Comparative transcriptome analysis during developmental stages of direct somatic embryogenesis in *Tilia amurensis* Rupr

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*Tilia* species are valuable woody species due to their beautiful shape and role as honey trees. Somatic embryogenesis can be an alternative method for mass propagation of *T. amurensis*. However, the molecular mechanisms of *T. amurensis* somatic embryogenesis are yet to be known. Here, we conducted comparative transcriptional analysis during somatic embryogenesis of *T. amurensis*. RNA-Seq identified 1505 differentially expressed genes, including developmental regulatory genes. Auxin related genes such as *YUC*, *AUX/IAA* and *ARF* and signal transduction pathway related genes including *LEA* and *SERK* were differentially regulated during somatic embryogenesis. Also, B3 domain family (*LEC2, FUS3*), *VAL* and *PKL*, the regulatory transcription factors, were differentially expressed by somatic embryo developmental stages. Our results could provide plausible pathway of signaling somatic embryogenesis of *T. amurensis*, and serve an important resource for further studies in direct somatic embryogenesis in woody plants.

*Tilia* is one of the very valuable species because they are excellent in timber materials and have beautiful tree shape in landscape. Also, there is a high demand for afforestation since they play an important role as honey trees in the summer season. But, the germination rate of *T. amurensis* is very low due to hard seed coats, immaturity of the embryo, and difficulty of penetration of moisture1,2. In addition, *T. amurensis* has multi-year or long-term dormancy type seedlings that take two to three years to germinate. Such low germination rate and time-consuming seedlings types, despite the high demand, make it difficult to propagate and nurture *Tilia* spp. Therefore, it is necessary to study alternative methods for mass propagation of *T. amurensis* in order to supply the seedlings to the honey farm.

Somatic embryogenesis is one of the biotechnological tools which makes somatic embryos (SE), similar in morphology to zygotic embryo. SE is bipolar structure which has both shoot apex and root apex3. SE can be obtained from somatic explants such as leaf, hypocotyl and zygotic embryo. SE has powerful advantages as mass propagation and allows study of morphology, physiology, and molecular mechanisms of embryo development4,5. Also, SE studies can provide insight into cell differentiation, totipotency, and plant regeneration6.

To induce somatic embryo, somatic cells must be switched to embryogenic cells which has totipotency. This process accompanies complex mechanisms such as internal, external stimuli recognition and regulatory networks2. Molecular mechanism of somatic embryogenesis initiation is unclear yet, but it is known that several genes are specially activated or repressed during somatic embryogenesis4,8. In order to induce SE from somatic cells, plant growth regulator (PGR) treatment is generally regarded as requisite. More than 80% of the SE induction protocol uses PGR, and most of them are auxins8. Exogenous auxins trigger auxin-related genes such as *YUCCA*(*YUC*), *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*), *AUXIN RESPONSE FACTOR* (*ARF*) and cause endogenous auxin level changes10. Thus, hormone-responsive genes and signal transduction pathway-related genes need to be considered when designing SE induction protocols.

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genes or transcription factors that regulate hormone biosynthesis and signaling are considered to be representative regulatory genes associated with somatic embryogenesis. Because SE-regulatory network appears to have a high degree of complexity, overall RNA-Seq screening was adopted for transcriptome analysis. Here, we identified the genes that regulate the induction and maturation of *T. amurensis* somatic embryos through RNA-Seq screening, and described their expression patterns during somatic embryo development.

**Results**

**Somatic embryogenesis for RNA-Seq analysis.** To investigate gene expression patterns during somatic embryogenesis, we prepared tissue samples according to the developmental stages. After 8 weeks of culture, somatic embryos were directly induced from the surface of zygotic embryos. To analyze, samples were classified into three stages; C, SE, and D with three biological replicates. Stage C represents control, zygotic embryo, which is extracted from seeds and used as explant (Fig. 1a). Stage SE represents early somatic embryo which is just induced from explants (Fig. 1b). Stage D stands for matured somatic embryo, separated from SE stage and developed on medium without PGR until it looks like the control stage.

**De novo assembly of *Tilia* transcriptome.** We conducted RNA-Seq of the C, SE and D stages with three biological replications and obtained a total of $15 \times 10^6 - 27 \times 10^6$ high-quality reads per sample. In total, 192,666 transcripts and 44,350 unigenes (166,007,076 bp) were generated by Trinity assembler with high quality assembly parameters (GC contents = 42.78% and N50 = 1128 bp). After using CAP3 to merge similar genes, unigene counting reduced to 35,851 and the complete BUSCO coverage was increased from 87.4 to 87.8%.

**Species composition results from BLASTx of *T. amurensis* transcriptome to** (a) *Refseq* plant protein and (b) non-redundant protein databases.

**Identification of DEGs and GO enrichment analysis.** The correlation was measured to investigate the relationship among the biological samples. Biological replicates were closely clustered in correlation heatmap (Supplementary figure S1 and S2). In total, 4592 genes were turned out to be differentially expressed genes
(DEGs) in multiple comparison of C, SE and D stages with a cutoff of fold change > 4 and $p$-value < $1 \times 10^{-5}$. Among them, 1505 DEGs in the sub-clusters where SE stage showed distinctive expression patterns were selected and used for further DEG analysis (Supplementary figure S3).

We annotated DEGs to Transcription Factor/Transcription Regulator (TF/TR) family identifier in order to figure out TF/TR genes present during somatic embryogenesis (Fig. 3). The proportion of TF/TR genes relative to the total DEGs was about 9.83% (148 in 1505). ZINC-FINGER (ZNF) and APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family TFs occupied one quarter of all TF/TRs. The AUX/IAA, GRAS, WRKY, MYB and NAC family each counted for 5% to 7% of identified transcripts.

The total of 75 and 18 unigenes were assigned with at least one gene ontology (GO) terms and Kyoto encyclopedia of genes and genomes (KEGG) pathways, respectively (Fig. 4, Supplementary Table 1). The DEGs were

**Figure 3.** Differentially expressed TF/TR genes and classification of TF/TR families.

**Figure 4.** GO and KEGG enrichment analyses. (a) Assignment of DEGs into the GO categories (b) Clustering of DEGs into KEGG pathways.
related to biological processes, cellular components and molecular functions. Developmental process, nucleus, auxin-activated signaling pathway and transcription regulation categories were significantly enriched. In addition, plant hormone signal transduction in KEGG pathway was enriched most.

Expressions of 75 genes which were assigned with GO terms were divided into four patterns; highly expressed in C, SE, and D stage respectively and gradually increased during embryogenesis (Fig. 5a and Supplementary Table 1). SE-high group included auxin-response protein IAA11 and IAA20, late embryogenesis abundant protein D-113, LRR RECEPTOR-LIKE SERINE/THREONINE-PROTEIN KINASE and ABC TRANSPORTER (Supplementary Table 1). Also, gradually increased group included LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASES, CYCLIN and BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED RECEPTOR KINASE 1 (SERK3). In order to define the relationship of these genes, the networks were constructed using the Cytoscape GeneMANIA app (Fig. 5b,c). Input genes and neighboring genes plugged into each other with co-expression, shared protein domain, physical interaction, predicted and co-localization relationships. The SE-high group network formation focused on the IAA and LATE EMBRYOGENESIS ABUNDANT PROTEIN (LEA) genes. Also, gradually increased group formed one large network, containing kinases such as RECEPTOR-LIKE KINASE (RLK) and LEUCINE-RICH RECEPTOR-LIKE PROTEIN KINASE (FXC).

Figure 5. (a) Expression heatmap of GO assigned genes. Colors represent row-scaled TMM-normalized TPM values. (b, c) Gene functional interaction network by GeneMANIA of SE-high group and Gradually increased group. Black circle, input genes. Gray circle, neighborhood genes.

Gene expression during development stages of somatic embryogenesis. To investigate key elements in somatic embryogenesis signaling pathways, we compared gene expression among stages (Fig. 6). Genes associated with signaling of auxin were highly expressed in SE or showed gradual increase in expression during somatic embryogenesis, whereas most of gibberellin oxides involved in signaling of gibberellic acid (GA) were less expressed in SE and D relative to control. B3 domain containing genes that affect these phytohormone signaling and BRASSINOSTEROID INSENSITIVE-1 ASSOCIATED RECEPTOR KINASE 1 (SERK3) were also increased during somatic embryogenesis. Transcription factor PICKLE (PKL) and VIVIPAROUS1/ABI3-LIKE
which were known to inhibit B3 domain containing genes showed different expression patterns. PKL were expressed high in SE and occasionally in D, whereas VAL1 and VAL2 commonly showed low expression in SE and high expression in D relative to control.

**qRT-PCR conformation of expression levels of DEGs.** To validate RNA-Seq results, we carried out qRT-PCR for fifteen genes from Fig. 6, eight genes that showed high expression in SE samples and eight genes that showed low expression in SE samples. The qRT-PCR measurements showed moderate correlation with the RNA-Seq results when values of all samples were used in analysis individually (r = 0.38, p-value < 0.001, Fig. 7a). Correlation between fold change value of SE and D stage versus control by qPCR and RNA-Seq was much higher (r = 0.65, p-value < 0.001, Fig. 7b). That indicated RNA-Seq results was reliable. Comparison of the expression change patterns for each gene in qPCR and RNA-Seq also supported this (Supplementary figure S4).

**Discussion**

*Tilia amurensis* is one of the honey sources and ornamental tree species in South Korea. The somatic embryogenesis, the way of rapid and efficient propagation for woody plant, is required in *T. amurensis* because of its inefficient reproduction manner. Previous researches on somatic embryogenesis of *T. amurensis* were mainly focused on condition for induction or morphological change^12^, but rarely on the molecular process. It is needed...
to understand molecular mechanism of somatic embryogenesis for improvement of propagation protocols. So far, the large unsequenced genome and heterozygosity have limited functional genomic analyses in *T. amurensis*. Recently, NGS-based transcriptome analyses allow the gene discovery and expression studies in non-model species. Here, we investigated transcriptome profile in order to reveal the key elements that regulate the somatic embryogenesis and to contribute to improve the strategies for *Tilia* in vitro culture. This is the first study reporting transcriptome data in *T. amurensis* and a total of 35,851 unigenes were assembled de novo. We conducted CAP3 assembly following Trinity assembly to merge similar genes, still found that genes have multiple copies as shown in Fig. 6. Provided that *Tilia amurensis* is diploid, it is suggested that the tree species has gene duplications in their genome. Although somatic embryogenesis is usually divided into embryogenic callus and somatic embryo developmental stages in the great majority of studies, we divided it into control (zygotic embryo from seed), SE in globular stage, and developed embryo from isolated SE (Fig. 1). Because, the SE directly emerged from the surface of somatic cell, not going through embryogenic callus stage in *T. amurensis*, which is called ‘direct somatic embryogenesis’. Direct somatic embryogenesis requires profound researches since it has low somaclonal variation rate so that be a desirable approach to obtain somatic embryo identical to parents.

We investigated DEGs to find key genetic factors during somatic embryogenesis. As the result, we identified 1505 DEGs that were considered to be involved in somatic embryo induction and maturation. Then, we performed GO term clustering, network analysis and expression comparison. The series of analyses indicated that transcription regulation, signal transduction and phytohormone signaling were mainly activated during somatic embryogenesis as well as zygotic embryogenesis.

The transcription factors and regulators play important roles in development process. In this study, we identified 148 TF/TR genes that were differentially expressed over somatic embryogenesis (Fig. 3). These TF/TR families were associated with functions in embryogenesis and cell differentiation ZNF, MYB, bHLH, B3 and b-ZIP), meristem maintenance or identity (GRAS and NAC) and hormone signaling (AP2/ERF and AUX/IAA). ZNF family proteins, charging largest portion of TF annotation, are involved in development processes and differentiation. Among them, VAL plays an important role in cell differentiation and is necessary for the development and maintenance of meristems. In the current study, gene VAL showed low expression in SE compared to control as in *Quercus suber*. That indicates VAL negatively controlled the somatic embryogenesis, which supported by a lot of molecular evidences. In addition, B3 domain containing transcription factors are proven to be involved in embryogenesis and induction of somatic embryo. In this study, B3 domain containing genes, such as LEAFY COTYLEDON2 (LEC2), FUS3 and ABI3, generally increased over somatic embryogenesis. The results were consistent with the previous study where *LEC1* and *FUS3* were up-regulated in embryogenic tissue compared to non-embryogenic tissue in *Arabidopsis thaliana*. The result that these TF/TR genes differentially expressed during somatic embryogenesis means the somatic embryogenesis was regulated epigenetically.

The cellular signal transduction associated proteins, such as LEUCINE-RICH REPEAT CONTAINING RECEPTOR-LIKE KINASE (LRR-RLK) and LEA plays important roles during somatic embryogenesis. For instance, SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) has been reported to be expressed specifically during somatic embryogenesis, which allowed it to be a marker for somatic embryogenesis. In this study, B3 domain containing genes, such as LEAFY COTYLEDON2 (LEC2), FUS3 and ABI3, generally increased over somatic embryogenesis. The results were consistent with the previous study where *LEC1* and *FUS3* were up-regulated in embryogenic tissue compared to non-embryogenic tissue in *Arabidopsis thaliana*. The result that these TF/TR genes differentially expressed during somatic embryogenesis means the somatic embryogenesis was regulated epigenetically.

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(Fig. 6, Supplementary Table 1). Moreover, LEA genes were considered to be involved in somatic embryogenesis in cotton, white spruce and sweet orange13-33. In present study, LEA showed high expression in SE stage (Fig. 6, Supplementary Table 1). The results indicate that cellular signal transduction associated genes including SERR and LEA regulated somatic embryogenesis in T. amurensis. Somatic embryogenesis is controlled by hormonal and metabolic signals27. Many genes play the significant roles in somatic embryogenesis actually by regulating plant hormones such as auxin and GA34. Auxin biosynthesis and signaling genes such as YUC, AUX/IAA and ARFs are considered to be required or be an important determinant for somatic embryo induction35,36. YUC expression were restricted to embryogenic tissues in cotton36. Also, AUX/IAA genes were actively expressed in the embryogenic callus compared to non-embryogenic callus in cotton and Catalpa bungei37,38. ARF genes were more abundant during the regeneration process in A. thaliana39, but they are classified as activating and repressing genes40. In this study, these auxin related genes were up-regulated during somatic embryogenesis (Figs. 5b and 6, Supplementary Table 1). On the other hand, GA has been reported to decrease somatic embryo induction, although the mechanism that GA regulates somatic embryogenesis is not clear, yet. For example, gibberellin inhibitors improved embryogenic tissue initiation in carrot and conifer41,42. Also, the expression of GIBBERELLIN2-BETA-DIOXYGENASE6 (GA2ox6), the GA signaling gene, was negatively correlated to production of somatic embryogenesis in A. thaliana43. In this study, GA signaling genes were down-regulated during somatic embryogenesis. The results indicate phytohormone auxin and GA signaling genes had the role during somatic embryogenesis. Therefore, auxin and GA signaling genes significantly affected the induction and maturation of somatic embryo in T. amurensis.

How genes above influence each other and how the genes regulate somatic embryogenesis can be indirectly inferred through the gene network and expressions of them. First, PKL repress the B3 containing genes with the help of the VAL proteins22. Accordingly, the expressions of PKL and VAL gene were predicted to be opposite to that of B3 gene. Lower expression of VAL and higher expression of B3 genes at SE stage than control suggest that VAL genes affected on expression of B3 genes, leading to enhanced somatic embryogenesis. Then, B3 genes are involved in auxin pathway. For instance, LEC1 was found to up-regulate YUC10 in A. thaliana44. And LEC2 up-regulates YUC2, YUC4 and IAA30 in A. thaliana45,46. In this study, auxin related genes were up-regulated at SE and D stages, where B3 genes showed higher expression compared to control (Fig. 6). Various expression patterns of auxin signaling genes during somatic embryogenesis might arise from the different role of the genes; YUCs are involved in biosynthesis and AUX/IAA and ARF in signaling of auxin. Otherwise, that might be related to repression of ARF genes by AUX/IAA genes in auxin signaling pathway. Besides, LEA genes co-express with IAA genes, although it does not suggest regulatory relationship (Fig. 5b)47. Meanwhile, The B3 domain containing genes are involved in GA signaling48. For instance, LEC2 and FUS3 express repression of GIBBERELLIN3-BETA-DIOXYGENASE2 (GA3ox2) and GA3ox149,49. Gibberellin oxidases, which catabolize biologically active GA, were down-regulated during somatic embryogenesis in this study (Fig. 6). This indicates gibberellin oxidases were negatively regulated by B3 genes for induction of somatic embryo.

The up- and down-regulated genes used in Fig. 7 were selected as having a large difference in RNA-Seq TMM value by stage (|z-score|> 2.3 for SE stage). The genes included the genes related to development such as EXTEN1 and higher expression of B3 genes at SE stage than control suggest that of B3 genes. Lower expression of ARFs genes are involved in GA signaling45. For instance, T. amurensis and GA signaling genes had the role during somatic embryogenesis. Therefore, auxin and GA signaling genes significantly affected the induction and maturation of somatic embryo in T. amurensis.

Materials and methods

Plant materials. Somatic embryos were induced from the immature zygotic embryos of T. amurensis which were collected from a tree of clonal seed orchard (established by Korea Forest Seed & Variety Center and located in Hwasung-si, South Korea) in August 2019. Seeds were sterilized in 70% (v/v) ethanol for 1 min followed by disinfecting in 2% (v/v) sodium hypochlorite solution for 8 min, and were rinsed 5 times in sterile distilled water at clean-bench. Somatic embryogenesis procedure was accomplished on MS (Murashige and Skoog) media with 2,4-D 1.0 mg/L following previous reports12. Somatic embryogenesis was induced in the dark room which is controlled temperature in 25 ± 2 ℃ and humidity 40%. After somatic embryos were induced, they were separated from explants and cultured on MS media without any plant regulator. All media were adjusted to pH 5.8 and sterilized for 15 min at 120 ℃. Media were solidified with gelrite 0.3% (w/v) on petri plates (9 cm in diameter).

RNA extraction and sequencing. Total RNA was extracted from C, SE and D using the HiYield Total RNA Mini Kit (Plant) following manufacturer's instruction. The purity of each RNA sample was assessed by Thermo Scientific NanoDrop and Agilent 4200 TapeStation. Library preparation and sequencing were performed using Illumina HiSeq by Macrogen (Seoul, Korea).

Bioinformatic analysis of sequence data. Raw RNA-Seq reads were filtered and trimmed for low-quality regions using PRINSEQ-lite (v0.20.4). Then, with clean reads, de novo assembly of Tilia amurensis embryo transcriptome was conducted using Trinity (v2.8.5). For delicate assembly, annotation using TransDecoder and merging unigenes with CAP3 were followed44. Then genome coverage of assembled unigenes were tested by Benchmarking Universal Single-Copy Orthologs (BUSCO). BLAST search against both Plant Refseq protein and GenBank non-redundant protein sequences were carried out to assign the putative functions to assembled genes using BLASTx program (v2.10.0)45. To identify putative transcription factors and regulators, iTAK online (v1.6) was used43. Reads for each sample were counted using method RSEM48 and DEGs were identified using edgeR57 with the cut-off p-value < 1e-5 and log2FC > 2. Functional annotation by gene ontology (GO) and
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Gene-specific primers (Supplementary Table 3) were designed for the cDNAs sequences with Primer3 (<v0.4.0>, http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized commercially. RNA samples were adjusted to concentration of 30 ng/µl and first-strand cDNA was generated using TOPscript RT DryMIX (Enzynomics). Then qRT-PCR was performed on a Bio-Rad CFX96, using the TOPreal qPCR RX Premix Enzymomics). PCR was carried out as follows: initial denaturing at 95 °C for 10 min, 40cycles consisting of 95 °C for 10 s, 55 °C for 15 s, and 72 °C for 15 s. The reference gene for normalization was ubiquitin gene showing stable expression. The experiments were carried out with three technical repetitions for each sample. Pearson's correlation coefficient of log-scaled qPCR and RNA-Seq values were calculated using R (v3.6.2).

Conclusions

We could reveal the molecular mechanism of somatic embryogenesis by analyzing the transcriptomes and comparing the expression levels. Somatic embryogenesis was triggered by the process that transcription factors, interacting with signaling transduction genes, regulated phytohormones in *Tilia amurensis*. Since there have been only few studies about direct somatic embryogenesis, this study would provide valuable insights of molecular mechanism for direct somatic embryogenesis in woody plant.

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Conceptualization, H.K., S.K., J.P., K.K. and D.S.; methodology, H.K., C.L., S.K. and J.P.; data analysis, H.K., C.L. and D.S.; writing—original draft preparation, H.K. and C.L.; writing—review and editing, S.K., J.P., K.K. and D.S.; data and figure preparation, H.K. and C.L. All authors have read and agreed to the published version of the manuscript.

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