

Deciphering growth stage-specific transcriptomic changes in KIOM-patented *in vitro* propagated *Asparagus cochinchinensis*

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Abstract *Asparagus cochinchinensis* is an endangered medicinal plant widely used in Korea, China, and Japan. Although *in vitro* micropropagation techniques have enabled successful regeneration of *A. cochinchinensis*, the molecular mechanisms underlying developmental transitions during propagation and acclimatization remain poorly understood. Herein, we performed *de novo* transcriptomic profiling across three key developmental stages (callus, *in vitro* plantlets, and acclimatized plants) to understand gene expression changes related to growth regulation and secondary metabolite biosynthesis. We obtained approximately 21 million clean reads through paired-end sequencing. *De novo* assembly generated 42,193 unigenes, and 12,850 differentially expressed genes were identified. Annotation against the Kyoto Encyclopedia of Genes and Genomes pathway database revealed distinct pathways for auxin biosynthesis at each stage. According to the transcript expression levels, the callus stage utilized the indole acetamide pathway and then

shifted to the indole pyruvate pathway during growth in *in vitro* chambers. These results indicated an adaptive transition of plant cells during development based on environmental conditions. Furthermore, one-month acclimatized plants activated environmental adaptation genes related to pathogen infection, such as plant-pathogen interactions and the mitogen-activated protein kinase signaling pathway, highlighting that plants require prolonged periods to reach a stable physiology. In addition, upregulation of genes involved in steroid and flavonoid biosynthesis was observed during the callus stage, highlighting early activation of secondary metabolite production pathways. The current findings provide fundamental insights relevant to the future development of *A. cochinchinensis* studies, especially for regeneration systems and mass production of pharmacologically active metabolites from *A. cochinchinensis*.

Keywords *Asparagus cochinchinensis*, transcriptome, auxin, secondary metabolite, adaptation

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Introduction

Asparagus cochinchinensis, a climbing perennial herb belonging to the *Liliaceae* family, has been a widely used medicinal plant for almost 2,000 years (Lee et al. 2019; Pahwa et al. 2022). It is widespread in temperate regions such as Korea, China, and Japan, and in tropical areas such as Vietnam and Laos (Lee et al. 2019; Wang et al. 2022). Since ancient times, *A. cochinchinensis* had been used as a medicinal resource, with clinical applications that have been recorded in the Pharmacopoeia of the People's Republic of China as a traditional Chinese medicine (TCM) (Wang et al. 2022). *A. cochinchinensis* root is an attractive medicinal ingredient that is commonly used to treat asthma, cough, constipation, and nerve and inflammatory diseases either

in combination with other medicinal herbs or applied individually (Chang et al. 2005; Jalsrai et al. 2016; Lee et al. 2015, 2017; Sung et al. 2016, 2017; Wang et al. 2022). In the last few decades, investigation of the active components of *A. cochinchinensis* that contribute to its therapeutic effects has identified various key chemical compounds, including steroidal saponins, C₂₁-steroids, lignans, polysaccharides, and amino acids (Chen et al. 2015; Choi et al. 2019; Li et al. 2012; Liu et al. 2021; Zhu et al. 2021).

The numerous therapeutic advantages of *A. cochinchinensis* have led to its excessive exploitation as a medicinal resource, rendering it endangered within its native habitats (Xiang-Hui et al. 2010). In Korea and Japan, *A. cochinchinensis* plants are found along seashores, whereas in China, they can easily grow on the slopes of forests and in uncultivated areas (Lee et al. 2019). In addition, the suburban advancement and migration of invasive species into their natural habitats have resulted in a decline of *A. cochinchinensis* populations, and their limited germination ability has exacerbated the problem (Xiang-Hui et al. 2010).

In a previous study, the successful *in vitro* micropropagation of *Asparagus cochinchinensis* was established using an indirect organogenesis approach, with the protocol formally registered as a Korean Patent (no: 10-2021-0108051, August 17, 2021, Korea Institute of Oriental Medicine) (Kim et al. 2021). While this protocol enables reliable plant production, the physiological and molecular changes that occur across developmental stages during *in vitro* propagation and acclimatization remain largely unexplored. Understanding these transitions is crucial to improving propagation efficiency and optimizing ex vitro performance.

In this study, we aimed to characterize the transcriptomic

dynamics of *A. cochinchinensis* across key developmental stages: callus formation, *in vitro* plantlet development, and acclimatization, compared to wild-type plants. We applied a *De novo* transcriptomic approach to profile gene expression changes associated with cellular dedifferentiation, organogenesis, and adaptation to external environments. By identifying stage-specific regulatory networks and biological pathways, we sought to uncover the molecular mechanisms underlying *in vitro* regeneration and acclimatization processes.

We hypothesized that each developmental stage would exhibit distinct transcriptional signatures reflecting physiological requirements, such as activation of auxin biosynthesis during callus and *in vitro* plant stages and upregulation of stress response pathways during acclimatization. To our knowledge, this represents the first comprehensive transcriptomic profiling of *A. cochinchinensis* during *in vitro* propagation. The findings will contribute foundational knowledge for advancing mass propagation strategies, improving acclimatization success, and enhancing the production of valuable secondary metabolites in this endangered medicinal plant.

Materials and Methods

Sample preparation

The callus (1_2), *in vitro* (3_2), and acclimatized KIOM-1 (4_2) *A. cochinchinensis* were obtained as described in a previous report by Kim et al. (2021) (Fig. 1). The callus on 4 weeks cultures on Murashige and Skoog (MS) medium supplemented with benzylaminopurine (BAP) (1 mg/L) and 1-naphthalene-

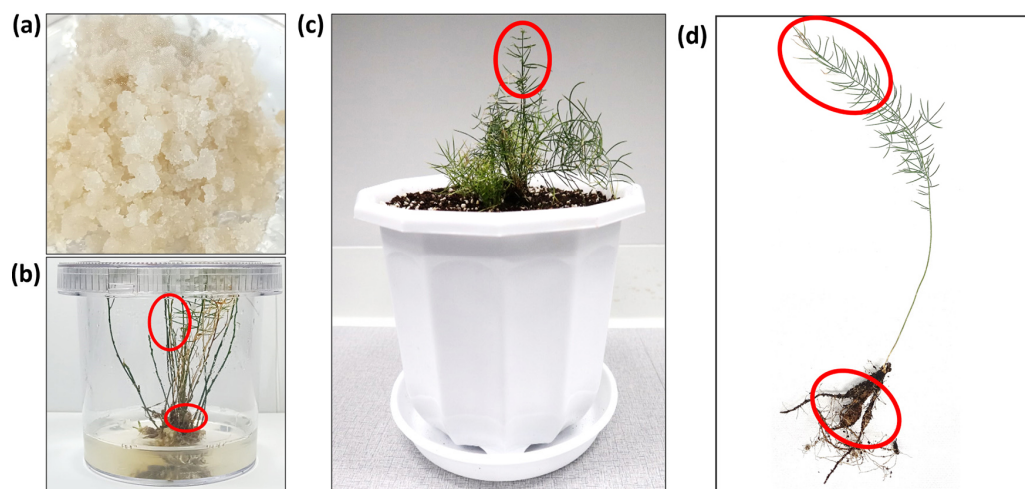


Fig. 1 *Asparagus cochinchinensis* at different stages of propagation. (a) callus (1_2), (b) *in vitro* plant (3_2), (c) KIOM-1 (4_2), and (d) wild-type (5_2). Red circle indicates sample part for RNA sequencing

acetic acid (NAA) (1 mg/l). The 9 weeks of *in vitro* plant was harvested for RNA-sequencing. The acclimatized *A. cochinchinensis* were sampled after six weeks of adaptation to sterilized soil in an indoor-controlled environment for RNA sequencing. The wild type (5_2) *A. cochinchinensis* was grown from seeds collected from Jeonju Botanic Garden, South Korea, in October 2018 (Kim et al. 2021). The two months of wildtype was used for RNA sequencing (Fig. 1). The mixture of aerial and underground part was used for RNA sequencing analysis which indicated with red circle on Fig. 1.

RNA sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA), and RNA quality was evaluated using the TapeStation 4000 system (Agilent Technologies, Amstelveen, The Netherlands). RNA concentration was measured using an ND-2000 spectrophotometer (Thermo Inc., Wilmington, DE, USA). RNA-Seq libraries were prepared using the NEBNext Ultra II Directional RNA-Seq Kit (New England BioLabs, UK). To isolate mRNA, we used a poly (A) RNA Selection Kit (Lexogen, Vienna, Austria). Purified mRNA was then used for cDNA synthesis and fragmentation, according to the manufacturer's instructions. Library indexing was performed using Illumina indices 1–12, and PCR amplification was used for enrichment. The quality of the final libraries, including fragment size distribution, was checked using a TapeStation HS D1000 ScreenTape (Agilent Technologies, Santa Clara, CA, USA). Library quantification was performed using a library quantification kit on a StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Finally, high-throughput sequencing was conducted on a NovaSeq 6000 platform (Illumina, San Diego, CA, USA) using 100 bp paired-end reads.

Transcriptome assembly

The kraken2 (Wood et al. 2019) was used with the standard database to remove foreign contaminants from the sequences. Then adapter and low-quality sequences were filtered using Fastp (Chen et al. 2018) to generate clean reads. *De novo* transcriptome assembly was performed with Trinity (v. 2.15.1) (Grabherr et al. 2011) using the default settings. Subsequently, TransDecoder (v. 5.7.0) was used to predict the coding regions of the assembled transcripts using the single_best_only option (Haas 2022). Next, we used the CD-HIT program (v. 4.8.1) (Fu et al. 2012) with a 0.99 sequence identity threshold option to reduce transcript redundancy.

Assessment of gene completeness

Completeness of the unigene assembly was assessed and then validated using the BUSCO program (v5) (Seppey et al. 2019), which curates single-copy orthologs derived from OrthoDB, which is present in all species within a specific lineage. CLC Genomics Workbench ver. 7.0.3 was used to check the contiguity and completeness of the draft genome, which was validated by mapping the RNA sequences from the same strain using default parameters.

Functional annotation and gene ontology (GO) classification

The Functional annotation was performed using the BLAST program (E-value less than 1×10^{-5}) between assembled transcripts with SwissProt (<http://www.ebi.ac.uk/uniprot/>), non-redundant (NR) (2023.12.08), and eggNOG (<http://eggno-mapper.embl.de/>) databases. Subsequently, OmicsBox (<https://www.biobam.com/omicsbox/>) was used to retrieve GO data for all unigenes. Investigation of the metabolic pathways of the unigenes was conducted in accordance with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/kegg/>). Moreover, total unigenes from the *De novo* RNA assembly were quantified using Salmon (Patro et al. 2017). Unigenes were processed based on the TMM+CPM normalization method using the EdgeR package (Robinson et al. 2009). Differentially expressed genes (DEG) were identified using edgeR in the Bioconductor package with a biological coefficient of variation (BCV) of 0.6. Venn diagrams of the DEGs were generated using the jvenn package (Bardou et al. 2014). GO Term plot were generated using the SRplot tools (Tang et al. 2023).

Validation of RNA-Seq using qRT-PCR

Validation of the selected unigenes was performed by extracting total RNA using TRIzol (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized as described previously (Lyu et al. 2011). Quantitative PCR (qPCR) was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and TOPreal SYBR Green qPCR Premix (Enzynomics, Daejeon, Korea). Actin was used as the internal quantitative control, and threshold values (Ct values) were normalized using the $2^{-\Delta\Delta Ct}$ formula for quantitative analysis. Eight unigenes were targeted for qPCR analysis (Table S1 Primer sequence for qRT-PCR for selected unigenes Table S1).

Table 1 Sequencing *A. cochinchinensis* transcriptome at four stages

Sample	Accession Number	Number of raw reads	Number of clean reads	Size of clean reads (bp)	GC%	Q20 (%)	Q30 (%)
1_2_1	SAMN48975769	27,267,942	21,072,895	2,123,283,841	48	97.75	93.62
3_2_1	SAMN48977749	26,514,762	18,717,505	1,885,776,990	49	97.83	93.81
4_2_1	SAMN48977753	26,544,370	16,452,413	1,659,589,923	51	97.67	93.44
5_2_1	SAMN48977863	27,415,493	13,145,692	1,325,871,817	54	97.55	93.30

Table 2 Statistics of transcriptome assembly and predicted unigenes

Type	Transcripts	Unigenes
Total Sequences	58,667	42,193
Total Base	48,750,366	29,579,337
Average length (bp)	830.97	835.35
N50 length (bp)	336	840

Results

Sequencing, *De novo* transcriptome assembly, and annotation

Paired-end sequencing of cDNA libraries generated 21,072,895, 18,717,505, and 16,452,413 clean reads for the 1_2, 3_2, 4_2 and 5_2 samples, respectively, with more than 93% of Q30 bases. For the WT sample, 13,145,692 clean reads with more than 93% of Q30 bases were acquired (Table 1). The GC content ranged between 48% and 54% among the samples (Table 1). Although we prepared replicates for each sample, the RNA quality remained insufficient for further analysis despite multiple extraction attempts. This limitation in replication will be a constraint of the present study.

Filtered reads from the samples were obtained after assembly using Trinity (v. 2.15.1), which generated 58,667 transcripts with an N50 of 336 bp. There were 42,193 assembled unigenes with around 835.35 bp on average and 840 bp of N50 length (Table 2). The assembled unigenes were compared to public databases and showed the highest coverage in the NR NCBI database (94.72%), followed by eggNOG (81.03%) and SwissProt (49.63%; Table S2).

Genome completeness analysis using BUSCO software revealed that 93.1% of the detected unigenes of *A. cochinchinensis* were complete and single copies. Further, 4.2% and 2.7% of unigenes were fragmented and missing, respectively, among the BUSCO orthologs (Table 3). The high percentage of complete genes with low percentages of fragmented and missing genomes indicated that the assembly results were of good quality (Seppey et al. 2019).

The E-value distribution of 35% of the top hits ($< 1 \times 10^{-50}$)

Table 3 Statistics of genome completeness using BUSCO

C:93.1%[S:75.5%,D:17.6%],F:4.2%,M:2.7%,n:425	
396	Complete BUSCOs (C)
321	Complete and single-copy BUSCOs (S)
75	Complete and duplicated BUSCOs (D)
18	Fragmented BUSCOs (F)
11	Missing BUSCOs (M)
425	Total BUSCO groups searched

showed significant homology with the sequence entries in the NR NCBI database (Fig. 2a). Annotation using the NCBI NR database identified 28,516 homologous sequences (Table S3). Moreover, the unigene sequences exhibited the greatest similarity to the gene sequences of *Iris palida* (3,083), followed by *Asparagus officinalis* (1,613), *Phoenix dactylifera* (1,331), *Cocos nucifera* (1,275), and *Elaeis guineensis* (750) using BLASTP (Table S3, Fig. 2b).

The OmicBox program revealed the GO classification of the assembled transcripts, which were classified into three major GO categories: 22,659 unigenes assigned to biological processes, 24,034 unigenes belonging to cellular components, and 25,061 unigenes annotated to molecular functions (Fig. 3a). ‘Protein metabolic process’ and ‘organonitrogen compound biosynthetic process’ were highly represented groups within the biological process category with around 4,760 and 3,869 unigenes, respectively (Fig. 3a). For the cellular component category, ‘protein-containing complex’ (6,190 unigenes) and ‘nucleus’ (5,553 unigenes) groups constituted the largest proportion. Lastly, 5,620 unigenes related to hydrolase activity and 5,516 unigenes related to transferase activity were dominant in the molecular function category (Fig. 3a).

KEGG pathway analysis identified 144 active biological pathways containing 7683 unigenes (Table S4). The most annotated pathways belonged to ‘purine metabolism’ (1758), followed by ‘thiamine metabolism’ (1565), ‘human immunodeficiency virus 1 infection’ (700), ‘glycolysis/gluconeogenesis’ (515), and ‘starch and sucrose metabolism’ (482) (Fig. 3b, Table S4).

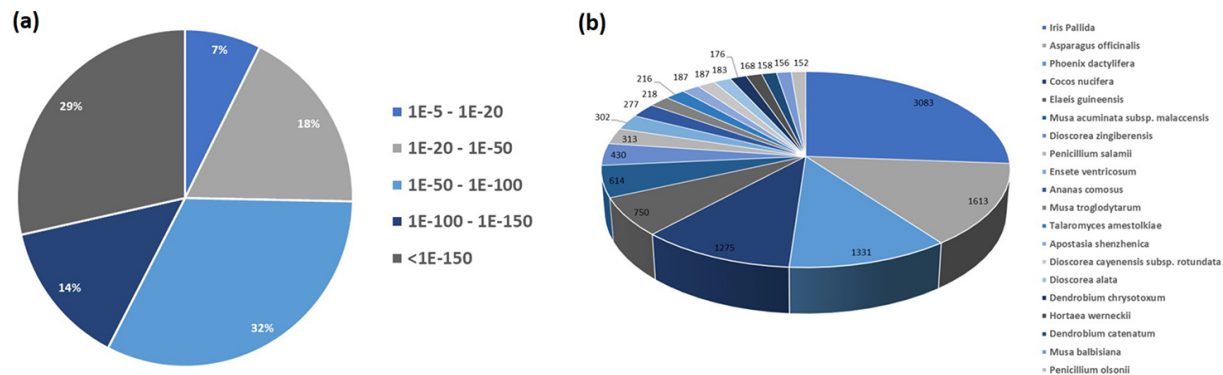


Fig. 2 Functional annotation summary of assembled transcripts based on NCBI NR protein database. (a) Distribution of E-values from BLASTP hits, indicating reliability of sequence matches. (b) Species distribution of functional annotation with NR BLASTP NCBI database

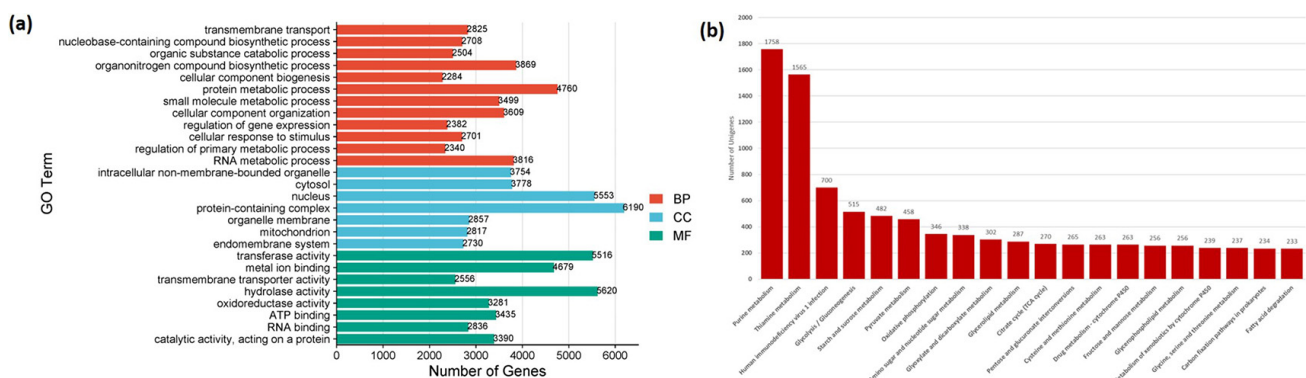


Fig. 3 Functional annotation of assembled unigenes using Gene Ontology (GO) and KEGG pathway databases. (a) Results of gene ontology classification are classified into three main categories: biological process (BP), cellular component (CC), and molecular function (MF). (b) Distribution of top 20 KEGG pathways containing most assembled transcripts

The *De novo*-assembled transcriptome, which was expressed during propagation and in the wild-type plantations, was used as a reference to identify the relationships among all samples. Correlation analysis based on the TMM + CPM normalization value distinguished between the *in vitro*-propagated samples and wild type. Moreover, the *in vitro* propagation samples were very similar ($r > 0.86$; a).

DEGs were identified during development stages of *A. cochinchinensis* using the edgeR software from Bioconductor package, with a biological coefficient of variation (BCV) of 0.6 as the threshold, to obtain an understanding of transcript expression related to growth between *in vitro* propagated plants compared to wild-type at the control stages. Unigenes with a fold change (FC) of expression level > 2 (false discovery rate (FDR) = 0.01) were identified as significant DEGs, comprising 12,850 unigenes (Fig. 4b).

Furthermore, GO enrichment analysis of DEGs revealed that the dominant biological processes varied across comparisons. For example, “regulation of cellular process” was most enriched in 5_2/1_2 (2,990 genes), while “transmembrane transport”

predominated in 5_2/3_2 (572 genes) and 5_2/4_2 (391 genes) (Fig. 4c). A similar trend was observed for cellular component categories, with “nucleus,” “cytosol,” and “mitochondrion” being the most represented terms in 5_2/1_2, 5_2/3_2, and 5_2/4_2, respectively (Fig. 4c). In contrast, “hydrolase activity” consistently appeared as the most enriched molecular function across all comparisons, with 2,992, 785, and 393 genes in 5_2/1_2, 5_2/3_2, and 5_2/4_2, respectively (Fig. 4c).

There were 726 unigenes that were annotated into the KEGG pathway analysis, where eleven unigenes were related to plant hormone signal transduction (ko04075), especially those involved in the response to auxin compounds, including *AUX/IAA* (K14484), *GH3* (K14487), *SAUR* (K14488), and *AHA 1/2* (K01535) (Fig. 5). Moreover, several unigenes also upregulated in others plant hormone signal transduction such as Gibberellin (1 unigene), Brassinosteroid (2 unigenes), salicylic acid (1 unigene) and PSK (5 unigenes) (Fig. S1). The expression of auxin-related genes in 1_2 stages was high compared to other stages; in particular, 4_2 plants showed the opposite pattern (Fig. 5).

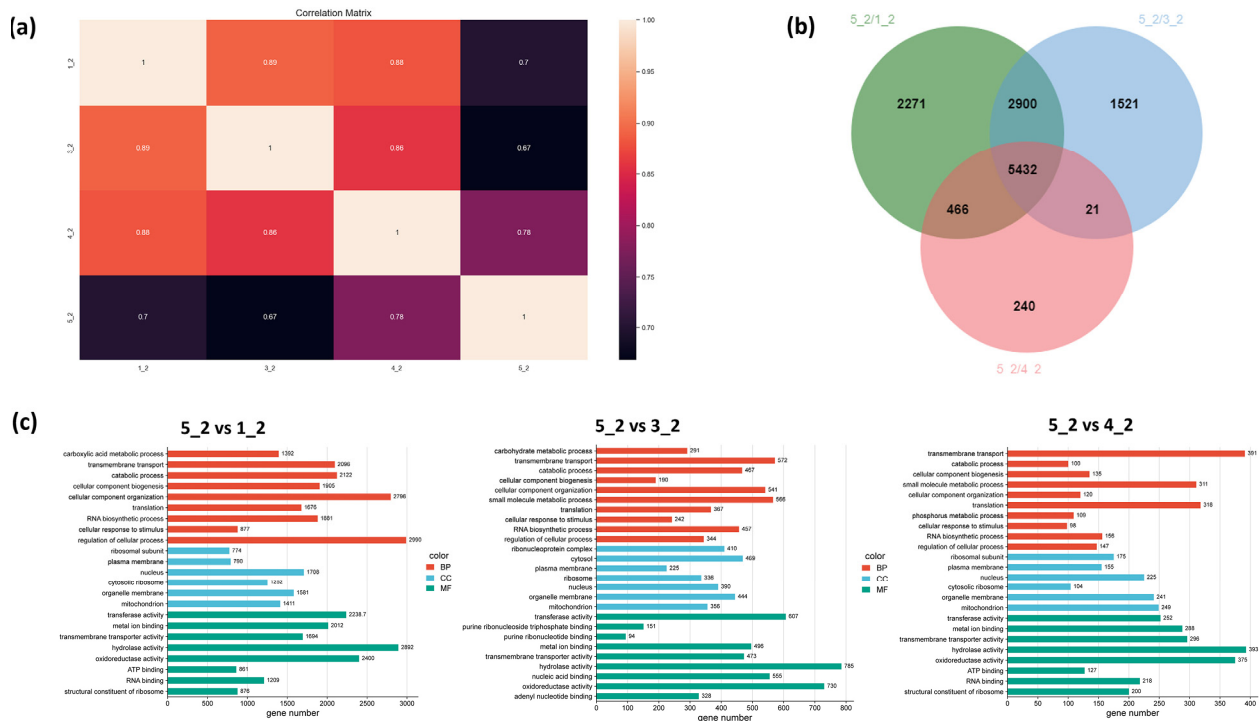


Fig. 4 Correlation of transcriptome assemblies and overlap of differentially expressed genes across samples. (a) Heatmap of Pearson correlation coefficients between assembled transcriptomes of four sample groups. 5_2 shows weaker correlation with others, indicating a distinct transcriptional profile. (b) Venn diagram showing overlap of differentially expressed genes (DEGs) from comparisons between sample 5_2 and each of other three samples. (c) GO term of DEGs of each comparison sample

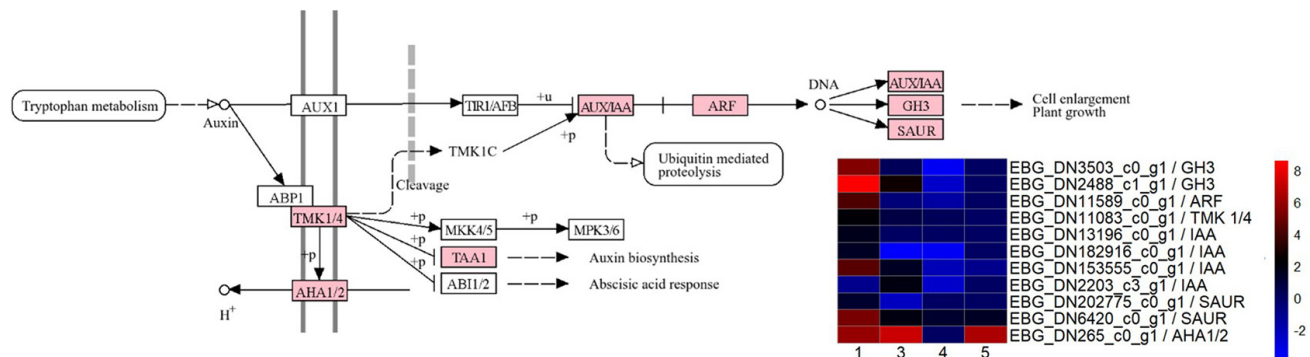


Fig. 5 Differential expression of genes involved in auxin signal transduction pathway. Schematic representation of auxin signaling components mapped via KEGG, with differentially expressed genes (DEGs) highlighted in pink. Heatmap shows log2-CPM of selected DEGs across four sample conditions, with red indicating upregulation and blue indicating downregulation. 1: callus stage, 3: *in vitro* plant, 4: acclimatized plant, and 5: wild-type plant

Validation of RNA-Seq using qRT-PCR

To validate the unigene expression levels, selected unigenes that were DEGs related to auxin biosynthesis were analyzed using qRT-PCR. As illustrated in Fig. 6, the expression profiles of the selected unigenes acquired using qPCR were identical to those derived from RNA sequencing data. According to a paired t-test, a p-value of 0.2764 was obtained, indicating that the difference between the results was not statistically significant. These results indicated that the qRT-PCR results were identical to the

RNA-sequencing data.

Discussion

In this study, we aimed to elucidate the auxin biosynthesis pathways at each developmental stage of *in vitro*-propagated *Asparagus cochinchinensis* and found that different stages utilize distinct pathways. Indole-3-acetic acid, frequently known as indoleacetate (IAA), is the most common auxin produced by

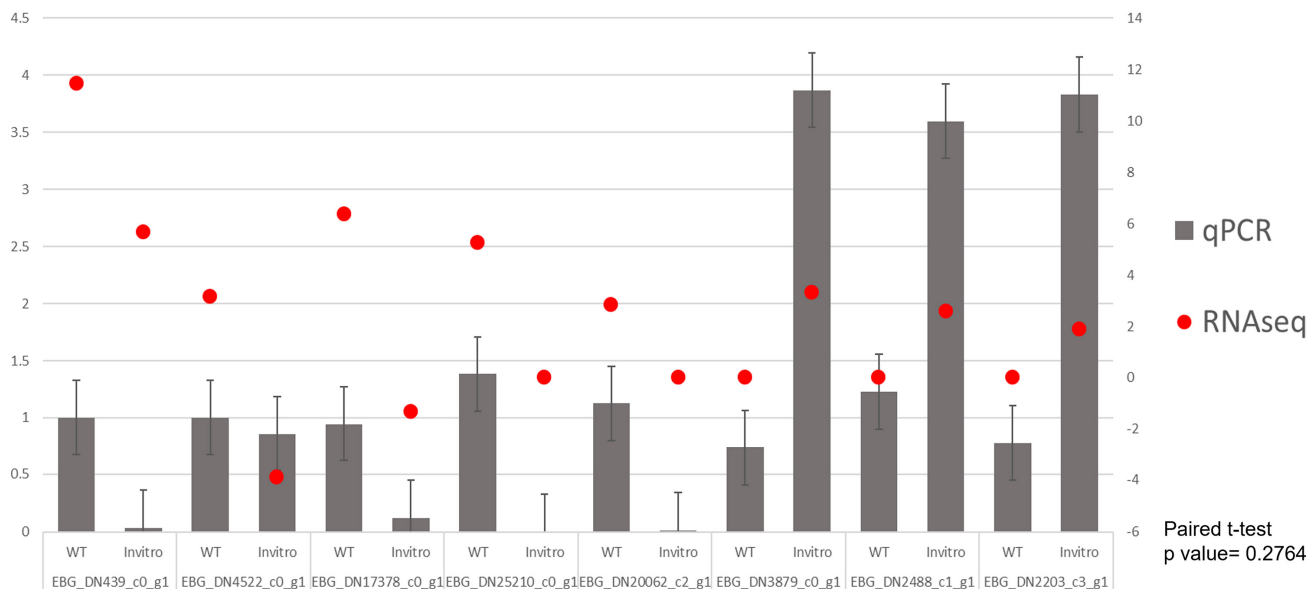


Fig. 6 Validation of RNA-Seq results using qRT-PCR. Expression levels of selected unigenes were compared between RNA-Seq (red dots) and qRT-PCR (gray bars with error bars) in wild-type (WT) and *in vitro*-grown samples. Overall, qRT-PCR results show expression patterns consistent with RNA-Seq data, supporting transcriptome profiling reliability

plants and plays an important role in the regulation of plant growth and development. The amino acid tryptophan (TRP) is the primary precursor component in the formation of IAA, which occurs through two main pathways: TRP-dependent and TRP-independent (Guilfoyle 1999; Mashiguchi et al. 2011; Wang et al. 2015). Within the TRP-dependent pathway, there are at least four well-characterized routes: indole-3-pyruvate (IPA), indole-3-acetamide (IAM), indole-3-acetaldoxime (IAOx), and tryptamine (TAM) (Bao et al. 2024; Guilfoyle 1999; Morffy and Strader 2020). According to DEG analysis, the 1_2 cell stage utilized the IAM pathway, as indicated by the upregulation of the gene encoding amidase (*amiE*/K01426) by 4.39 fold-change and downregulated tryptophan aminotransferase (*TAA*) (K16903) by almost 8.97 fold-change. In contrast, the IPA pathway appeared to be the predominant route for auxin biosynthesis in *in vitro* plants, as indicated by the significantly higher expression of the tryptophan aminotransferase (*TAA*) gene compared to those for other routes (Table S5, Fig. 7). This finding is further supported by the low expression level of amidase (*amiE*), which plays a role in the conversion of IAM to IAA, indicating that the synthesis of IAM is lower than that of IPA in the *in vitro* plants. These results align with the findings of Mano and Nemoto (2012), who reported that IPA and IAM are the major routes of IAA biosynthesis.

Notably, the present study found that indole-3-pyruvate monooxygenase (*YUCCA*), an enzyme that converts IPA into IAA, was downregulated. This pathway involves a well-

established two-step production of auxin from tryptophan that is dominant in *in vitro* plants but not in the wild-type (Fig. 7) (Mashiguchi et al. 2011; Zhao 2014). Low gene expression observed in the downstream IPA pathway suggests the involvement of an alternative auxin production route. To the best of our knowledge, no reports exist of the isolation of IPA decarboxylase from plants, the enzyme presumed to be responsible for transforming IPA into IAAla, despite the presence of IAAla in various plant species (Abu-Zaitoon et al. 2019; Brown and Purves 1976; Kutáček and Terziivanova-dimova 1991; Ludwig-Müller and Hilgenberg 1990; Mashiguchi et al. 2011). Abu-Zaitoon et al. (2019) hypothesized that IAAla could be synthesized from either TAM or IAOx. However, no scientific evidence has identified the specific intermediate enzyme(s) responsible for IAAla biosynthesis via the IPA, TAM, or IAOx pathways in plants (Mano and Nemoto 2012). Notably, the annotation results of this study demonstrated the absence of genes involved in these three pathways of IAAla production. Additionally, the presence of molybdenum in Murishage and Skoog (MS) medium can enhance aldehyde oxidase activity because the enzyme requires molybdenum as a cofactor for its catalytic function, thereby facilitating the conversion of IAAla into IAA (Wu et al. 2022). This possibly explains the high expression level of ALDH in 1_2 and 3_2 plants, to enhance the production of IAA (Fig. 7).

In this study, the expression level of auxin-related signal transduction genes were comparable between cultivated and 5_2

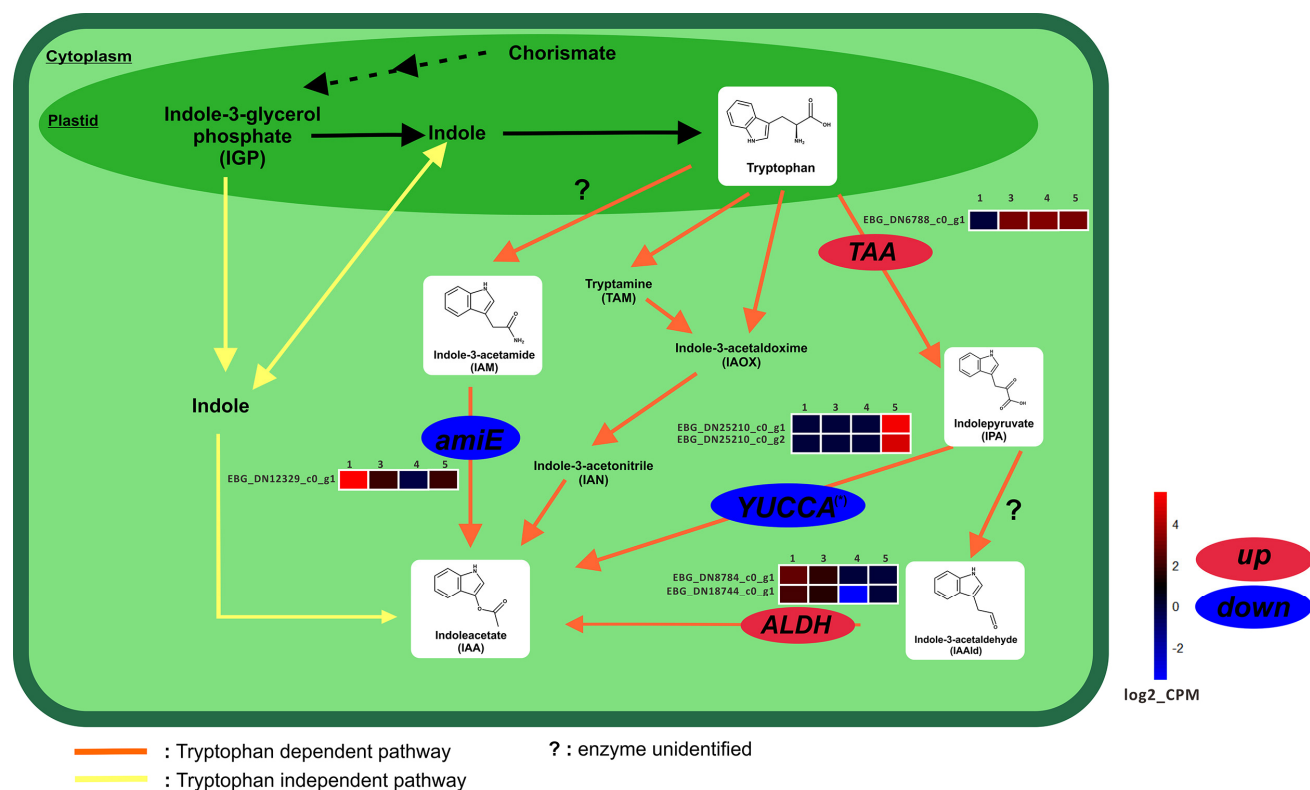


Fig. 7 Predicted scheme of auxin biosynthesis in *in vitro* plants of *A. cochinchinensis* culture. Schematic representation of tryptophan-dependent auxin (IAA) biosynthesis pathways through indole pyruvate (IPA) pathway based on differentially expressed genes (DEGs) identified from RNA sequencing data

plants, except at the 1_2 stage, and they were upregulated in 3_2, and 4_2 plants, where they showed low expression levels (Fig. 5). Moreover, the unigenes predicted to encode Arabidopsis H⁺-ATPase isoforms 1 and 2 (AHA 1/2) exhibited the lowest expression across all developmental stages (Table S5, Fig. 5). AHA 1/2 are plasma membrane H⁺-transporting ATPases that play a crucial role in the regulation of proton efflux within plant cells (Hoffmann et al. 2019; Yuan et al. 2017). Their activity is influenced by intracellular auxin concentrations and environmental factors (Miao et al. 2021). Given the observed downregulation of auxin-related genes, external environmental factors may have contributed to the reduced expression of AHA 1/2 (Table S5, Fig. 5).

Furthermore, DEG analysis of unigenes revealed pathogen infection-related genes as key contributors to the adaptation process of 4_2 plants grown in soil (Table 4). There were 17 unigenes related to plant-pathogen interactions (map04626), six unigenes related to the mitogen-activated protein kinase (MAPK) signaling pathway (map04016), and 4 unigenes shared both plant-pathogen interactions and MAPK signaling pathways. The majority of unigenes in MAPK signaling contribute to plant protection and adaptation to pathogen infection (K20725,

K13449, K20714, K17686, K14515, K20547, K20718, K02183, and K13447), including WRKY (K13425), which exhibited a 0.42 fold-change. WRKY is a transcription factor that is activated mostly during pathogen attack (by bacteria, viruses, or fungi) in plants during the acclimatization period (Rurek and Smolibowski 2024). Moreover, further investigation is required to reveal the time period required for *in vitro* propagated *A. cochinchinensis* to reach a stable physiology, which is important for maximizing the survival rate and preparing a suitable environment to reduce acclimation stress for the plant. Investigating dynamic transcript expression across different sampling times over an extended observation period may provide detailed insights into the plant's adaptation processes.

In this study, DEG analysis revealed upregulation of three unigenes related to steroid biosynthesis (map00100) and eight unigenes related to flavonoid biosynthesis (map00941) at the 1_2 stage (Table 5, Fig. S2, Fig. S3). These metabolites are important compounds produced by *A. cochinchinensis* that contribute to its various pharmacological activities. For example, methyl protodioscin, a major compound in 70% ethanol extract of *A. cochinchinensis*, exhibited strong inhibition of the production of proinflammatory cytokines such as IL-6, IL-8, and

Table 4 Plant-pathogen interaction and MAPK signaling-related genes that were differentially expressed during acclimatization

Unigene ID	Fold change	Annotation	KO ID
EBG_DN6040_c0_g1	0.42	WRKY transcription factor 22	K13425
EBG_DN9868_c0_g1	0.23	basic endochitinase B	K20547
EBG_DN15793_c0_g3	2.35	P-type Cu ⁺ transporter	K17686
EBG_DN172912_c0_g1	8.30	LRR receptor-like serine/threonine-protein kinase ERECTA	K20718
EBG_DN18909_c1_g3	3.00	serine/threonine-protein kinase OXI1	K20714
EBG_DN79629_c0_g2	-2.73	EIN3-binding F-box protein	K14515
EBG_DN13893_c0_g1	6.44	respiratory burst oxidase	K13447
EBG_DN1385_c0_g3	-0.03	calmodulin	K02183
EBG_DN192490_c0_g1	-2.68	pathogenesis-related protein 1	K13449
EBG_DN153983_c0_g1	2.84	MAP kinase substrate 1	K20725
EBG_DN9518_c0_g1	1.21	cyclic nucleotide gated channel	K05391
EBG_DN1097_c0_g1	2.30	calcium-binding protein	K13448
EBG_DN4895_c0_g1	2.20	calcium-dependent protein kinase	K13412
EBG_DN51_c0_g2	3.15	calcium-binding protein	K13448
EBG_DN7298_c0_g1	1.58	elongation factor Tu	K02358
EBG_DN505_c0_g2	2.49	calcium-dependent protein kinase	K13412
EBG_DN7530_c0_g1	8.83	cyclic nucleotide gated channel	K05391
EBG_DN7915_c0_g1	0.95	glycerol kinase	K00864
EBG_DN8361_c0_g1	7.91	3-ketoacyl-CoA synthase	K15397
EBG_DN5594_c0_g1	3.29	disease resistance protein RPS2	K13459
EBG_DN5179_c0_g1	-0.57	EIX receptor 1/2	K13466
EBG_DN193501_c0_g1	3.57	3-ketoacyl-CoA synthase	K15397
EBG_DN6319_c0_g1	2.01	3-ketoacyl-CoA synthase	K15397
EBG_DN33640_c0_g1	1.57	calcium-binding protein	K13448
EBG_DN6319_c0_g2	5.65	3-ketoacyl-CoA synthase	K15397
EBG_DN102003_c0_g1	0.10	calcium-dependent protein kinase	K13412
EBG_DN192583_c0_g1	4.28	EIX receptor 1/2	K13466

tumor necrosis factor- α (TNF- α), which contribute to inflammation, especially in the respiratory tract (Lee et al. 2015). Since plants produce secondary metabolites at low concentrations, massive production using cell suspension approaches may be more efficient than isolation from the original plant. This discovery may be an important finding for the future production of secondary metabolites from the callus of *A. cochinchinensis*. The present study revealed an increased expression of upstream enzymes involved in steroid biosynthesis, suggesting a potential elevation of steroid backbone-derived secondary metabolites, such as steroidal saponins and C21-steroids (Fig. S2). Similarly, genes associated with flavonoid biosynthesis were up-regulated, indicating an enhanced accumulation of flavonoid compounds within plant cells (Fig. S3). Consistently, De Bellis et al. (2022) reported the extract of *Cydonia oblonga* Mill reported that extracts from *Cydonia oblonga* Mill. Calli were enriched with

secondary metabolites, including 5-O-caffeoylquinic acid (5-CQA), 5-p-coumaroylquinic acid (5-p-CoQA), maslinic acid, and corosolic acid. However, further exploration of genes related to steroid-saponin compounds or other targeted compounds in *A. cochinchinensis* is required to gain a comprehensive understanding of the biosynthetic pathways of targeted metabolites. Metabolic engineering of plants related to these secondary metabolites could be a potential strategy for exploring genes associated with targeted compounds (Jia et al. 2025; Zhao et al. 2022).

Despite the valuable insights gained from this study, several limitations should be acknowledged. The sample size was relatively limited, and biological replicates were not included, which may affect the robustness of the findings. Additionally, the observation period was relatively short, and functional validation of candidate genes was not performed. However, to minimize potential biases, statistical corrections were applied,

and the transcriptomic results were carefully validated against existing literature. Future studies incorporating larger sample sizes, extended observation periods, and functional analyses such as gene knockout or overexpression experiments would help to strengthen and expand upon these findings.

Conclusion

Transcriptomic analysis is a highly effective approach for understanding the expression levels of genes during *in vitro*

propagation of *A. cochinchinensis*. In this study, paired-end sequencing generated 42,193 unigenes. This is also the first study to conduct comparative transcriptomic analysis within the developmental stages of *in vitro* propagated *A. cochinchinensis*, which provides basic knowledge of transcription and hormone regulation at different stages. Moreover, the distinct auxin biosynthesis pathway at each stage provides a foundation for the future improvement of *A. cochinchinensis* studies (Fig. 8). Functional validation of key genes through gene editing techniques could offer comprehensive insights into the auxin biosynthesis pathways at specific developmental stages. Activated

Table 5 List of differentially expressed genes related to steroid and flavonoid biosynthesis at callus stage with wild type as control

Unigene ID	Fold Change	Annotation	KO ID
Flavonoid			
EBG_DN140882_c0_g1	11.19	chalcone synthase	K00660
EBG_DN3637_c0_g1	7.34	flavonol synthase	K05278
EBG_DN1063_c0_g2	5.30	flavonoid 3'-monooxygenase	K05280
EBG_DN10041_c0_g1	4.49	chalcone synthase	K00660
EBG_DN1063_c0_g1	1.67	flavonoid 3'-monooxygenase	K05280
EBG_DN1856_c0_g2	13.07	chalcone isomerase	K01859
EBG_DN779_c0_g1	6.06	naringenin 3-dioxygenase	K00475
EBG_DN2667_c0_g1	4.93	caffeoyl-CoA O-methyltransferase	K00588
Steroid			
EBG_DN10670_c0_g1	8.90	Delta24-sterol reductase	K09828
EBG_DN3985_c0_g2	6.86	Delta7-sterol 5-desaturase	K00227
EBG_DN578_c0_g2	4.32	Plant 4alpha-monomethylsterol monooxygenase	K14424

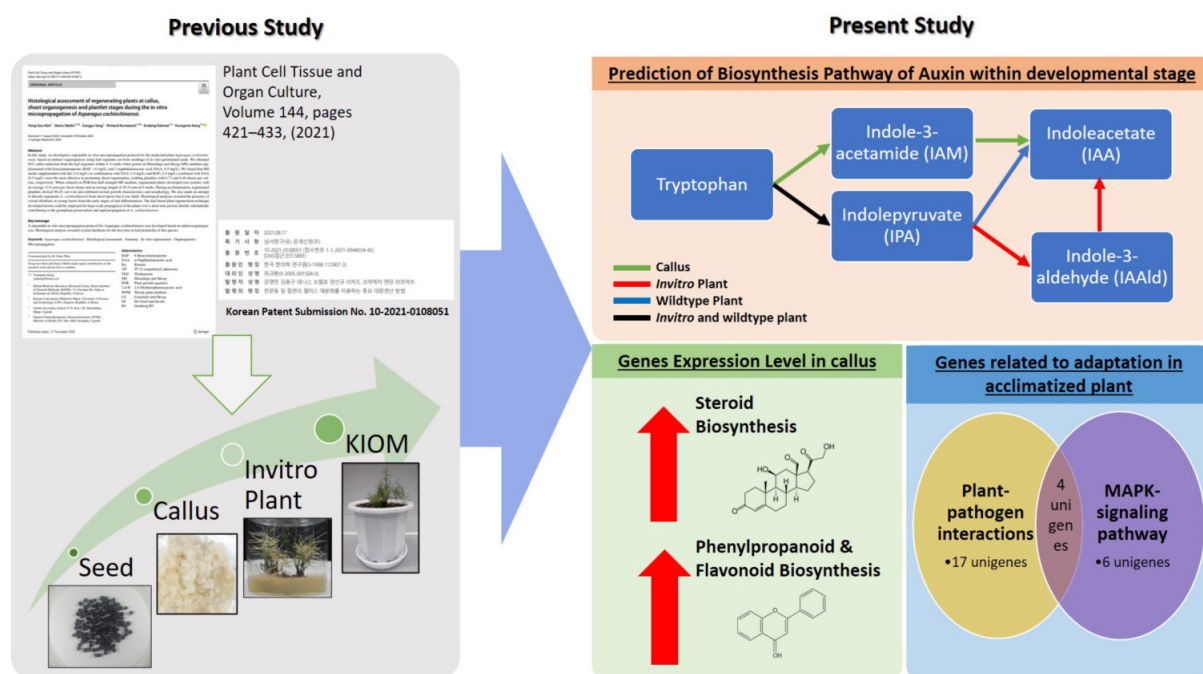


Fig. 8 Summary of *Asparagus cochinchinensis* study in our lab

genes related to the environmental adaptation of acclimatized *A. cochinchinensis* could be important for improving the survival rate during the acclimatization process in the future (Fig. 8).

Author Contributions

A.H.: Conceptualization, data curation, formal analysis, visualization and writing original draft preparation. J.H.L.: qRT-PCR validation, C.L.: formal analysis, software and visualization, R.G.: provide resources and manuscript review and editing, E.R.: provide resources and manuscript review and editing, D.P.: methodology, software, supervision and manuscript review and editing, Y.K.: Conceptualization, funding acquisition, manuscript review and editing.

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