Notably, *S. viridis* lacks genes from the *PIN3/4/7* clade, which is predominantly found in eudicots (**Fig. 1A**). Conversely, the *PIN10* subfamily, a monocot-specific sister clade to *PIN3/4/7* (**Fig. 1A**), consists of two paralogues (*SvPIN10a* and *SvPIN10b*), located on chromosomes 3 and 5, respectively (**Fig. 1B**), and likely arose from a segmental duplication (**Fig. 1C**).

Table 1: Identified PIN proteins of *Setaria viridis*.

(Sv) Gene ID	(Sv) Name	(Sv) Protein length	(At) Name	(Os) Name
Sevir.4G101000.2	*SvPIN1a*	678	*AtPIN1*	*OsPIN1a*
Sevir.1G323500.1	*SvPIN1b*	595		*OsPIN1b*
Sevir.7G323600.1	*SvPIN1c*	559		*OsPIN1c*

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Sevir.8G024250.1	*SvPIN1d*	561		*OsPIN1d*
Sevir.4G158100.1	*SvPIN2*	630	*AtPIN2*	*OsPIN2*
Sevir.2G275402.1	*SvPIN5a*	271	*AtPIN5*	*OsPIN5a*
Sevir.5G435500.1	*SvPIN5b*	365		*OsPIN5b*
Sevir.6G228200.2	*SvPIN5c*	426		*OsPIN5c*
Sevir.7G333600.1	*SvPIN5d*	470		*OsPIN5d*
Sevir.5G298200.1	*SvPIN8*	362	*AtPIN8*	*OsPIN8*
Sevir.5G356100.2	*SvPIN9*	422		*OsPIN9*
Sevir.5G249500.1	*SvPIN10a*	621	*AtPIN3/4/7*	*OsPIN10a*
Sevir.3G137700.1	*SvPIN10b*	591		*OsPIN10b*

Gene IDs according to the primary transcript in the Phytozome v13 database. (Sv) *S. viridis*; (At) *A. thaliana*; (Os) *O. sativa*. Protein length in amino acid units.

The *PIN5* subfamily includes four paralogues (*SvPIN5a*, *SvPIN5b*, *SvPIN5c* and *SvPIN5d*), located on chromosomes 2, 5, 6 and 7, respectively (**Fig. 1B**). *SvPIN5a*, *SvPIN5b* and *SvPIN5c* likely originated from segmental duplications (**Fig. 1C**), while *SvPIN5d* is a dispersed genomic duplicate. No orthologues of *PIN6* were identified in any of the grass genomes, including *S. viridis*. The *PIN8* and *PIN9* subfamilies each contain a single gene (*SvPIN8* and *SvPIN9*, respectively), both located on chromosome 5 (**Fig. 1B**). Collinearity analysis indicated that both genes originated from dispersed duplication events. Notably, the *PIN9* subfamily is another monocot-exclusive clade. Alongside the phylogenetic reconstruction of the *PIN-FORMED* family, we investigated the exon-intron composition of each gene and the conserved motifs in the amino acid sequences to corroborate the differences between canonical and non-canonical proteins.

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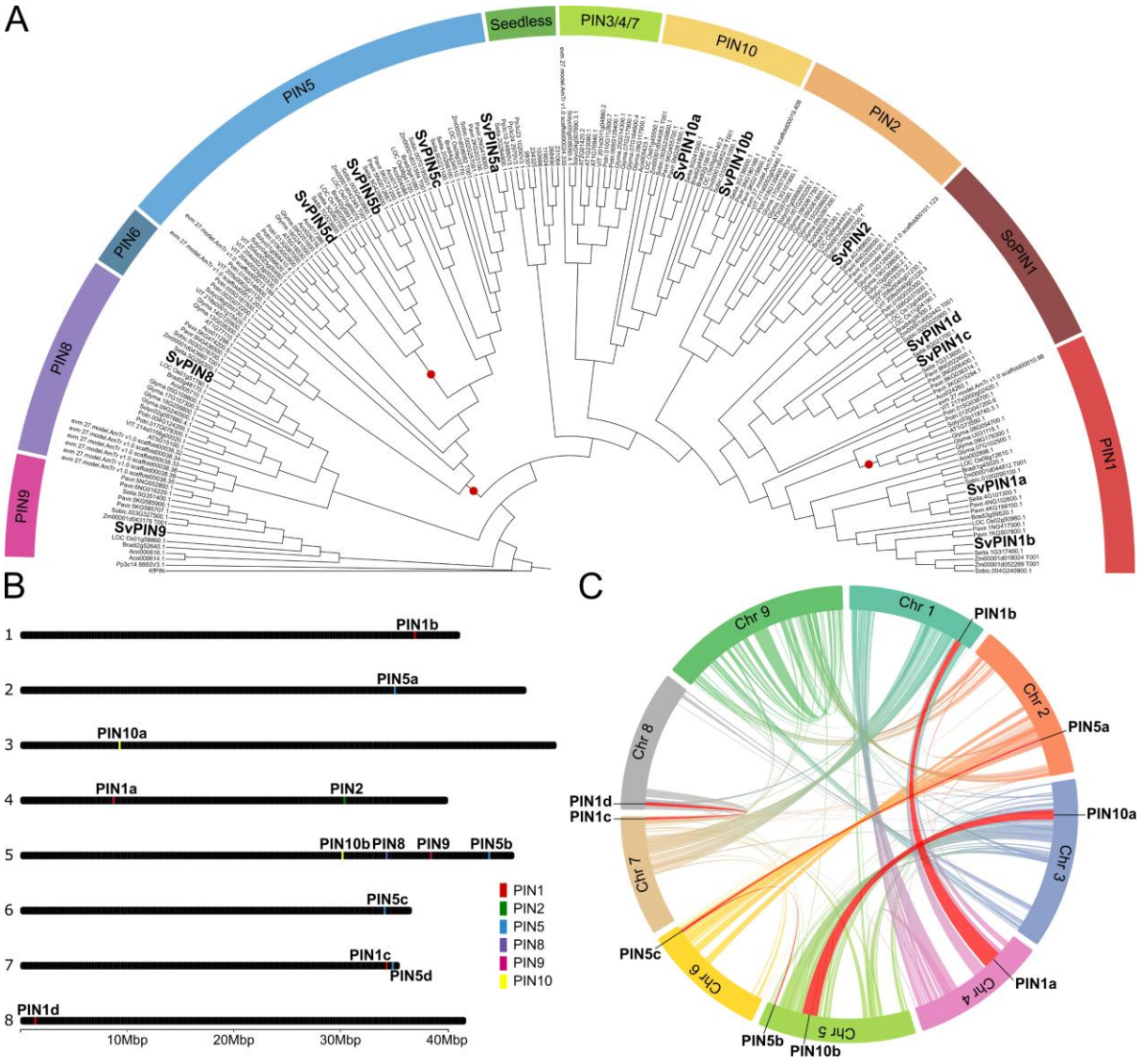


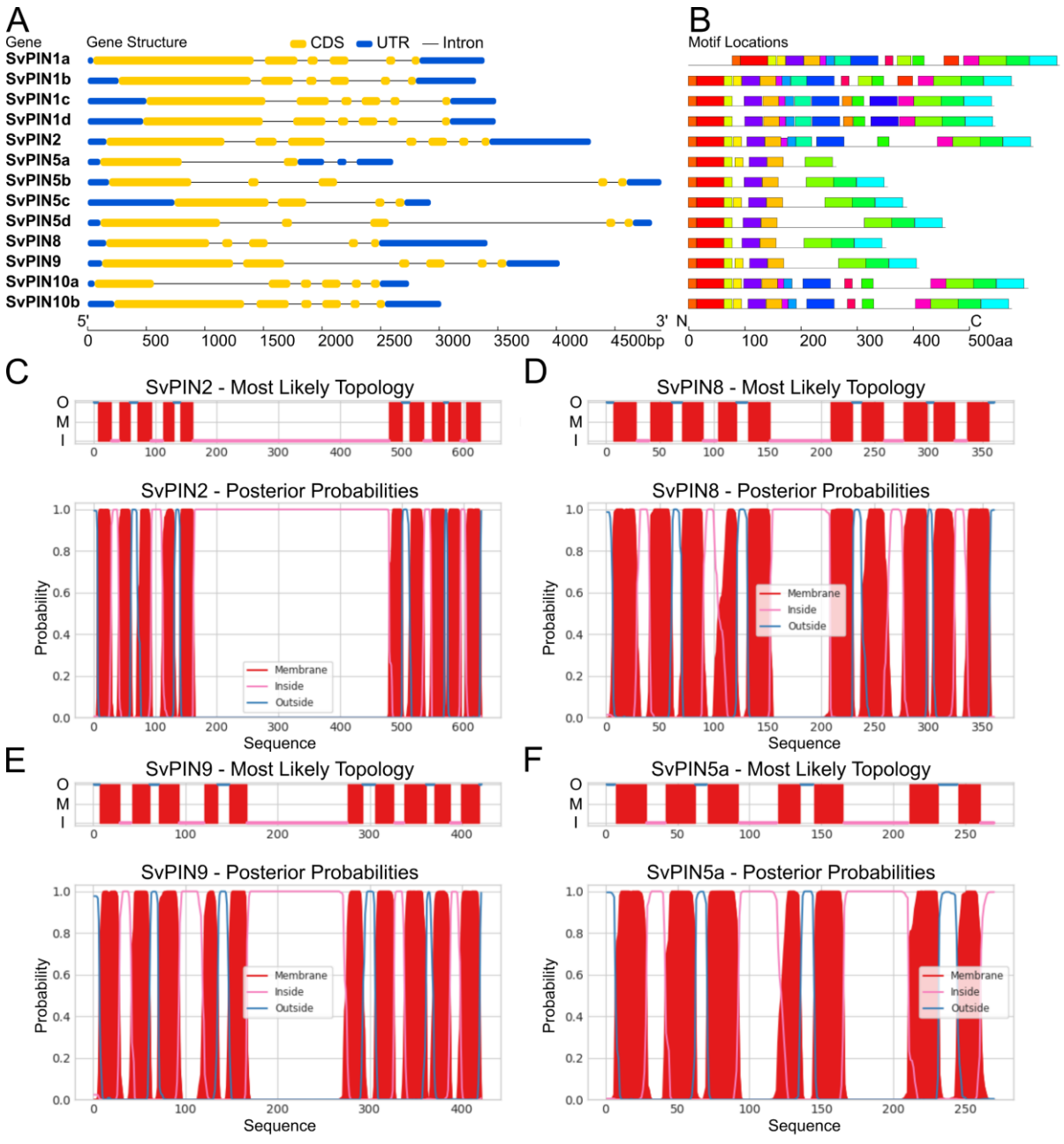
Fig. 1: *Setaria viridis PIN-FORMED* family phylogenetic reconstruction, chromosomal location and syntenic analysis. (A) Maximum likelihood tree showcasing the evolutionary relationship of *PIN* subfamilies across land plants. The phylogeny encompasses representatives of mosses (*P. patens*), lycophytes (*S. moellendorfii*) and major groups of angiosperm species, focusing on grasses. *S. viridis* orthologues are highlighted in bold. Branches with support lower than 70% are marked with a red dot. The charophyte *K. flaccidum PIN* orthologue (*KfPIN*) was used as the outgroup. **(B)** Location of *SvPIN* genes in *S. viridis* chromosomes. Only the chromosomes containing *SvPIN* homologues are represented. **(C)** Collinearity map of *S. viridis* chromosomes and the identified syntenic blocks. *SvPIN1a*, *SvPIN1b*, *SvPIN1c*, *SvPIN1d*, *SvPIN5a*, *SvPIN5b*, *SvPIN5c*, *SvPIN10a* and *SvPIN10b* are represented within their respective duplicated regions. Duplicated regions are highlighted in red.

Gene Structure and Conserved Motifs Distinguish Canonical and Non-Canonical PINs

PIN-FORMED proteins are known for their highly conserved transmembrane domains, which are crucial for auxin transportation. Gene structure analysis showed that most *SvPIN* genes contain six exons, with a large initial exon, followed by a mid-sized exon and several smaller downstream exons (**Fig. 2A**). Interestingly, *SvPIN5a* lacks several downstream exons and encodes a significantly shorter transcript. When analyzing conserved protein motifs, we found high conservation across *S. viridis* PINs, particularly at their N- and C-termini, where the transmembrane helixes are located, while the center portion of the protein is more variable (**Fig. 2B**). Notably, the canonical PINs (PIN1, -2 and -10) present more conserved central motifs, while the non-canonical PINs (PIN5, -

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8 and -9) exhibit less conservation. SvPIN5a was the only protein lacking the two C-terminal motifs that are present in all other family members, potentially due to its shorter transcript and likely compromising its tridimensional structure. To further corroborate the motif analysis, the transmembrane domains and protein structures were predicted using deep learning algorithms. These analyses revealed 10 highly conserved transmembrane helixes flanking the central hydrophilic loop in all proteins, except for PIN5a (**Fig. 2C-F**). Canonical PINs showed a larger hydrophilic loop than non-canonical PINs, reinforcing their identities. SvPIN9 has an intermediary-sized central loop (**Fig. 2E)**. Owing to the lack of the last two conserved motifs, SvPIN5a displayed an atypical predicted structure, with only seven transmembrane helixes (**Fig. 2F; Supplementary Fig. S3**). These combined results not only reinforce the divisions between canonical and non-canonical proteins but also point to SvPIN5a being functionally compromised. To investigate whether the 13 identified *PIN* genes are functional, gene expression analyses were performed throughout plant development and in callus cultures.



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Fig. 2: *Setaria viridis PIN-FORMED* gene structure, conserved motifs and transmembrane domain analyses. (A) *SvPIN* gene structure, showcasing the exon and intron distribution within the primary transcripts. Canonical PINs display transcripts with shorter introns, contrary to non-canonical PINs. *SvPIN5a* has a significantly smaller transcript and only two exons, reflected in its protein structure. **(B)** SvPIN proteins conserved motifs. Canonical PINs have more conserved motifs in the central hydrophilic loop portion of the protein compared to non-canonical PINs. The absence of the two C-terminal motifs in the SvPIN5a structure likely compromises its transporter function. The consensus sequence for each identified motif is available in **Supplementary Fig. S4**. **(C-F)** Transmembrane domain predictions for different PIN proteins. O = Outside; M = Membrane; I = Inside. **(C)** SvPIN2 and **(D)** SvPIN8 represent canonical and non-canonical proteins, respectively, displaying the significant size different between the hydrophilic loops of each group. **(E)** SvPIN9 showcases the intermediary-sized loop of a non-canonical protein derived from a canonical ancestor. **(F)** SvPIN5a lacks three transmembrane domains, possibly owing to the loss of the last two C-terminal conserved motifs that are present in every other PIN protein.

Expression Profiling Highlights Tissue- and Stage-Specific Roles for SvPIN Genes

PIN-FORMED genes are shown to regulate a wide range of processes and therefore exhibit diverse expression patterns in multiple tissues and throughout all stages of development. To evaluate the role of PINs during *S. viridis* development, we analyzed five key developmental stages, from 8 days after imbibition (DAI) seedlings to 42 DAI flowering panicles (P1.10 to I7.10, Junqueira et al., 2020). The *SvPIN1* subfamily was mostly expressed in seedlings, shoots and panicles. Notably, all *SvPIN1* genes had very low expression in developed leaves (**Fig. 3A-D**). *SvPIN1a* was highly expressed in mature roots (R5.50) and shoots (C5.50), while *SvPIN1b* expression peaked during panicle development (I5.50). *SvPIN1c* was mostly expressed in shoot and panicle tissue throughout stages 1.70, 5.50 and 7.10. Similarly, *SvPIN1d* expression peaked in developing shoots (C1.70), and it was expressed twice as much as other *SvPIN1* genes in seedlings. *SvPIN2* was moderately expressed in seedlings and throughout all stages of root development (R1.30, R1.70 and R5.50) and peaked in flowering panicles (**Fig. 3E**). The *SvPIN5* subfamily (**Fig. 3F-H**) showed a peculiar expression profile. *SvPIN5a* expression remained undetected in all tissues and stages investigated, further pointing to a non-functional gene. Meanwhile, *SvPIN5b* was highly expressed in developed leaves (F1.30, F1.70 and F5.50), unlike any other *SvPIN*. Furthermore, *SvPIN5b* was moderately expressed in seedlings. *SvPIN5c* and *SvPIN5d* had similar expression profiles, being moderately expressed in seedlings and peaking during panicle development. Notably, *SvPIN5d* was the most expressed gene in flowering panicles. *SvPIN8* was mostly expressed in seedlings, shoots and panicles, while expression levels in roots and leaves remained low (**Figure 3I**). *SvPIN9* stood out for its peculiar expression profile (**Figure 3J**). While expression remained low in almost all tissues, *SvPIN9* had the highest expression peaks of all *SvPIN* genes in developing (R1.70) and mature roots (R5.50). Additionally, *SvPIN9* was moderately expressed in seedlings. Finally, the *SvPIN10* subfamily was mostly expressed in the reproductive tissues during panicle development and flowering (**Figure 3K-L**). *SvPIN10b* additionally showed high expression levels during shoot development. To test whether the observed *SvPIN* expression patterns are hormone-sensitive, we applied 2,4-D and kinetin treatments to callus cultures and compared the hormonal regulation of *SvPIN* genes with their developmental profiles.

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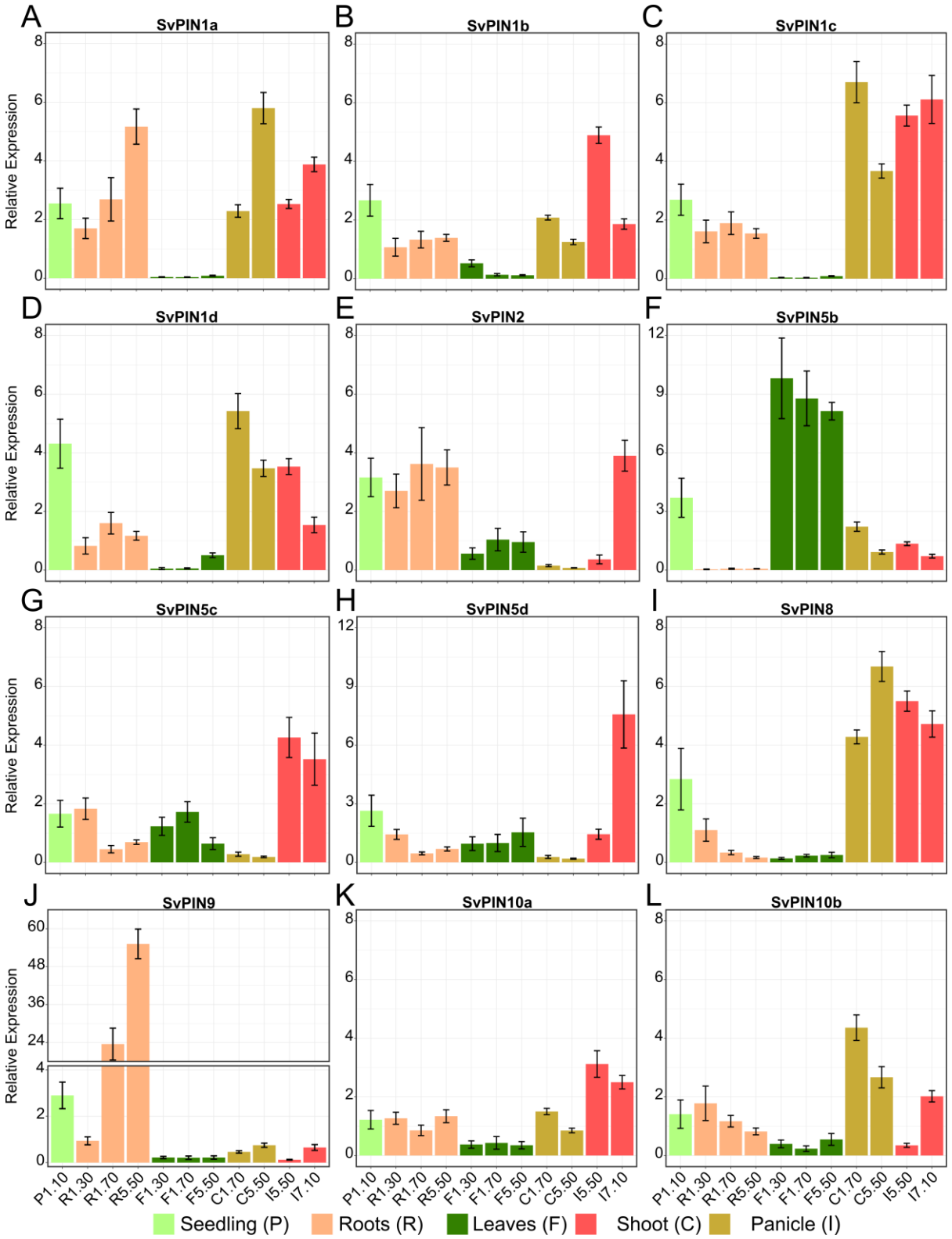


Fig. 3: Relative expression of *Setaria viridis PIN-FORMED* genes throughout development. (A-D) *SvPIN1* subfamily gene expression profile. **(E)** *SvPIN2* gene expression profile. **(F-H)** *SvPIN5* subfamily gene expression profile. *SvPIN5a* expression was undetected in all tissues. **(I)** *SvPIN8* gene expression profile. **(J)** *SvPIN9* gene expression profile. Y-axis was cut to fit the relative expression bars of R1.70 and R5.50 samples. **(K-L)** *SvPIN10* subfamily gene expression profile. Expression values are represented as relative expression in comparison to reference genes *Si035045* and *Si034613*. Each bar represents an average between three biological replicates and error bars represent the standard deviation of each sample. P = Seedling; R = Roots; F = Leaves; C = Shoot; I = Panicle.

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Phytohormone Treatments Reveal Differential Regulation of SvPIN Genes in Calli

Plant calli, when induced by exogenous phytohormones, demonstrably develop into vascular-rich masses of undifferentiated tissue (Sugimoto et al. 2010), thus being interesting targets for the study of *PIN-FORMED* genes. *SvPIN* expression was responsive to different levels of 2,4-D and Kinetin during callus growth. The control calli rarely developed leaf-like structures and remained mostly unchanged during the course of the experiment (**Fig. 4A**). Calli treated with higher 2,4-D and lower Kinetin frequently developed pale, leaf-like structures, and often achieved larger mass than others (**Fig. 4B**). *SvPIN1b*, *SvPIN2*, *SvPIN5d*, *SvPIN8* and *SvPIN10a* were significantly upregulated in 2,4-D-treated calli (**Fig. 4D**). None of the *PIN-FORMED* genes were downregulated under high 2,4-D treatment. Conversely, low 2,4-D and high Kinetin levels resulted in calli developing fine, hair-like structures on the surface, reminiscent of young roots (**Fig. 4C**). Nearly all *SvPIN* genes had their expression repressed by this treatment, with the exception of *SvPIN8*. *SvPIN2*, *SvPIN5c* and *SvPIN5d* were the most downregulated genes (**Fig. 4D**). *SvPIN5a* expression remained undetected in the callus assays, corroborating the pseudogenization hypothesis. This analysis shows the hormone-sensitivity of *SvPINs* and how high concentrations of auxin lead to increased *PIN* expression and leaf initiation in undifferentiated tissue. To further support the expression data, we analyzed the cis-element composition of *PIN* promoters in *S. viridis*.

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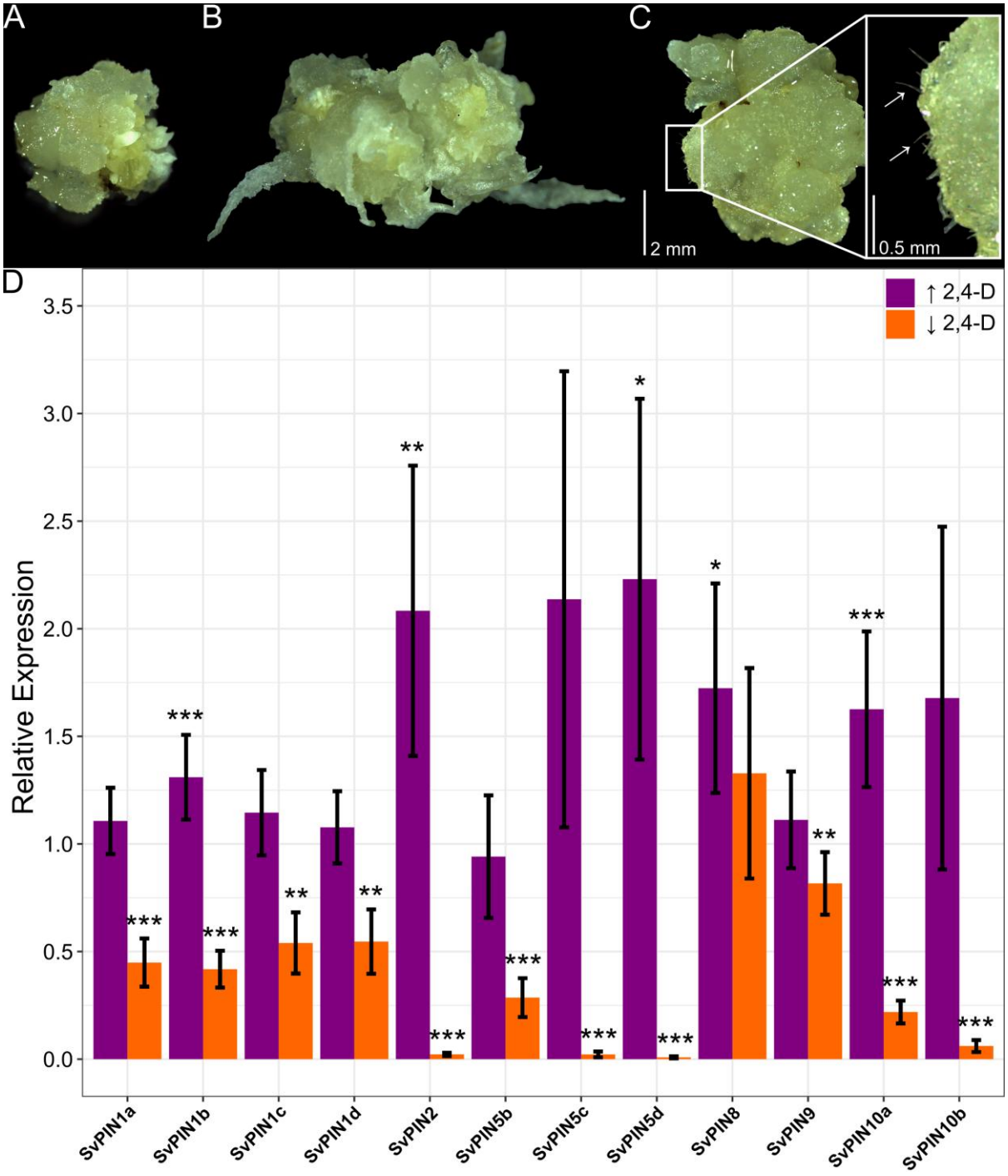


Fig. 4: Differential expression of *Setaria viridis PIN-FORMED* genes in morphogenic callus under different 2,4-D and Kinetin concentrations. (A) Control morphogenic callus, cultivated in standard CIM. **(B)** Morphogenic callus under high 2,4-D, low Kinetin. Compared to control calli, these grew larger and developed leaf-like structures on their surface. **(C)** Morphogenic callus under low 2,4-D, high Kinetin. These retained a similar size compared to control calli, and developed fine, hair-like structures, similar to young roots, indicated by the arrows. **(D)** Relative expression of *SvPIN* genes under high (↑2,4-D) and low (↓2,4-D) concentrations of 2,4-D, respectively. Expression values are represented as relative expression in comparison to control expression levels and reference genes *Si035045* and *Si034613*. Each bar represents an average between three biological replicates and error bars represent the standard deviation of each sample. Asterisks represent significance according to the fixed reallocation randomization test, with $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***).

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Promoter Analysis Reveals a Wide Range of CAREs in SvPIN Genes

Cis acting regulatory elements (CAREs) are important aspects of gene expression and can offer insights into the potential roles of gene when paired with expression data. Promoter analysis identified 200 unique CAREs across all *SvPIN* genes (**Table S1**), categorized into eight functional groups: (1) Stress Response; (2) Light Response; (3) Tissue Expression; (4) Phytohormone Response; (5) Nutrient Response; (6) Defense Related; (7) Anaerobic Response; and (8) Others. CARE distribution was largely homogenous, though duplicate pairs, such as *SvPIN1a/1b* and *SvPIN1c/1d*, showed slightly divergent promoter compositions. In contrast, *SvPIN5a/5b* promoters shared remarkable similarities in CARE composition (**Fig. 5**). CARE enrichment analysis identified 20 significantly enriched CAREs in some promoters when compared to the rest of the family (**Table S2**). The *SvPIN1d* promoter was enriched in light-responsive motif S000486, linked to photomorphogenesis. *SvPIN5b* promoter was enriched in S000277, a motif related to endosperm-specific expression. *SvPIN5d* had the highest number of enriched motifs, including light response and defense related S000042, tissue expression-related elements S000512 and S000314 associated with root hairs and leaf expression, respectively, nutrient-responsive elements S000501 and S000507, and dehydration response elements S000414, S000415 and S000407. The *SvPIN9* promoter was enriched in phytohormone-responsive element S000390, linked to salicylic acid response. Finally, *SvPIN10a* was enriched in S000254, a tissue expression-related element associated with expression in pollen grains. These results align with some of the observations derived from expression data, further corroborating the role of *PINs* in different aspects of plant development.

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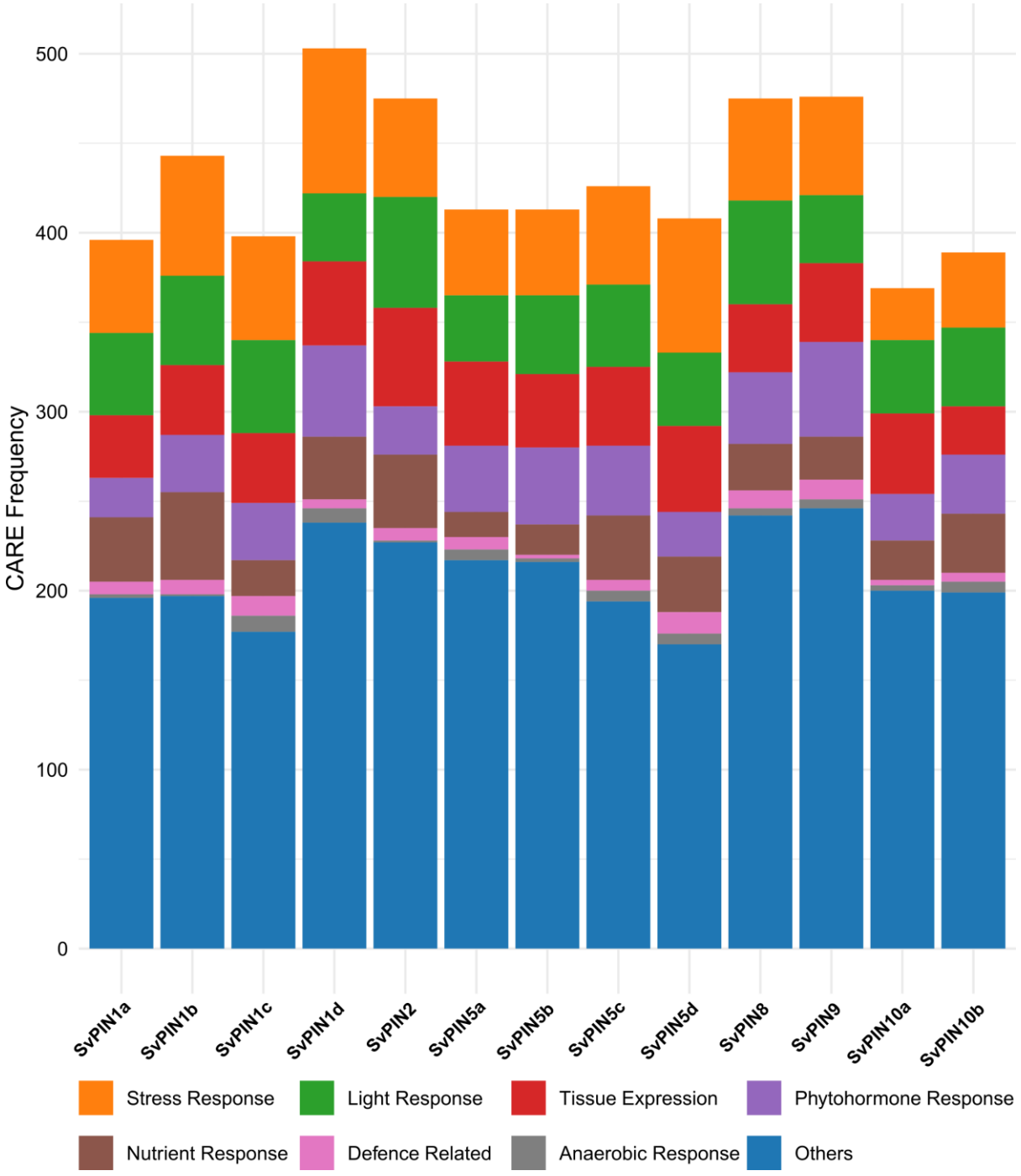


Fig. 5: Cis acting regulatory elements (CAREs) in *SvPIN* promoters. CAREs distribution was relatively homogenous among *SvPIN* homologues. Closely related genes also displayed similarities in CARE composition in their promoters, likely due to conserved functional redundancies in the family. CAREs were grouped based on their annotated function in the NewPLACE database. A list with all identified CAREs is available in **Supplementary Table S2**.

Discussion

Auxin is a central regulator of plant development, orchestrating processes from embryogenesis to organogenesis (Leyser 2018). One unique aspect of auxin is its polar transport, which is mediated by PIN-FORMED proteins and is crucial for vein patterning during vascular development and several other processes (Balzan et al. 2014; Wakeman and Bennett 2023). Although PINs have been extensively characterized in C_3 model plants, such as rice

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and *Arabidopsis*, their role in C_4 plants remain a significant gap in knowledge, particularly in C_4 grasses. Investigating their role is crucial, given the importance of the specialized Kranz vascular anatomy to C_4 photosynthetic efficiency (Sedelnikova et al. 2018). In fact, leveraging vascular development may be a key step towards introducing C_4 metabolism into staple crops (Ermakova et al. 2020). To address this, our study provides a comprehensive characterization of the *PIN-FORMED* family throughout the development of the model C_4 monocot *S. viridis*.

The evolutionary history of PIN proteins provides context for their functional diversity. Believed to transport auxin since the emergence of streptophyte algae, PINs were likely crucial for the colonization of terrestrial environments (Viaene et al. 2013; Bennett 2015; Bogaert et al. 2022). Within land plants, the family diversified significantly, undergoing functional innovations key to terrestrial adaptation (Zhang et al. 2020). An early divergence during the rise of the embryophytes led to the division of PINs into canonical (long, plasma membrane-localized) and non-canonical (short, ER-localized) types based on transmembrane domain structure and the length of the hydrophilic loop (Viaene et al. 2013; Viaene et al. 2014; Bennett 2015), a finding corroborated by our phylogenetic reconstruction. Further diversification was particularly important in flowering plants, where polar auxin transport and auxin maxima regulation are essential for flower development and patterning (Zhang et al. 2020). This may explain the early divergence of moss and lycophyte branches from other canonical PINs, forming a distinct “Seedless” clade in our phylogenetic tree.

We identified 13 *PIN-FORMED* genes in *S. viridis*, a number similar to other grasses like *Oryza sativa* (12 orthologues, Manna et al., 2022) and *Zea mays* (14 orthologues, Yue et al., 2015), suggesting conservation within Poaceae. This number is typically higher in monocots than in eudicots, especially grasses, likely due to duplication events (Balzan et al. 2014; O’Connor et al. 2017). For instance, the *PIN1* clade underwent triplication in grasses (Wakeman and Bennett 2023), giving rise to the *PIN1a*, *PIN1b* and *PIN9* clades. Furthermore, the sister clade to *PIN1*, often named *PIN11* or *SISTER-OF-PIN1* (*SoPIN1*), is a separate group absent only in Brassicaceae (O’Connor et al. 2014). While the *PIN1a/1b* duplication is widespread in grasses, our phylogenetic analysis supports the finding that the *PIN1c/1d* duplication within the *SoPIN1* clade occurs only in certain taxa, including *S. viridis*, *S. italica*, and *O. sativa* (O’Connor et al. 2014).

The *PIN-FORMED* family in monocots also features two exclusive clades: *PIN9* and *PIN10*. Although *PIN9* shares an origin with *PIN1*, it is a monocot-specific clade that underwent significant structural changes, resulting in a novel non-canonical group (Bennett 2015), which explains its clustering with other non-canonical *PINs* in our phylogeny. Interestingly, its presence in pineapple (*Ananas comosus*) (Zhao et al. 2021), indicates that its divergence from *PIN1* likely predates the rise of Poaceae. Similarly, the *PIN10* clade diverged significantly from its ancestral *PIN3/4/7* lineage, leading to its initial classification as a distinct group(Bennett 2015). Our phylogenetic reconstruction confirms that *PIN10* shares its most recent common ancestor with the eudicot-exclusive *PIN3/4/7* clade. *S. viridis* has two *PIN10* paralogues, *SvPIN10a* and *SvPIN10b*, and phylogenetic and collinearity analyses support a grass-specific duplication event. *PIN10* is also present in pineapple (Zhao et al. 2021) suggesting its divergence from *PIN3* predates grasses. This grass-specific diversification within *PINs* has been proposed as a driver behind developmental innovations found in Poaceae (Wakeman and Bennett 2023).

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Among non-canonical *PINs*, grasses appear to have lost *PIN6* but underwent a triplication of *PIN5* (Wakeman and Bennett 2023). Some species, including *S. viridis*, possess four *PIN5* paralogues. Our collinearity analysis indicates that *PIN5a/b/c* originated from segmental duplications. The expansion of *SvPIN5* may have created functional redundancy among the paralogues. Notably, *SvPIN5a* shows a shorter transcript with fewer exons and appears to have lost two C-terminal motifs, resulting in an abnormal structure with only seven transmembrane domains. These unique characteristics, coupled with its absence of expression in all organs and calli under our experimental conditions, suggest that *SvPIN5a* may be undergoing a process of pseudogenization.

The expression patterns of *PIN* genes in *S. viridis* plants and calli provide crucial insights into their functional roles. The *PIN1* subfamily, which emerged alongside flowering plants, is known to play important roles in floral development (Zhang et al. 2020). In line with this, our observed expression patterns suggest collective roles for *SvPIN1* genes in seedling, shoot, and panicle development. PIN1a and PIN1b are known to cooperate during organ initiation, directing PAT from auxin maxima created by SoPIN1 (O'Connor et al. 2014). While these genes have distinct roles in *B. distachyon* inflorescence development, their loss-of-function mutants exhibit mild phenotypes, suggesting functional redundancy (O'Connor et al. 2014; O'Connor et al. 2017). Similar expression patterns of *SvPIN1a* and *SvPIN1b* imply conserved redundancy in *S. viridis* panicle development, though *SvPIN1a* appears to have a more prominent role in shoot and roots. Conversely, the importance of the *SoPIN1* clade is highlighted by the severe phenotypes of its mutants. *B. distachyon sopin1* knockouts resemble *Arabidopsis pin1* mutants (O'Connor et al. 2017), and rice *ospin1c-ospin1b* double mutants fail to develop inflorescences (Li et al. 2019). Additionally, *ZmPIN1d* is highly expressed in maize tassels and ears (Forestan et al. 2012). The high expression of *SvPIN1c* and *SvPIN1d* in shoots and panicles suggests similarly essential functions in *S. viridis*. Furthermore, high *SvPIN1d* expression in seedlings, coupled with CARE enrichment analysis highlighting the light-responsive element S000486, previously associated with cotyledon-specific expression in rice seedlings (Jiao et al. 2005), points to a potential role in early development.

Under high auxin, only *SvPIN1b* showed significant induction, while all *SvPIN1* genes were repressed by low auxin conditions. This aligns with findings in rice where *OsPIN1a* and *OsPIN1b* are more auxin-responsive than *OsPIN1c* and *OsPIN1d* (Li et al. 2019). While all four genes respond to auxin gradients, CARE analysis detected auxin-responsive elements exclusively in the *SvPIN1b* promoter, which could explain its stronger response. A recent study found multiple auxin-responsive CAREs in the promoters of rice *PIN-FORMED* genes (Manna et al. 2022), so their potential presence in other *SvPIN1* promoters cannot be ruled out.

The single *PIN2* orthologue in *S. viridis*, *SvPIN2*, displayed distinctive expression in seedlings, roots and flowering panicles. This pattern is consistent with known PIN2 functions in *Arabidopsis*. AtPIN2 mediates root gravitropism by directing PAT to root elongation zones (Balzan et al. 2014; Retzer et al. 2019) and contributes to cotyledon auxin maxima formation (Pérez-Henríquez et al. 2025). In *S. viridis*, *SvPIN2* expression in seedlings may relate to post-germination development, while its pattern in reproductive tissues matches *ZmPIN2* activity in maize (Forestan et al. 2012). Although PIN2 regulates rice tillering (Wang et al. 2009; Chen et al. 2012), the absence of *SvPIN2* induction in our shoot samples warrants future investigation into tillering in C_4 grasses. Supporting its putative role in root development, the *SvPIN2* promoter contained several elements related to root and root-hair expression, such as S000314, S000482 and S000512. Furthermore, *SvPIN2* exhibited one of the strongest inductions and repressions under high and low auxin, respectively, indicating that auxin gradients are a

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key regulatory input for *PIN2* in *S. viridis*. This is consistent with IAA-mediated regulation of *PIN2* in *A. thaliana* (Pérez-Henríquez et al. 2025), suggesting conserved regulatory mechanisms between monocots and eudicots.

A notable divergence in expression is observed in the grass-specific *PIN10* clade. Its function has shifted from the roles of its eudicot counterpart, *PIN3*, which mediates lateral auxin transport and root tropisms (Friml et al. 2002; Zhang et al. 2013), and apical hook formation (Žádníkova et al. 2010). The apical hook absence and the differences in root architecture in monocots may explain this divergence. Instead, *SvPIN10a* and *SvPIN10b* were primarily expressed in developing and flowering panicles, with *SvPIN10b* also highly expressed in shoots during panicle initiation. This aligns with the established role of PIN10 in grass inflorescence development (Wakeman and Bennett 2023), as seen in rice and maize (Wang et al. 2009; Forestan et al. 2012). Corroborating this reproductive role, the promoter of *SvPIN10a* was found to be enriched in pollen-specific element S000254 (Hamilton et al. 1998).

When it comes to *PIN5*, the absence of *SvPIN5a* expression, combined with its truncated transcript and anomalous protein structure missing transmembrane domains, strongly supports the pseudogenization hypothesis, likely stemming from redundancy within the subfamily. Usually, PIN-FORMED proteins display five transmembrane helices on both sides of the hydrophilic loop (Ung et al. 2022). The missing three helices on the C-terminus of SvPIN5a correlate with the missing motifs in our motif analysis and may indicate that this is a non-functional protein. The remaining three *SvPIN5* paralogues were expressed mainly in leaves and reproductive tissues, contrasting with the role of *AtPIN5* in root development (Mravec et al. 2009; Ganguly et al. 2014). The high expression of *SvPIN5b* in leaves suggests a potential involvement in monocot vein patterning possibly by coordinating intracellular auxin transport in association with PIN1 (Sawchuk et al. 2013). This parallels the expression of *OsPIN5a* in rice (Wang et al. 2009) and result warrants further investigation into its role in C4 grass vascular development. Similarly, the expression of *SvPIN5c* and *SvPIN5d* in reproductive tissues mirrors *ZmPIN5c* expression in developing maize kernels (Forestan et al. 2012), suggesting an involvement in seed development. This is supported by the enrichment of embryogenesis-associated element S000042 (Chandrasekharan et al. 2003) in the *SvPIN5d* promoter, which may explain its peak expression during late reproductive stages when the first seeds start to develop.

Finally, the expression of the remaining *PINs* also reveals functional insights. *SvPIN8* was highly expressed in shoots and inflorescences, matching expression patterns in rice and maize (Matthes et al. 2019), but contrasting with *Arabidopsis*, where *AtPIN8* is predominantly expressed in pollen grains and during the formation of the pollen tube, while showing very low expression in the inflorescence itself (Ding et al. 2012; Bosco et al. 2012). This indicates a departure from the role of PIN8 in eudicots, since expression in grasses seems to be greatly reduced in pollen grains (Wakeman and Bennett 2023). *SvPIN9* showed elevated expression in mature roots, a pattern consistent with observations in rice and maize plants (Wang et al. 2009; Forestan and Varotto 2012). The increase in *SvPIN9* expression between stages 1.70 and 5.50 coincides with the development of a larger root system, suggesting a potential role in lateral root formation. Additionally, given that PIN9 has been implicated in rice tillering, especially in the waterlogged conditions of flooded paddy fields (Nguyen et al. 2018; Hou et al. 2021), this further highlights the importance of studying tillering in C_4 grasses. Collectively, these expression profiles not only affirm the conserved roles of several *PIN* orthologues in fundamental developmental processes

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but also highlight clade-specific functional innovations within grasses, providing a foundational framework for understanding auxin transport in the C_4 context.

Conclusion

Our analysis of the *PIN-FORMED* family in *Setaria viridis* reveals key evolutionary and functional insights into auxin transport in C_4 grasses. We identified 13 *SvPIN* genes and uncovered lineage-specific duplications, underscoring a history of functional diversification. Distinct expression patterns indicate specialized, with *SvPIN1* and *SvPIN10* in panicle development, while *SvPIN2* and *SvPIN9* underly root development. Notable findings like the pseudogenization of *SvPIN5a* and the distinct hormonal responsiveness of *SvPIN1b* and *SvPIN2* highlight a complex regulatory landscape. Our findings lay a foundational groundwork for future investigations aiming to leverage auxin transport pathways to improve C_4 crop architecture and contribute to more sustainable agriculture practices.

Author Contribution Statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by JMFE, JTL and JDFG. The first draft of the manuscript was written by JMFE, and all subsequent versions of the manuscript were reviewed by all authors. Funding was secured by MAF. All authors read and approved of the final manuscript.

Data Availability

All data generated and analyzed during this study is included in this published article and its supplementary files.

References

Adamowski M, Friml J (2015) PIN-dependent auxin transport: Action, regulation, and evolution. Plant Cell 27:20–32. https://doi.org/10.1105/tpc.114.134874

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Anand L (2018) chromoMap: Interactive Genomic Visualization of Biological Data. CRAN: Contributed Packages. https://doi.org/10.32614/CRAN.package.chromoMap

Bailey TL, Elkan C (1994) Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc Int Conf Intell Syst Mol Biol 2:28–36

Balzan S, Johal GS, Carraro N (2014) The role of auxin transporters in monocots development. Front Plant Sci 5:1–12. https://doi.org/10.3389/fpls.2014.00393

Bennett T (2015) PIN proteins and the evolution of plant development. Trends Plant Sci 20:498–507. https://doi.org/10.1016/j.tplants.2015.05.005

Bogaert KA, Blomme J, Beeckman T, De Clerck O (2022) Auxin's origin: do PILS hold the key? Trends Plant Sci 27:227–236. https://doi.org/10.1016/j.tplants.2021.09.008

Borrell AK, Wong ACS, George-Jaeggli B, Van Oosterom EJ, Mace ES, Godwin ID, Liu G, Mullet JE, Klein PE, Hammer GL, McLean G, Hunt C, Jordan DR (2022) Genetic modification of PIN genes induces causal mechanisms of stay-green drought adaptation phenotype. J Exp Bot 73:6711–6726. https://doi.org/10.1093/jxb/erac336

Bosco CD, Dovzhenko A, Liu X, Woerner N, Rensch T, Eismann M, Eimer S, Hegermann J, Paponov IA, Ruperti B, Heberle-Bors E, Touraev A, Cohen JD, Palme K (2012) The endoplasmic reticulum localized PIN8 is a pollen-specific auxin carrier involved in intracellular auxin homeostasis. The Plant Journal 71:860–870. https://doi.org/10.1111/j.1365-313X.2012.05037.x

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454 Brutnell TP, Wang L, Swartwood K, Goldschmidt A, Jackson D, Zhu X-G, Kellogg E, Van Eck J (2010) Setaria
455 viridis : A Model for C4 Photosynthesis. Plant Cell 22:2537–2544. https://doi.org/10.1105/tpc.110.075309
456 Chandrasekharan MB, Bishop KJ, Hall TC (2003) Module-specific regulation of the β-phaseolin promoter during
457 embryogenesis. The Plant Journal 33:853–866. https://doi.org/10.1046/j.1365-313X.2003.01678.x
458 Chen Y, Fan X, Song W, Zhang Y, Xu G (2012) Over-expression of OsPIN2 leads to increased tiller numbers,
459 angle and shorter plant height through suppression of OsLAZY1. Plant Biotechnol J 10:139–149.
460 https://doi.org/10.1111/j.1467-7652.2011.00637.x
461 Darwin C (1883) The 'Power of movement in plants.'--1880. In: Darwin F (ed) The life and letters of Charles
462 Darwin, including an autobiographical chapter, Vol 3 (7th thousand rev.). John Murray, London, pp 329–
463 338. https://doi.org/10.1037/13004-011
464 Ding Z, Wang B, Moreno I, Duplá Ková N, Simon S, Carraro N, Reemmer J, Pě Nčí K A, Chen X, Tejos R,
465 SkÅpa P, Pollmann S, Mravec J, Petrá Š Ek J, ZaÅimalova E, Honys D, Rolčí K J, Murphy A, Orellana A,
466 Geisler M, Friml J (2012) ER-localized auxin transporter PIN8 regulates auxin homeostasis and male
467 gametophyte development in Arabidopsis. Nat Commun 3. https://doi.org/10.1038/ncomms1941
468 Ermakova M, Danila FR, Furbank RT, von Caemmerer S (2020) On the road to C4 rice: advances and
469 perspectives. Plant Journal 101:940–950. https://doi.org/10.1111/tpj.14562
470 Finn RD, Clements J, Eddy SR (2011) HMMER web server: Interactive sequence similarity searching. Nucleic
471 Acids Res 39:29–37. https://doi.org/10.1093/nar/gkr367
472 Forestan C, Farinati S, Varotto S (2012) The maize PIN gene family of auxin transporters. Front Plant Sci 3:1–
473 23. https://doi.org/10.3389/fpls.2012.00016
474 Forestan C, Varotto S (2012) The role of PIN auxin efflux carriers in polar auxin transport and accumulation and
475 their effect on shaping maize development. Mol Plant 5:787–798. https://doi.org/10.1093/mp/ssr103
476 Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3
477 mediates tropism in Arabidopsis. Nature 415:806–809. https://doi.org/10.1038/415806a
478 Ganguly A, Park M, Kesawat MS, Cho HT (2014) Functional analysis of the hydrophilic loop in intracellular
479 trafficking of Arabidopsis PIN-FORMED proteins. Plant Cell 26:1570–1585.
480 https://doi.org/10.1105/tpc.113.118422
481 Goodstein DM, Shu S, Howson R, Neupane R, Hayes RD, Fazo J, Mitros T, Dirks W, Hellsten U, Putnam N,
482 Rokhsar DS (2012) Phytozome: A comparative platform for green plant genomics. Nucleic Acids Res
483 40:1178–1186. https://doi.org/10.1093/nar/gkr944
484 Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to
485 estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. Syst Biol 59:307–
486 321. https://doi.org/10.1093/sysbio/syq010
487 Gutierrez S, Tyczynski WG, Boomsma W, Teufel F, Winther O (2022) MembraneFold: Visualising
488 transmembrane protein structure and topology. bioRxives. https://doi.org/10.1101/2022.12.06.518085
489 Hallgren J, Tsirigos KD, Pedersen MD, Almagro Armenteros JJ, Marcatili P, Nielsen H, Krogh A, Winther O
490 (2022) DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks.
491 bioRxiv. https://doi.org/10.1101/2022.04.08.487609
492 Hamilton DA, Schwarz YH, Mascarenhas JP (1998) A monocot pollen-specific promoter contains separable
493 pollen-specific and quantitative elements. Plant Mol Biol 38:663–669.
494 https://doi.org/10.1023/A:1006083725102
495 Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2008) qBase relative quantification
496 framework and software for management and automated analysis of real-time quantitative PCR data.
497 Genome Biol 8. https://doi.org/10.1186/gb-2007-8-2-r19
498 Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database.
499 Nucleic Acids Res 27:297–300. https://doi.org/10.1093/nar/27.1.297
500 Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS (2018) UFBoot2: Improving the Ultrafast
501 Bootstrap Approximation. Molecular biology and evolution. Mol Biol Evol 35:518–522.
502 https://doi.org/10.5281/zenodo.854445
503 Hou M, Luo F, Wu D, Zhang X, Lou M, Shen D, Yan M, Mao C, Fan X, Xu G, Zhang Y (2021) OsPIN9, an
504 auxin efflux carrier, is required for the regulation of rice tiller bud outgrowth by ammonium. New
505 Phytologist 229:935–949. https://doi.org/10.1111/nph.16901

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506 Hu B, Jin J, Guo AY, Zhang H, Luo J, Gao G (2015) GSDS 2.0: An upgraded gene feature visualization server.
507 Bioinformatics 31:1296–1297. https://doi.org/10.1093/bioinformatics/btu817
508 Jiao Y, Ma L, Strickland E, Deng XW (2005) Conservation and Divergence of Light-Regulated Genome
509 Expression Patterns during Seedling Development in Rice and *Arabidopsis* [W]. Plant Cell 17:3239–3256.
510 https://doi.org/10.1105/tpc.105.035840
511 Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C, McWilliam H, Maslen J, Mitchell A, Nuka G, Pesseat
512 S, Quinn AF, Sangrador-Vegas A, Scheremetjew M, Yong SY, Lopez R, Hunter S (2014) InterProScan 5:
513 Genome-scale protein function classification. Bioinformatics 30:1236–1240.
514 https://doi.org/10.1093/bioinformatics/btu031
515 Junqueira NEG, Bezerra ACM, Cattem MVO, Medici LO, Alves-Ferreira M, Macrae A, Ortiz-Silva B, Reinert F
516 (2020) Phenology of the genetic model Setaria viridis (Poaceae) according to the BBCH-scale of
517 development. Botanical Journal of the Linnean Society 192:224–241.
518 https://doi.org/10.1093/botlinnean/boz070
519 Kalyaanamoorthy S, Minh BQ, Wong TKF, Von Haeseler A, Jermiin LS (2017) ModelFinder: Fast model
520 selection for accurate phylogenetic estimates. Nat Methods 14:587–589.
521 https://doi.org/10.1038/nmeth.4285
522 Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular evolutionary genetics analysis across
523 computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096
524 Lambret-Frotté J, De Almeida LCS, De Moura SM, Souza FLF, Linhares FS, Alves-Ferreira M (2015) Validating
525 internal control genes for the accurate normalization of qPCR expression analysis of the novel model plant
526 setaria viridis. PLoS One 10:1–22. https://doi.org/10.1371/journal.pone.0135006
527 Leyser O (2018) Auxin Signaling. Plant Physiol 176:465–479. https://doi.org/10.1104/pp.17.00765
528 Li Y, Zhu J, Wu L, Shao Y, Wu Y, Mao C (2019) Functional Divergence of PIN1 Paralogous Genes in Rice. Plant
529 Cell Physiol 60:2720–2732. https://doi.org/10.1093/pcp/pcz159
530 Lüth VM, Rempfer C, van Gessel N, Herzog O, Hanser M, Braun M, Decker EL, Reski R (2023) A Physcomitrella
531 PIN protein acts in spermatogenesis and sporophyte retention. New Phytologist 237:2118–2135.
532 https://doi.org/10.1111/nph.18691
533 Manna M, Rengasamy B, Ambasht NK, Sinha AK (2022) Characterization and expression profiling of PIN auxin
534 efflux transporters reveal their role in developmental and abiotic stress conditions in rice. Front Plant Sci
535 13. https://doi.org/10.3389/fpls.2022.1059559
536 Martins PK, da Cunha BADB, Kobayshi AK, Molinari HBC (2019) Setaria viridis as a Model Plant for Functional
537 Genomic Studies in C4 Crops. Methods Mol Biol 1864:49–65. https://doi.org/10.1007/978-1-4939-8778-
538 8_4
539 Matthes MS, Best NB, Robil JM, Malcomber S, Gallavotti A, McSteen P (2019) Auxin EvoDevo: Conservation
540 and Diversification of Genes Regulating Auxin Biosynthesis, Transport, and Signaling. Mol Plant 12:298–
541 320. https://doi.org/10.1016/j.molp.2018.12.012
542 Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R (2020) IQ-TREE
543 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. Mol Biol Evol
544 37:1530–1534. https://doi.org/10.1093/molbev/msaa015
545 Mravec J, Skůpa P, Bailly A, Hoyerová K, Křeček P, Bielach A, Petrášek J, Zhang J, Gaykova V, Stierhof YD,
546 Dobrev PI, Schwarzerová K, Rolčík J, Seifertová D, Luschnig C, Benková E, Zažímalová E, Geisler M,
547 Friml J (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5
548 transporter. Nature 459:1136–1140. https://doi.org/10.1038/nature08066
549 Nguyen TN, Tuan PA, Mukherjee S, Son S, Ayele BT (2018) Hormonal regulation in adventitious roots and
550 during their emergence under waterlogged conditions in wheat. J Exp Bot 69:4065–4082.
551 https://doi.org/10.1093/jxb/ery190
552 Núñez Siri J, Neufeld E, Parkin I, Sharpe A (2020) Using Simulated Annealing to Declutter Genome
553 Visualizations
554 O'Connor DL, Elton S, Ticchiarelli F, Hsia MM, Vogel JP, Leyser T (2017) Cross-species functional diversity
555 within the PIN auxin efflux protein family. Elife 6:1–29. https://doi.org/10.7554/eLife.31804

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O'Connor DL, Runions A, Sluis A, Bragg J, Vogel JP, Prusinkiewicz P, Hake S (2014) A Division in PIN-Mediated Auxin Patterning during Organ Initiation in Grasses. PLoS Comput Biol 10:21–24. https://doi.org/10.1371/journal.pcbi.1003447

Okada K, Ueda J, Komaki MK, Bell CJ, Shimura Y (1991) Requirement of the auxin polar transport system in early stages of Arabidopsis floral bud formation. Plant Cell 3:677–684. https://doi.org/10.2307/3869249

Park YJ, Lee HJ, Gil KE, Kim JY, Lee JH, Lee H, Cho HT, Vu LD, Smet I De, Park CM (2019) Developmental programming of thermonastic leaf movement. Plant Physiol 180:1185–1197. https://doi.org/10.1104/pp.19.00139

Pérez-Henríquez P, Nagawa S, Liu Z, Pan X, Michniewicz M, Tang W, Rasmussen C, Cui X, Van Norman J, Strader L, Yang Z (2025) PIN2-mediated self-organizing transient auxin flow contributes to auxin maxima at the tip of Arabidopsis cotyledons. Nat Commun 16:1380. https://doi.org/10.1038/s41467-024-55480-8

Petrášek J, Friml J (2009) Auxin transport routes in plant development. Development 136:2675–2688. https://doi.org/10.1242/dev.030353

Pfaffl MW (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:36e–336. https://doi.org/10.1093/nar/30.9.e36

Retzer K, Akhmanova M, Konstantinova N, Malínská K, Leitner J, Petrášek J, Luschnig C (2019) Brassinosteroid signaling delimits root gravitropism via sorting of the Arabidopsis PIN2 auxin transporter. Nat Commun 10. https://doi.org/10.1038/s41467-019-13543-1

Ruonala R, Ko D, Helariutta Y (2017) Genetic Networks in Plant Vascular Development. Annu Rev Genet 51:335–359. https://doi.org/10.1146/annurev-genet-120116-024525

Sawchuk MG, Edgar A, Scarpella E (2013) Patterning of Leaf Vein Networks by Convergent Auxin Transport Pathways. PLoS Genet 9. https://doi.org/10.1371/journal.pgen.1003294

Scarpella E, Barkoulas M, Tsiantis M (2010) Control of leaf and vein development by auxin. Cold Spring Harb Perspect Biol 2:1–18. https://doi.org/10.1101/cshperspect.a001511

Sebastian J, Wong MK, Tang E, Dinneny JR (2014) Methods to promote germination of dormant Setaria viridis seeds. PLoS One 9:1–7. https://doi.org/10.1371/journal.pone.0095109

Sedelnikova O V., Hughes TE, Langdale JA (2018) Understanding the Genetic Basis of C 4 Kranz Anatomy with a View to Engineering C 3 Crops. Annu Rev Genet 52:249–270. https://doi.org/10.1146/annurev-genet-120417-031217

Sugimoto K, Jiao Y, Meyerowitz EM (2010) Arabidopsis Regeneration from Multiple Tissues Occurs via a Root Development Pathway. Dev Cell 18:463–471. https://doi.org/10.1016/j.devcel.2010.02.004

Ung KL, Schulz L, Kleine-Vehn J, Pedersen BP, Hammes UZ (2023) Auxin transport at the endoplasmic reticulum: roles and structural similarity of PIN-FORMED and PIN-LIKES. J Exp Bot 74:6893–6903. https://doi.org/10.1093/jxb/erad192

Ung KL, Winkler M, Schulz L, Kolb M, Janacek DP, Dedic E, Stokes DL, Hammes UZ, Pedersen BP (2022) Structures and mechanism of the plant PIN-FORMED auxin transporter. Nature 609:605–610. https://doi.org/10.1038/s41586-022-04883-y

Untergasser A, Nijveen H, Rao X, Bisseling T, Geurts R, Leunissen JAM (2007) Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35:71–74. https://doi.org/10.1093/nar/gkm306

Viaene T, Delwiche CF, Rensing SA, Friml J (2013) Origin and evolution of PIN auxin transporters in the green lineage. Trends Plant Sci 18:5–10. https://doi.org/10.1016/j.tplants.2012.08.009

Viaene T, Landberg K, Thelander M, Medvecka E, Pederson E, Feraru E, Cooper ED, Karimi M, Delwiche CF, Ljung K, Geisler M, Sundberg E, Friml J (2014) Directional auxin transport mechanisms in early diverging land plants. Current Biology 24:2786–2791. https://doi.org/10.1016/j.cub.2014.09.056

Vosolsobě S, Skokan R, Petrášek J (2020) The evolutionary origins of auxin transport: what we know and what we need to know. J Exp Bot 71:3287–3295. https://doi.org/10.1093/jxb/eraa169

Wakeman A, Bennett T (2023) Auxins and grass shoot architecture: how the most important hormone makes the most important plants. J Exp Bot 74:6975–6988. https://doi.org/10.1093/jxb/erad288

Wang JR, Hu H, Wang GH, Li J, Chen JY, Wu P (2009) Expression of PIN genes in rice (Oryza sativa L.): Tissue specificity and regulation by hormones. Mol Plant 2:823–831. https://doi.org/10.1093/mp/ssp023

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Wang Y, Tang H, Debarry JD, Tan X, Li J, Wang X, Lee TH, Jin H, Marler B, Guo H, Kissinger JC, Paterson AH (2012) MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. Nucleic Acids Res 40. https://doi.org/10.1093/nar/gkr1293

Went F (1926) On growth-accelerating substances in the coleoptile of Avena sativa. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen 30:10–19

Wickham H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York

Wong ACS, Van Oosterom EJ, Godwin ID, Borrell AK (2023) Integrating stay-green and PIN-FORMED genes: PIN-FORMED genes as potential targets for designing climate-resilient cereal ideotypes. AoB Plants 15. https://doi.org/10.1093/aobpla/plad040

Yue R, Tie S, Sun T, Zhang L, Yang Y, Qi J, Yan S, Han X, Wang H, Shen C (2015) Genome-Wide Identification and Expression Profiling Analysis of ZmPIN, ZmPILS, ZmLAX and ZmABCB Auxin Transporter Gene Families in Maize (Zea mays L.) under Various Abiotic Stresses. PLoS One 10:e0118751. https://doi.org/10.1371/journal.pone.0118751

Žádníkova P, Petrášek J, Marhavý P, Raz V, Vandenbussche F, Ding Z, Schwarzerová K, Morita MT, Tasaka M, Hejatko J, Van Der Straeten D, Friml J, Benková E (2010) Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana. Development 137:607–617. https://doi.org/10.1242/dev.041277

Zhang K-X, Xu H-H, Yuan T-T, Zhang L, Lu Y-T (2013) Blue-light-induced PIN3 polarization for root negative phototropic response in Arabidopsis. The Plant Journal n/a-n/a. https://doi.org/10.1111/tpj.12298

Zhang Y, Rodriguez L, Li L, Zhang X, Friml J (2020) Functional innovations of PIN auxin transporters mark crucial evolutionary transitions during rise of flowering plants. Sci Adv 6:1–15. https://doi.org/10.1126/sciadv.abc8895

Zhao H, Maokai Y, Cheng H, Guo M, Liu Y, Wang L, Chao S, Zhang M, Lai L, Qin Y (2021) Characterization of auxin transporter AUX, PIN and PILS gene families in pineapple and evaluation of expression profiles during reproductive development and under abiotic stresses. PeerJ. https://doi.org/10.7717/peerj.11410

Zhao S, Fernald RD (2005) Comprehensive algorithm for quantitative real-time polymerase chain reaction. Journal of Computational Biology 12:1047–1064. https://doi.org/10.1089/cmb.2005.12.1047

Zwiewka M, Bielach A, Tamizhselvan P, Madhavan S, Ryad EE, Tan S, Hrtyan M, Dobrev P, Vanková R, Friml J, Tognetti VB (2019) Root Adaptation to H 2 O 2 -Induced Oxidative Stress by ARF-GEF BEN1- and Cytoskeleton-Mediated PIN2 Trafficking. Plant Cell Physiol 60:255–273. https://doi.org/10.1093/pcp/pcz001

Statements and Declarations

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Competing interests

The authors declare that there are no conflicts of interest.