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Evolutionary adaptations to the hormonal regulation of vascular tissue development

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Abstract

Vascular tissues provide long distance transport and physical support in the vascular plant lineage, providing a significant adaptive advantage. Although the crosstalk between auxin and cytokinin in promoting both vascular cell proliferation and differentiation has been well studied in angiosperms such as *Arabidopsis thaliana*, little is known about this regulation in other vascular plant lineages. Here, we found that unlike the hormonal cross talk found in all other species under study, the lycophyte *Selaginella moellendorffii* shows clear task separation with auxin driving vascular cell proliferation only, and cytokinin specifically triggering cell differentiation. Using a cross-species transcriptomics approach, we found members of the *AUXIN/INDOLE-ACETIC ACID (AUX/IAA)* and *CYTOKININ OXIDASE (CKX)* gene families exhibited divergent expression patterns in response to auxin and cytokinin treatments. Despite these regulatory differences, we show that AUX/IAA and CKX proteins are functionally conserved between *Arabidopsis* and *Selaginella*. Taken together, our findings suggest an evolutionary adaptation to the hormonal regulation of vascular tissue development in which core protein functions are conserved, but regulatory circuits diverged in lycophytes.

Significance

Vascular tissues are a hallmark of land plants, enabling long-distance transport and structural support. Although the interplay between auxin and cytokinin during vascular development is well characterized in few model plants, it remains unclear how conserved these regulatory networks are in other lineages. Unlike the vascular plants studied to date where cross-talk between these two phytohormones is observed during vascular tissues development, here we demonstrate that the lycophyte *Selaginella moellendorffii* employs a unique task separation strategy, with auxin and cytokinin exclusively controlling cell proliferation and differentiation, respectively. This discovery provides insight into how fundamental tissue patterning principles can be modified and rewired over evolution, which could be important to explain the diversification of vascular architectures found in extant plants today.

Keywords

vascular plants, evolutionary adaptations, *Selaginella moellendorffii*, auxin and cytokinin, vascular development

Introduction

Multicellular land plants have shaped the earth's biosphere as we know it today¹ and can be divided into bryophytes (non-vascular plants) and tracheophytes (vascular plants)². Unlike green algae, dominant land plants use a complex vascular system to transport nutrients, hormones and other signaling molecules throughout the plant body and store carbon^{1,3}. Together with other innovations, this development of vascular tissues contributed to a significant advantage in competition, enabling vascular plants to ultimately dominate the planet. Vascular tissues consist of two specialized cell types: phloem cells, which function as conduits for sugar transport, and xylem cells, which distribute water throughout the plant⁴. These pools of cells are replenished by divisions of the intervening (pro)cambium cells^{5,6}. Research in the model species *Arabidopsis thaliana* has shown that phytohormone signaling is crucial in maintaining (pro)cambium identities and regulating its proliferation activity; leading to the establishment of functional vascular tissues⁷⁻⁹. During primary growth in root meristems, both auxin and cytokinin promote procambial cell proliferation and vascular cell differentiation.

Auxin is an essential phytohormone that plays a fundamental role in developmental processes during both embryogenesis and post-embryogenesis. Its signaling is initiated upon binding to the TIR1 (TRANSPORT INHIBITOR RESPONSE1)/AFB (AUXIN SIGNALING F-BOX) family of F-box receptors, which initiates the ubiquitin-mediated degradation of AUXIN/INDOLE-ACETIC ACID (AUX/IAA) transcriptional repressors¹⁰. This degradation releases AUXIN RESPONSE FACTOR (ARF)

transcription factors from inhibition, thereby activating gene expression. During vascular tissue development, BODENLOS/IAA12 disrupts the division orientation of pro-vascular initial cells by repressing the transcriptional activities of MONOPTEROS (MP/ARF5)^{11,12}. SHORT HYPOCOTYL 2 (SHY2/IAA3) acts as a negative regulator of secondary vascular formation and ectopic expression of stabilized SHY2 variants in the vascular cambium disrupts the differentiation of specialized vascular tissues¹³⁻¹⁵. Moreover, the interplay with auxin and cytokinin also plays an essential role in vascular tissues development^{5-7,16,17}. MP/ARF5 induces expression of the MP-dependent basic HELIX-LOOP-HELIX (bHLH) transcription factor *TARGET OF MONOPTEROS 5 (TMO5)*¹⁸, which forms a heterodimer complex with another bHLH TF called LONESOME HIGHWAY (LHW). This complex activates expression of *LOG LONELY GUY 3 (LOG3)*, *LOG4*, and *BETA GLUCOSIDASE 44 (BGLU44)*, thereby increasing active cytokinin levels^{5,19,20}. The TMO5/LHW complex also induces expression of the cytokinin-inducible *DOF2.1* TF, promoting vascular cell proliferation²¹. Additionally, cytokinin diffuses into adjacent procambium and phloem, stimulating cell division. This effect is counterbalanced by CYTOKININ OXIDASE3 (CKX3), which is regulated by SHORT ROOT (SHR) as another TMO5/LHW downstream target¹⁶. The phytohormones auxin and cytokinin were also shown to exhibit conserved functions on vascular tissue development across diverse tracheophyte species, such as *Populus* trees²² and *Oryza sativa*²³. Despite these anecdotal examples of a putative conserved role for auxin and cytokinin in regulating vascular proliferation and differentiation processes, it remains unclear whether this mechanism is indeed maintained throughout land plant evolution.

In this study, we observed that the hormonal control of vascular tissue development in the lycophyte *Selaginella moellendorffii* diverged from the complex auxin-cytokinin cross talk found in all other lineages under study. In *Selaginella*, auxin uniquely regulates cell proliferation, while cytokinin specifically triggers vascular cell differentiation. We further show that diverged responses of AUX/IAA and CKX family members to these phytohormones likely lay at the basis of this task separation, despite their conserved protein functions during vascular tissues development. As such, our work suggests that changes to the regulatory logic likely drove the evolutionary divergence of hormonal control during vascular tissue development across vascular plants.

Results

Selaginella has diverged hormonal functions during vascular tissue development

In tracheophytes, the presence of vascular tissues is a defining feature and its development is largely determined by the interplay between the phytohormones auxin and cytokinin. To explore the potential conservation of this interaction, we first investigated vascular tissues development in response to exogenous auxin and cytokinin treatments in a representative species from the dicot, monocot, fern and lycophyte clades (Fig. 1A). In the root of *Arabidopsis thaliana*, a 2-day auxin (1 μ M 1-naphthaleneacetic acid (NAA)) treatment promoted cell proliferation by increasing the total cell numbers in primary vascular tissues (Fig. 1B and F). Additionally, we observed that auxin treatment promoted the differentiation of xylem cells visualized by the presence of secondary cell wall accumulations (Fig. 1B and G). A similar effect was observed for a 2-day cytokinin (1 μ M 6-benzylaminopurine (BAP)) treatment in the root of *Arabidopsis* (Fig. 1B and F-G). This cross-talk in function between auxin and cytokinin signaling on vascular cell proliferation and differentiation (as typed by secondary cell wall accumulation) was also observed in the roots of the monocot *Triticum aestivum* (Fig. 1C and F-G) and the fern *Azolla filiculoides* (Fig. 1D and F-G). In contrast, when analyzing these responses in *Selaginella moellendorffii* as a lycophyte model species (Fig. 1A)²⁴⁻²⁶, we observed that a 7-day auxin treatment (1 μ M NAA) in *Selaginella* root promoted cell proliferation near the root tip (observed about 0.2 cm from root tip) (Fig. 1E-G), but inhibited differentiation into specialized vascular cells compared to the mock treated control (observed about 0.5 cm from root tip) (*SI Appendix*, Fig. S1A-B). To determine the identity of the auxin-induced proliferating cells, we first stimulated proliferation with auxin treatment (1 μ M NAA) and then transferred the roots to mock medium to allow differentiation to occur (*SI Appendix*, Fig. S1C). One day after auxin removal, the proliferating cells initiated specialized vascular cell differentiation (*SI Appendix*, Fig. S1C). Seven days after auxin removal, well-developed vascular tissues ultimately formed in the root center (*SI Appendix*, Fig. S1C-E); suggesting that normal differentiation took place after auxin removal, but in an enlarged vascular area compared to the mock control. Moreover, a cytokinin treatment (1 μ M BAP) applied to the root tip significantly promoted vascular cell differentiation without changing the total amount of vascular tissue cell numbers on a cross section compared to a mock treated control (Fig. 1E-G and *SI Appendix*, Fig. S1A-B) and reduced root meristem size (*SI Appendix*, Fig. S1F-G) as observed the longitudinal sections.

Taken together, our findings suggest that both auxin and cytokinin signaling can promote root vascular cell proliferation and differentiation in *Arabidopsis* (dicot), *Triticum* (monocot), and *Azolla* (fern) roots. However, in *Selaginella* (lycophyte), these hormones operate through independent pathways with auxin inducing vascular cell

proliferation only, while cytokinin specifically initiates vascular cell differentiation. This divergence highlights a unique regulatory mechanism in lycophyte vascular tissue development.

Characterizing auxin and cytokinin regulation in *Selaginella* vascular tissue development

As *Selaginella* exhibits a unique hormonal task separation, we next analyzed the response of vascular tissue development to auxin and cytokinin in more detail. Vascular tissues formation in the *Selaginella* root can be separated into several characteristic growth phases (*SI Appendix*, Fig. S2). A first stage can be characterized by cytoplasmic dense and rather equal sized cells above the apical initial cell(s) (Stage 1). Subsequently, cells in the center of the root become more specialized in size and shape and the first patterning is observable (Stage 2). Next, the first differentiated xylem and phloem cells are observed (Stage 3). Finally, mature phloem and xylem were present in the center of the *Selaginella* root (Stage 4) (*SI Appendix*, Fig. S2)²⁴.

After building on our characterization of vascular tissue development in *Selaginella*, we next investigated the modalities of how auxin treatments affect vascular tissue development in this model system. First, we applied different concentrations of 2,4-D (2,4-Dichlorophenoxyacetic acid) to sustain high auxin level in the roots, enabling us to investigate the functions of auxin in root vascular tissues development²⁷⁻²⁹. Auxin treatments (0.1 μ M 2,4-D and 1 μ M 2,4-D) in mature root (Stage 4) vascular tissues had no effect on the number of specialized vascular tissues (phloem and xylem) or total vascular tissue cells count (*SI Appendix*, Fig. S3A-B). By contrast, an auxin treatment in root tips (Stage 3) significantly promoted cell proliferation in a dose dependent manner, without inducing specialized vascular tissues differentiation (*SI Appendix*, Fig. S3C-E). This effect was observed for different types of auxin (NAA, indole-3-acetic acid (IAA), or 2,4-D) albeit with different strengths (*SI Appendix*, Fig. S3F-H). In line with expectations, blocking polar auxin transport using NPA (N-1-naphthylphthalamic acid) reduced the number of vascular cells (*SI Appendix*, Fig. S3F-H). Subsequently, we conducted a similar analysis to elucidate the precise role of cytokinin signaling pathways in regulating cell differentiation within the vascular tissues of *Selaginella* root systems. We first applied different concentrations of cytokinin (0.1 μ M BAP and 1 μ M BAP) to mature root (Stage 4) vascular tissues and root tips (Stage 2) and found that a 7-day cytokinin treatment had no effect on the number of specialized vascular tissues (phloem and xylem) or the total number of vascular cells (*SI Appendix*, Fig. S4A-B). However, a 1 μ M BAP treatment in root tips (Stage 2) is sufficient to induce vascular tissue differentiation (Fig. 1E-G) while this is not the case for lower cytokinin concentrations (*SI Appendix*, Fig. S4C-D). Furthermore, the cytokinin treatment was able to counteract the auxin-induced cell proliferation effect and enhance vascular cell

differentiation in the presence of auxin (*SI Appendix*, Fig. S4E-I). These findings suggest that both auxin and cytokinin operate in vascular stem cells in *Selaginella* but have distinct functions. More specifically, cytokinin signaling acts antagonistically to auxin to suppress vascular cell proliferation, and drive vascular cell differentiation.

***Selaginella* shows diverged transcriptional responses to auxin and cytokinin**

To uncover how auxin and cytokinin signaling might regulate vascular tissue development at the molecular level in the root of *Selaginella* and *Arabidopsis* treated with these hormones, we performed bulk RNA sequencing (RNA-seq). Firstly, we analyzed the whole-genome transcriptional changes in both *Selaginella* and *Arabidopsis* root tips after 2-hour treatments with mock, auxin (10 μ M NAA) or cytokinin (10 μ M BAP) (Fig. 2A-B, *SI Appendix*, Fig. S5A-B and Dataset S1). Moreover, given that that an auxin treatment in *Selaginella* is able to induce a population of undifferentiated vascular cells, which subsequently differentiate upon removal of auxin (*SI Appendix*, Fig. S1C-E), this offers a unique system to observe *de novo* establishment of vascular cells. We thus decided to take advantage of this effect to further narrow down potential regulators. In this second set-up, we conducted a time-series RNA-seq experiment following NAA removal at 0, 3, 6, 12, and 24 hours (*SI Appendix*, Fig. S5C and Dataset S1). By integrating data from both experimental conditions, we identified two distinct gene subsets regulating vascular tissue development in *Selaginella* roots: one promoting cell proliferation and another driving differentiation (*SI Appendix*, Fig. S5D-E). Considering that the diverged functions of auxin and cytokinin signaling in root vascular tissues of *Arabidopsis* and *Selaginella* are likely to be tightly connect to distinct transcriptional profiles, we hypothesized that the orthologous regulators should be differently regulated in our bulk RNA-seq datasets. Amongst the orthologous gene families differentially regulated between *Arabidopsis* and *Selaginella* (Dataset S1), members of the *AUX/IAA* and *CKX* gene families (*SI Appendix*, Fig. S5F and S6A-B) stood out as being highly regulated upon auxin and cytokinin treatment. Given their divergent role in auxin and cytokinin signaling in regulating vascular tissue development between *Arabidopsis* and *Selaginella*, we considered these as logical targets for deeper analysis. To further investigate this hypothesis, we first validated whether these genes are expressed in vascular cells at the correct developmental window. For the *Arabidopsis* orthologs, several selected genes have already been reported to be expressed in vascular tissues, including *AtIAA1*³⁰, *AtIAA3*/*SHY2*¹⁴, *AtIAA5*³¹, and *AtIAA19*³² from the *AUX/IAA* gene family, as well as *AtCKX1*³³ and *AtCKX3*¹⁶ from the *CKX* gene family. In order to validate the expression patterns for the *Selaginella* orthologs, we used hybridization chain reaction RNA fluorescence *in situ* hybridization (HCR RNA-FISH)³⁴ and designed probes for *SmIAAI*,

SmIAA3, and *SmCKX1*. We found that both *SmIAA1* and *SmIAA3* were expressed in the young vascular tissues of the root apical meristem (Fig. 2C). In contrast, the expression of *SmCKX1* in the root apical meristem appeared in later stages of vascular tissue development (Fig. 2C) in a mutually exclusive pattern compared to the *SmIAA* genes. In summary, our results show distinct transcriptomics responses in *AUX/IAA* and *CKX* gene families upon auxin and cytokinin treatment in roots of *Arabidopsis* and *Selaginella*, suggesting lineage-specific evolutionary adaptations in vascular tissue development.

IAA and CKX proteins are functionally conserved in *Selaginella* and *Arabidopsis*

Given the vascular expression of these auxin- and cytokinin-related genes, we next sought to investigate their evolutionary relationships by constructing a phylogenetic tree of *AUX/IAA* and *CKX* gene family members in *Selaginella* and *Arabidopsis*. The phylogenetic analysis of the *AUX/IAA* gene family revealed that *Selaginella* orthologs cluster together and separately from the orthologs in *Arabidopsis*. In contrast, the *CKX* gene family members from *Selaginella* and *Arabidopsis* showed intermixing in the phylogenetic tree (*SI Appendix*, Fig. S6B and S7B). However, a strong conservation at the level of the protein sequence was observed in several protein domains for both species (*SI Appendix*, Fig. S6A and S7A). These results suggested that the biochemical functions of the *AUX/IAA* and *CKX* orthologs are likely conserved, despite the observed differential responses to auxin and cytokinin at the transcript level.

Based on the protein sequence conservation presented in *AUX/IAA* and *CKX* family members from *Selaginella* and *Arabidopsis*, we next explored whether the protein functions themselves are also conserved. Given the *AUX/IAA* family is highly redundant, few phenotypes are observed in loss-of-function mutants. Indeed, our understanding of the *AUX/IAA* family in *Arabidopsis* mostly relies on gain of function mutants^{35,36}. A noticeable exception is the *shy2-31* loss-of-function mutant causing lateral root hydropatterning defects³⁷. We thus next analyzed the role of AtIAA3/SHY2 in vascular tissue development using a gain-of-function (*shy2-2*) and loss-of-function (*shy2-31*) mutant. *shy2-2* exhibited fewer vascular cells and delayed secondary cell wall deposition in primary xylem compared to the Col-0 control (*SI Appendix*, Fig. S8A-B). In contrast, *shy2-31* showed no difference from Col-0 (*SI Appendix*, Fig. S8A-B). Given functional redundancy among *AUX/IAA* proteins during vascular tissue development in *Arabidopsis* (*SI Appendix*, Fig. S8A-B)³⁵, we then generated *Arabidopsis* lines ectopically overexpressing AtIAA1, AtIAA5, and AtIAA19 under the ubiquitously expressed *UBI10* promoter (p*UBI10*::AtIAA1-GFP, p*UBI10*::AtIAA5-GFP, p*UBI10*::AtIAA19-GFP in Col-0). Similar to the *shy2-2* mutant, overexpression of these At*AUX/IAA* proteins inhibited vascular cell proliferation and secondary cell wall deposition in the primary xylem (Fig. 3A-B and *SI Appendix*, Fig. S8A-F).

Subsequently, we examined the AUX/IAA proteins from *Selaginella* (pUBI10::SmIAA1-GFP and pUBI10::SmIAA4-GFP in Col-0). Although a diverged hormonal regulatory pattern was found for *SmIAA1* and *SmIAA4* (*SI Appendix*, Fig. S6 and Dataset S1), we observed that the produced phenotypes were identical to those of AtAUX/IAA members, exhibiting similar impairments in vascular cell proliferation and primary xylem secondary cell wall deposition (Fig. 3C-D and *SI Appendix*, Fig. S8G-H).

We then investigated the functions of Arabidopsis CKX proteins. Fitting with previous studies showing that CKX family members play a crucial role in vascular tissue development in *Arabidopsis*^{16,38-40}, we found that the *ckx7* mutants impaired the xylem differentiation (*SI Appendix*, Fig. S9A-B). Conversely, ectopic overexpression of AtCKX7 suppressed vascular cell proliferation and significantly triggered xylem differentiation (Fig. 3E-F)⁴⁰. Similar to these results, ectopic overexpression of SmCKX1 (pUBI10::SmCKX1-GFP) and SmCKX2 (pUBI10::SmCKX2-GFP) in Arabidopsis, mirrored AtCKX7 function, limiting vascular cell proliferation and promoting xylem differentiation (Fig. 3G-H and *SI Appendix*, Fig. S9C-D), even though these two orthologs exhibited a different regulatory profile (*SI Appendix*, Fig. S6 and Dataset S1). These results suggest a functional conservation of AUX/IAA and CKX proteins between Arabidopsis and *Selaginella*, despite different regulation in response to auxin and cytokinin treatments at the transcript level.

Discussion

Although abundant morphological variations of vascular tissues exist across species, the differentiated vascular tissues (xylem and phloem cell types) are functionally conserved for long-distance transport of organic and inorganic compounds⁴¹⁻⁴³. Based on our results in model species of dicots, monocots and ferns, it is likely to assume that in most plant species, auxin and cytokinin act cooperatively in a complex cross-talk to regulate both cell proliferation and differentiation during vascular development in the root^{4,9,44-46}. As an interesting divergence from this general rule, the tasks of auxin and cytokinin are clearly separated in *Selaginella* as lycophyte model species with auxin uniquely promoting cell proliferation, while cytokinin specifically driving differentiation of vascular cell types (Fig. 4). Moreover, despite the functional conservation of AUX/IAA and CKX proteins in controlling vascular development as downstream regulators in these hormone signaling pathways^{9,16,35,40}, their expression and regulatory patterns diverge between Arabidopsis and *Selaginella* (Fig. 4). Taken together, these findings suggest that evolutionary innovations in vascular tissue development might have arisen through regulatory rewiring of core hormone signaling pathways, while their developmental roles at the protein level remained highly conserved. As such, the regulatory plasticity observed between *Selaginella* and

Arabidopsis appears to be mediated by lineage-specific promoter architectures, suggesting evolutionary tinkering with transcriptional control mechanisms while preserving core protein functions. These findings hint towards an evolutionary strategy in which conserved developmental pathways can achieve species-specific modulation through cis-regulatory diversification⁴⁷⁻⁴⁹. It would thus be interesting to further explore auxin- and cytokinin-related binding motifs across vascular plants through *in silico* analyses in order to uncover additional transcriptional networks that modulate developmental pathways.

Materials and Methods

Plant material and growth

Arabidopsis thaliana and *Triticum aestivum* plants were grown either on 1/2 MS medium supplemented with 1% sucrose and 0.8% plant agar under continuous light until sampling, or in soil under long-day conditions (16 hours light / 8 hours dark) for seed production. *Azolla filiculoides* plants were grown on liquid media as described in⁵⁰ under continuous light until sampling. *Selaginella moellendorffii* plants were grown on 1/2 MS supplemented with 1% sucrose, 0.01% myo-inositol, 0.05% MES, and 0.8% plant agar under continuous light until sampling. Chemical treatments were added as a mixture to the media and included: 2,4 D (0.1µM/1µM, Sigma, D-7299, diluted in DMSO), IAA (1µM, Duchefa, I0901, diluted in DMSO), NAA (1µM/10µM, Duchefa, N0903, diluted in DMSO), NPA (10µM, Duchefa, N0926, diluted in DMSO) or BAP (1µM/10µM, Duchefa, B0904, diluted in DMSO). For treatment, plants were initially grown on mock plates or in liquid media before being transferred to plates or liquid media containing the respective chemicals. The mutants and transgenic lines (T1 or T2 segregating homozygous populations) of *Arabidopsis thaliana* used in this study are listed in *SI Appendix*, Table S1. Genes used in this study are listed in *SI Appendix*, Table S5.

Molecular cloning

Protein-coding sequences from *Arabidopsis thaliana* and *Selaginella moellendorffii* root cDNA were amplified using primers listed in *SI Appendix*, Table S2. All transgenic constructs were assembled using Modular GreenGate technology⁵¹. If protein coding sequences contain BSA sites, they were replaced as described in⁵². *SI Appendix*, Table S3 lists all modules and constructs used in this study, while *SI Appendix*, Table S4 details the module assembly strategies. For plant transformation, *Agrobacterium tumefaciens* GV3101 was used, and *Arabidopsis thaliana* plants were transformed via the floral dip method. Heterozygous T2 or homozygous T3 lines were used for subsequent analyses.

Histology staining

Root samples were collected from the root junction region (0.5-1 cm) for plastic embedding. Plastic embedding and cross-sectioning were performed according to the protocol described in⁵³, using Technovit 7100 (Heraeus Kulzer, 64709003). Sections (5-8 μm thick) were stained with 0.1% toluidine blue (Sigma, T3260) and imaged using an Olympus BX51 microscope equipped with a digital imaging system.

RNA-FISH and Confocal Microscopy

HCR RNA-FISH in the root of *Selaginella moellendorffii* is conducted as mentioned in^{24,34,54}. Gene probes were designed and synthesized by Molecular Instruments (www.molecularinstruments.com). Leica Stellaris 5 LIAchroic upright confocal was used for acquiring the confocal images with specific settings. Fluorophores were imaged using 633-nm excitation and 650–755 nm detection, while Calcofluor White was imaged using 405-nm excitation and 410–525 nm detection.

RNA sequence and Gene cluster trend analysis

Paired-end 150 bp RNA sequencing was performed by BGI. Raw data processing and analysis were conducted using the Galaxy platform (<https://usegalaxy.be/>)⁵⁵. Sequence adapters were trimmed using Trimmomatic (default parameters), followed by quality assessment with FastQC. Reads were aligned to the TAIR10 reference genome (for *Arabidopsis thaliana*) and Genome *Selaginella moellendorffii* v1.0 using Salmon (quant mode, default parameters). Transcript abundance estimation and read counting were performed with tximport, and differential gene expression analysis was conducted using DESeq2 (Dataset S1). Gene cluster trend analysis was done using Mfuzz package (v2.68.0) with time-course gene expression matrix as input (Dataset S1).

Statistical analyses

All statistical analyses, data quantification, and visualization were performed in R (v4.0.3). Boxplots were generated using ggplot2 (v3.4.3). Count data were analyzed by fitting generalized linear (mixed) models with Poisson (stats package), quasi-Poisson (stats package), or negative binomial (stats package) distributions. Model selection was based on residual diagnostics performed using the DHARMA package (v0.4.6) with simulation-based approaches. Statistical inferences were drawn using the emmeans package (v1.5.5-1), with p-values from pairwise comparisons adjusted via the Holm-Bonferroni method. Significant differences ($\alpha = 0.05$) were visualized using compact letter displays generated with the multcomp package (v1.4-16). Complete datasets and statistical test results are provided in Dataset S2.

Cell number quantification and image analyses

Cell number quantification was performed using Fiji (v2.3.0; <https://fiji.sc/>)⁵⁶. For each genotype, we analyzed 5-20 root cross-sections obtained from independent plants. While 2-3 independent biological replicates were performed, data from a single representative experiment are shown here.

Phylogenetic and protein alignment analysis

Representative subfamily members of the CKX or IAA gene families from *Arabidopsis thaliana* or *Selaginella moellendorffii* were used as query sequences for BLASTP searches (E-value < 1×10^{-5}) against protein databases of multiple species. Retrieved orthologs were aligned using MAFFT, with sequences lacking a complete domain excluded. Candidate orthologs were then re-aligned to ensure only sequences with intact domains were retained. For phylogenetic analysis, the sequences were aligned using IQ-TREE, followed by Maximum Likelihood (ML) tree reconstruction with 1000 bootstrap replicates to assess branch support.

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Author contributions

Conceptualization, W.X. and B.D.R.; formal analysis, W.X., L.Y. and E.V.; funding acquisition, B.D.R. and L.Y.; investigation, W.X., L.Y. and E.V.; methodology, W.X. Y.K. and E.V.; supervision, B.D.R.; visualization, W.X. and E.V.; main writing, W.X. and B.D.R. All authors read and commented on the final version of the manuscript.

Declaration of interests

The authors declare no competing interests.

Data, Materials, and Software Availability.

Raw RNA-seq data can be accessed at NCBI with GEO number GSE301807⁵⁷. All other data are included in the article and/or supporting information.

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Figures

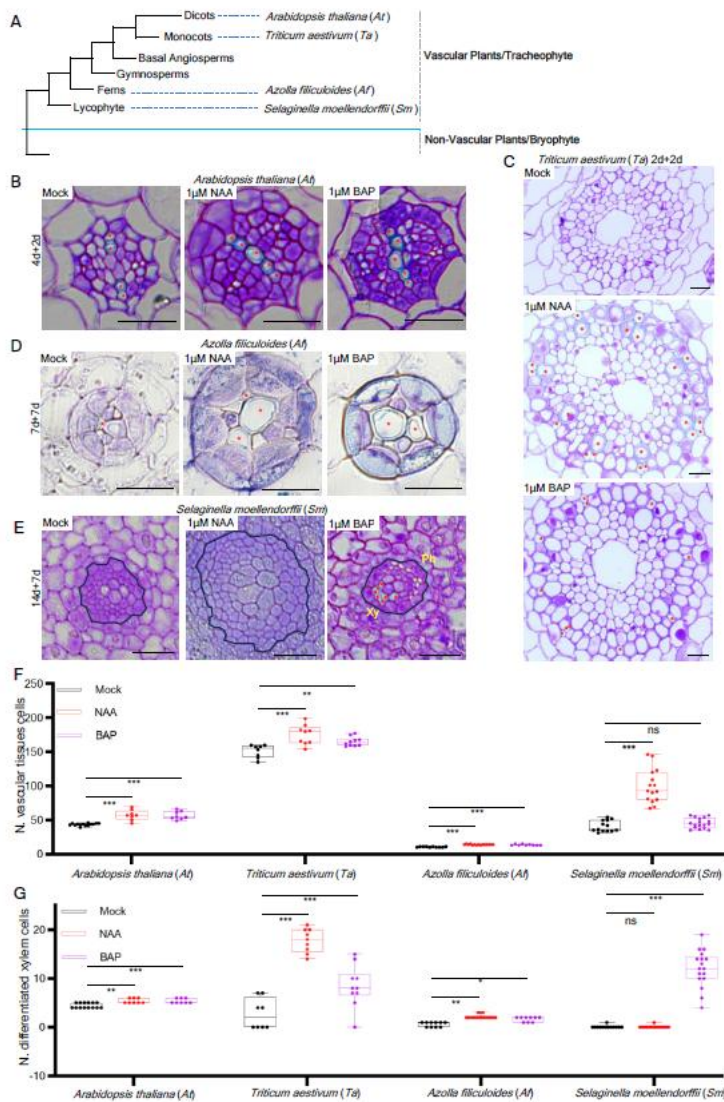


Fig. 1. Diverged hormonal functions during vascular tissue development

(A), Phylogenetic relationships of selected land plants. (B), Cross-section through the root of *Arabidopsis thaliana* (Col-0). 4-day-old plants were transferred to either mock (left panel), 1µM NAA (middle panel) or 1µM BAP (left panel) plates for 2 days before analysis. (C), Cross-section through the root of *Triticum aestivum*. 2-day-old plants were transferred to either mock (upper panel), 1µM NAA (middle panel) or 1µM BAP (lower panel) plates for 2 days before analysis. (D), Cross-section through the root of *Azolla filiculoides*. Plants were transferred to either mock (left panel), 1µM NAA (middle panel) or 1µM BAP (right panel) plates for 7 days before analysis. (E), Cross-section (~0.2 cm from root tip) through the root of *Selaginella moellendorffii*. 14-day-old plants were transferred to either mock (left panel), 1µM NAA (middle panel) or 1µM BAP (right panel) plates for 7 days before analysis. The blue circle indicated the vascular tissues. (F-G), Quantification of the number of vascular tissues cells and

differentiated xylem cells in panels B-E. Red asterisk in B-E indicates the differentiated xylem cell. Ph: Phloem, Xy: Xylem. Black scale bars: 20 μ m. Count data in G-H was modelled with a generalized mixed model (p-values were adjusted using Holm-Bonferroni correction; *: p<0.05, **: p<0.01, ***: p<0.001, ns: not significant).

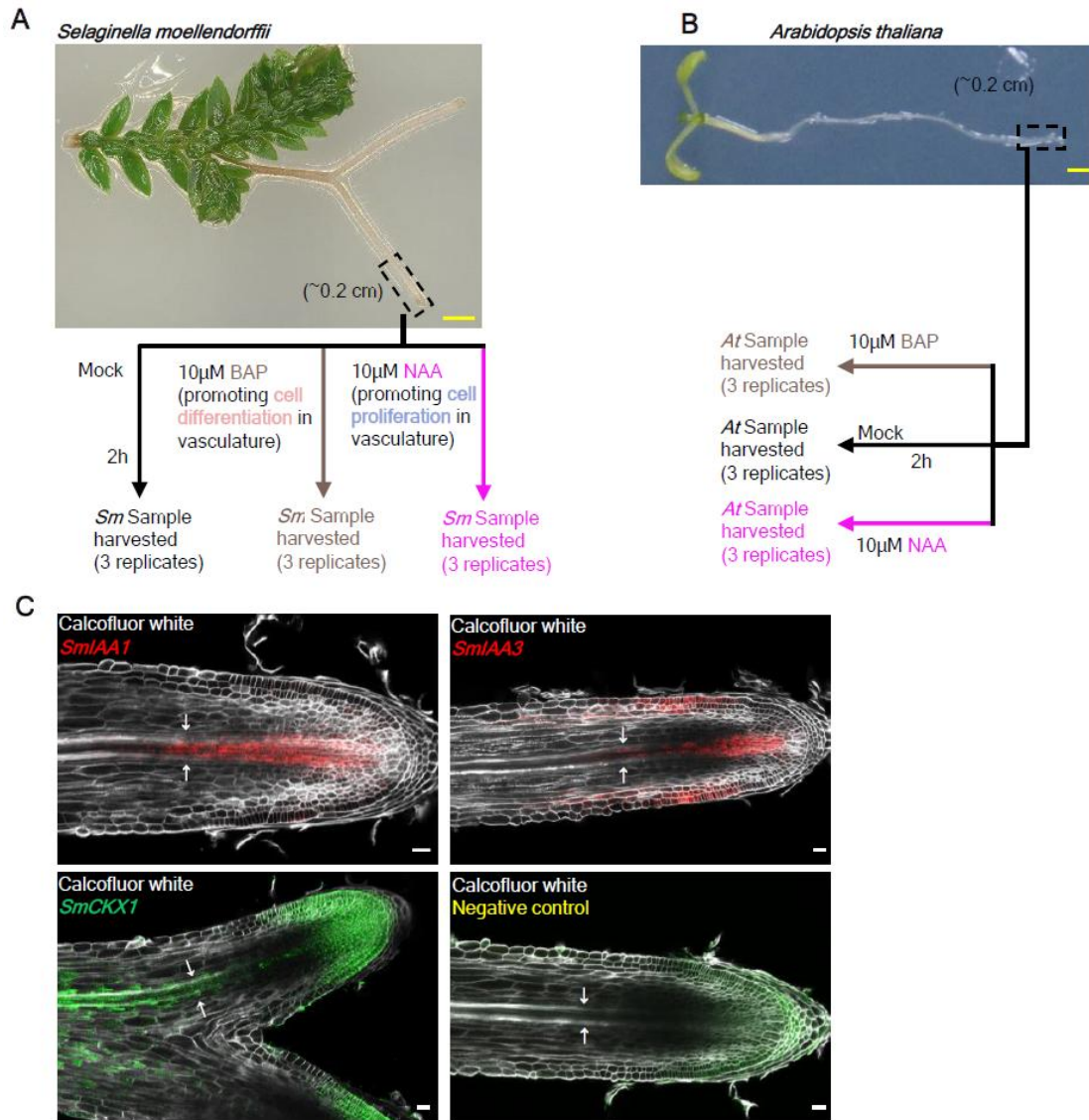


Fig. 2. Diverged transcriptional responses to auxin and cytokinin in *Selaginella*
(A-B), Overview of the RNA-seq experimental design. In *Selaginella moellendorffii*, 14-day-old plants were transferred for 2h on mock, 10µM NAA or 10µM BAP plates, and the root tips (~0.5 cm) were collected for each sample (A). In *Arabidopsis thaliana*, 4-day-old plants were transferred for 2h on mock, 10µM NAA or 10µM BAP plates, and the root tips (~0.5 cm) were collected for each sample (B). **(C)**, Whole-mount HCR RNA-FISH of *SmIAA1*, *SmIAA3*, and *SmCKX1* in the root tip of *Selaginella moellendorffii*. Note that the negative control (including the hairpin but no probes) is valid for experiments with both probes. The white arrow indicates the vascular tissues. White scale bars (for panels C-D): 20µm and yellow scale bars (for panels A-B) : 1mm.

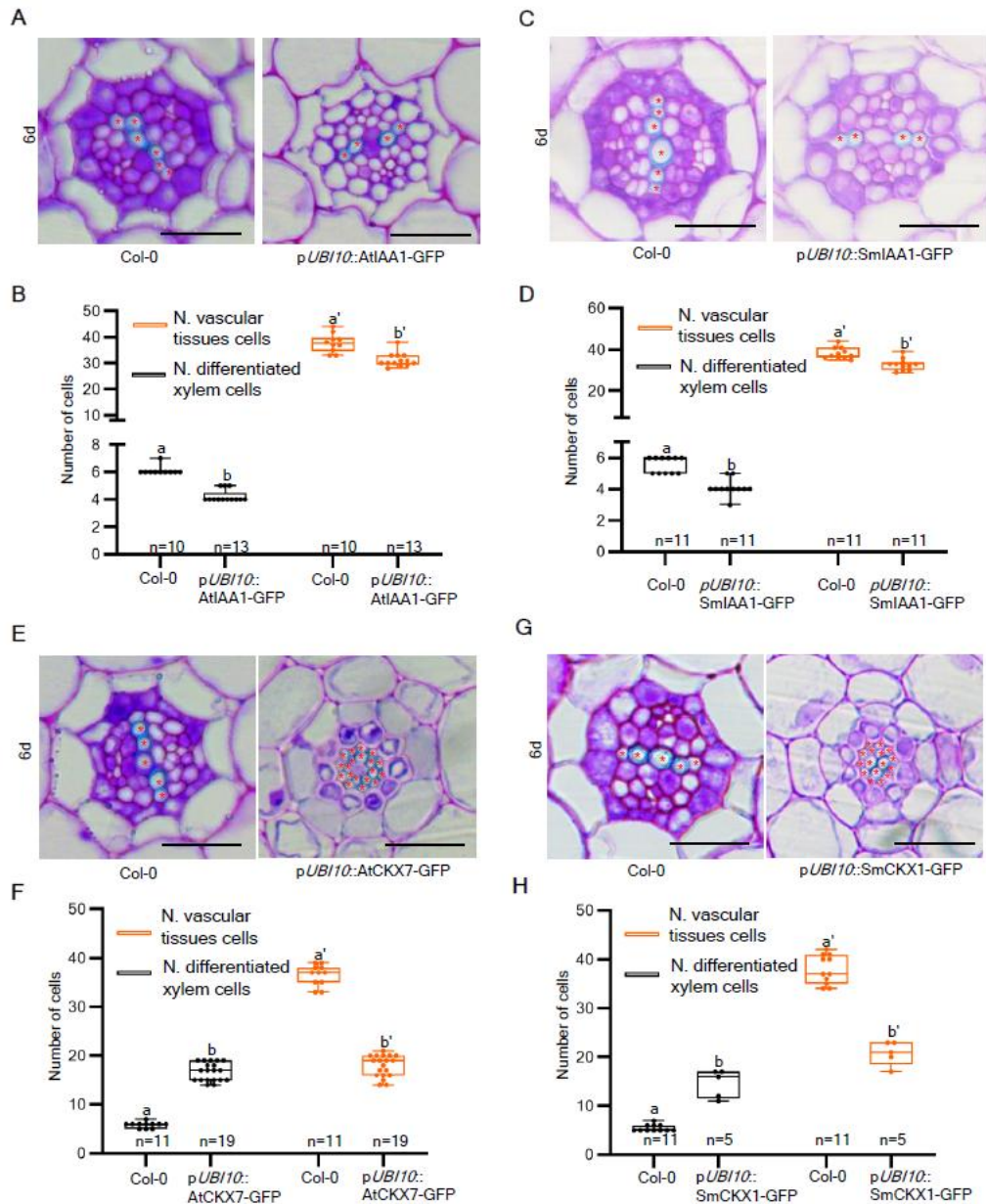


Fig. 3. Aux/IAA and CKX orthologs are functionally conserved

(A), Cross-section through 6-day-old Col-0 and pUBI10::AtIAA1-GFP roots. (B), Quantification of the number of vascular tissues cells and differentiated xylem cells in panel A. (C), Cross-section through 6-day-old Col-0 and pUBI10::SmIAA1-GFP roots. (D), Quantification of the number of vascular tissues cells and differentiated xylem cells in panel C. (E), Cross-section through 6-day-old Col-0 and pUBI10::AtCKX7-GFP roots. (F), Quantification of the number of vascular tissues cells and differentiated xylem cells in panel E. (G), Cross-section through 6-day-old Col-0 and pUBI10::SmCKX1-GFP roots. (H), Quantification of the number of vascular tissues cells and differentiated xylem cells in panel G. Red asterisk indicates the differentiated xylem cell. Black scale bars: 20 μ m. Count data was modelled with a generalized mixed model (p-values were adjusted using Holm-Bonferroni correction).

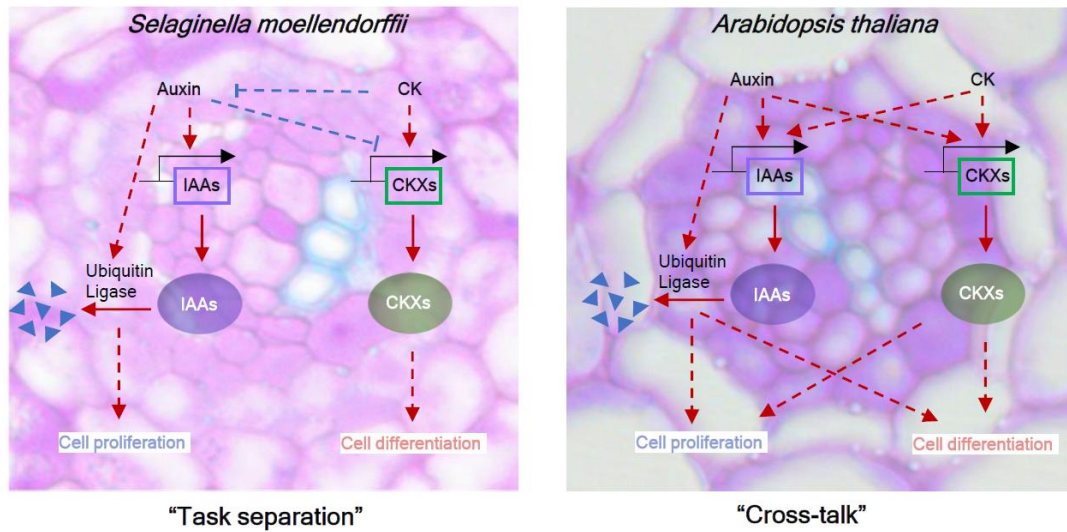


Fig. 4. Distinct evolutionary adaptations to hormonal regulation of vascular development

Schematic overview of the task separation in *Selaginella* where the phytohormones auxin and cytokinin have distinct functions (left) versus the hormonal cross-talk between these hormones in other vascular plant species (right).

Table S1. Plant material used in this study.

Plants	Background	Ref.	Obtained from
<i>shy2-2</i>	Col0	¹	Malcolm J. Bennett
<i>shy2-31</i>	Col0	¹	Malcolm J. Bennett
<i>ckx7</i>	Col0	²	Maurizio Di Marzo
pUBI10::AtIAA1-GFP	Col0	This study	This study
pUBI10::AtIAA5-GFP	Col0	This study	This study
pUBI10::AtIAA19-GFP	Col0	This study	This study
pUBI10::SmIAA1-GFP	Col0	This study	This study
pUBI10::SmIAA4-GFP	Col0	This study	This study
pUBI10::AtCKX7-GFP	Col0	This study	This study
pUBI10::SmCKX1-GFP	Col0	This study	This study
pUBI10::SmCKX2-GFP	Col0	This study	This study

Table S2. Primers used for molecular cloning.

Element	Primer	Sequence
AtIAA1	pGGC-AtIAA1-F	AACAGGTCTCAGGCTCAATGGAAGTCACCAAT GGGC
	pGGC-AtIAA1-R	AACAGGTCTCACTGATAAGGCAGTAGGAGCTTC GG
AtIAA5	pGGC-AtIAA5-F	AACAGGTCTCAGGCTCAATGGCGAATGAGAGT AATAATCTTG
	pGGC-AtIAA5-R	AACAGGTCTCACTGATCCTCTGTTACATGATCT CTTCATAATC
AtIAA19	pGGC-AtIAA19-F	AACAGGTCTCAGGCTCAATGGAGAAGGAAGGA CTCGG
	pGGC-AtIAA19-R	AACAGGTCTCACTGACTCGTCTACTCCTCTAGG CTGC
SmIAA1	pGGC-SmIAA1-F	AACAGGTCTCAGGCTCAATGACACTGGAAGCA GAGGC
	SmIAA1_NoBSA_ F	AACAGGTCTCACATGCGAAGGACGGAGC
	SmIAA1_NoBSA_ R	AACAGGTCTCACATGTTcAGACCACCCGC
	pGGC-SmIAA1-R	AACAGGTCTCACTGACTTGTGGCAGCGGTCAG TT
SmIAA4	pGGC-SmIAA4-F	AACAGGTCTCAGGCTCAATGGGGGAGGATTCCG CGAGC
	pGGC-SmIAA4-R	AACAGGTCTCACTGAGCTTGTAGCCTCGGCTTC TC
CKX7	pGGC-CKX7_F	AACAGGTCTCAGGCTCAATGATAGCTTACATAG AACCATACTTC
	CKX7_No-BSA_F	AACAGGTCTCAGTGGGACCGGAAGATATCG
	CKX7_No-BSA_R	AACAGGTCTCACCCTGGTtTCACCACAGC
	pGGC-CKX7_R	AACAGGTCTCACTGAAAGAGACCTATTGAAAA TCTTTTGAC
SmCKX1	pGGC-SmCKX1-F	AACAGGTCTCAGGCTCAATGTTGATCCTCTCGT GGGG
	SmCKX1_NoBSA _F	AACAGGTCTCAGACGAaACCGTCTCGTC
	SmCKX1_NoBSA _R	AACAGGTCTCACGTCAATGGTGGCGG
	pGGC-SmCKX1-R	AACAGGTCTCACTGACAGGGCGCTCGAGAGCTT G
SmCKX2	pGGC-SmCKX2-F	AACAGGTCTCAGGCTCAATGGGGAAAGTGAAG CTC
	pGGC-SmCKX2-R	AACAGGTCTCACTGAACGCTGGTCAGCAGCCTG

		TTT
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Table S3. GG vectors used in this study.

GG modules/vectors	Ref.	Obtained
pGG-C0	3	Jan Lohmann
pZ03	3	Jan Lohmann
pGG-A- <i>UBI10</i>	3	Jan Lohmann
pGG-B03 (B-dummy)	3	Jan Lohmann
pGG-C-AtIAA1	This study	This study
pGG-C-AtIAA5	This study	This study
pGG-C-AtIAA19	This study	This study
pGG-C-SmIAA1	This study	This study
pGG-C-SmIAA4	This study	This study
pGG-C-SmCKX1	This study	This study
pGG-C-SmCKX2	This study	This study
pGG-D-GFP	3	Jan Lohmann
pGG-E1 (pea RBCS terminator)	3	Jan Lohmann
pGG-F-FR (FASTRED)	4	Alexis Maizel

Table S4. GG Assembly strategy.

Final vector (in pZ03)	A	B	C	D	E	F
p <i>UBI10</i> ::AtIAA1-GFP	p <i>UBI10</i>	B3	AtIAA1	D-GFP	E1	FR
p <i>UBI10</i> ::AtIAA5-GFP	p <i>UBI10</i>	B3	AtIAA5	D-GFP	E1	FR
p <i>UBI10</i> ::AtIAA19-GFP	p <i>UBI10</i>	B3	AtIAA19	D-GFP	E1	FR
p <i>UBI10</i> ::SmIAA1-GFP	p <i>UBI10</i>	B3	SmIAA1	D-GFP	E1	FR
p <i>UBI10</i> ::SmIAA4-GFP	p <i>UBI10</i>	B3	SmIAA4	D-GFP	E1	FR
p <i>UBI10</i> ::AtCKX7-GFP	p <i>UBI10</i>	B3	AtCKX7	D-GFP	E1	FR
p <i>UBI10</i> :: SmCKX1-GFP	p <i>UBI10</i>	B3	SmCKX1	D-GFP	E1	FR
p <i>UBI10</i> :: SmCKX2-GFP	p <i>UBI10</i>	B3	SmCKX2	D-GFP	E1	FR

Table S5. Genes used in this study.

Gene Abbreviation	Gene Name	Gene Number
<i>AtIAA1</i>	<i>INDOLE-3-ACETIC ACID INDUCIBLE 1</i>	<i>AT4G14560</i>
<i>AtIAA5</i>	<i>INDOLE-3-ACETIC ACID INDUCIBLE 5</i>	<i>AT1G15580</i>
<i>AtIAA19</i>	<i>INDOLE-3-ACETIC ACID INDUCIBLE 19</i>	<i>AT3G15540</i>
<i>SmIAA1</i>	<i>INDOLE-3-ACETIC ACID INDUCIBLE-like 1</i>	<i>SMO137G0202</i>
<i>SmIAA4</i>	<i>INDOLE-3-ACETIC ACID INDUCIBLE-like 4</i>	<i>SMO351G0127</i>
<i>AtCKX7</i>	<i>CYTOKININ OXIDASE 7</i>	<i>AT5G21482</i>
<i>SmCKX1</i>	<i>CYTOKININ OXIDASE-like 1</i>	<i>SMO147G0128</i>
<i>SmCKX2</i>	<i>CYTOKININ OXIDASE-like 2</i>	<i>SMO141G0187</i>

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