Article

A Comparative Transcriptome Analysis Reveals the Molecular Mechanisms That Underlie Somatic Embryogenesis in Peaonia ostii ‘Fengdan’

Huiting Ci 1,†, Changyue Li 1,†, Theint Thinzar Aung 1, Shunli Wang 1, Chen Yun 1, Fang Wang 1, Xiuxia Ren 1,* and Xiuxin Zhang 1,2,*

1 Key Laboratory of Biology and Genetic Improvement of Horticultural Crops, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Ministry of Agriculture and Rural Affairs, Beijing 100081, China
2 National Agricultural Science and Technology Center, Chengdu 610213, China
* Correspondence: renxiuxia@caas.cn (X.R.); zhangxiuxin@caas.cn (X.Z.); Tel.: +86-010-8210-5944 (X.Z.)
† These authors contributed equally to this work.

Abstract: Low propagation rate is the primary problem that limits industry development of tree peony. In this study, a highly efficient regeneration system for tree peony using somatic embryogenesis (SE) was established. The transcriptomes of zygotic embryo explants (S0), non-embryonic callus (S1), embryonic callus (S2), somatic embryos (S3), and regenerated shoots (S4) were analyzed to determine the regulatory mechanisms that underlie SE in tree peony. The differentially expressed genes (DEGs) were identified in the pairwise comparisons of S1-vs-S2 and S1-vs-S3, respectively. The enriched DEGs were primarily involved in hormone signal transduction, stress response, and the nucleus (epigenetic modifications). The results indicated that cell division, particularly asymmetric cell division, was enhanced in S3. Moreover, the genes implicated in cell fate determination played central roles in S3. Hormone signal pathways work in concert with epigenetic modifications and stress responses to regulate SE. SERK, WOX9, BBM, FUS3, CUC, and WUS were characterized as the molecular markers for tree peony SE. To our knowledge, this is the first study of the SE of tree peony using transcriptome sequencing. These results will improve our understanding of the molecular mechanisms that underly SE in tree peony and will benefit the propagation and genetic engineering of this plant.

Keywords: somatic embryogenesis; tree peony; transcriptome analysis; hormone network; stress response; epigenetic modifications

1. Introduction

Tree peony (Paeonia Sect. Moutan DC.) is an important ornamental plant that is native to China. The plant is also known for its edible and medicinal values. Cortex Moutan is a famous Chinese traditional medicine listed in the Chinese Pharmacopeia [1] that is widely used to treat arthritis, traumatic injury, tumor, and nerve defects [2–5]. Tree peony is also recognized as an important oil plant owing to its rich content of unsaturated fatty acids, particularly α-linolenic acid [6,7]. Currently, the creation of new varieties and the mass production of uniform seedlings is an extremely urgent process owing to the rapid development of tree peony industry. However, it takes more than 10 years to breed a new variety and propagation coefficient of the traditional methods (division and grafting) is relatively low [8], which hinders the breeding process and industrial development.

Plant regeneration via somatic embryogenesis (SE) has substantial potential for mass multiplication, and it has been widely used for commercial plant micropropagation and transgenic plant production [9]. However, the regenerative capacity varies among different genotypes [10,11]. Thus, there is an urgent need to improve this situation.
studies were conducted on the plant regeneration of tree peony that has primarily focused on the following explants, including zygotic embryos, cotyledons, and hypocotyls [10,12,13]. Shoot organogenesis has also been successfully induced from the young leaves of peony [14]. Even though there has been some process in the plant regeneration of tree peony, the process still depends on the available genotypes. Moreover, abnormalities and low efficiency are still serious problems in the regeneration of tree peony plants [10]. Elucidation of the underlying molecular basis will significantly help to solve these problems. However, to date, little research has been reported on the molecular mechanism of plant regeneration in tree peony.

The regenerative capacity of plants is always controlled by the endogenous factors that include developmental stages and reactive oxygen species (ROS) levels, as well as the culture medium and environmental conditions [11,15]. Auxin gradients established by polar transport and biosynthesis in the localized region are vital for the induction of SE [16]. SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK), BABY BOOM (BBM), LEAFY COTYLEDON (LEC), FLUSCA3 (FUS3), CUP SHAPED COTYLEDONS (CUC), and WUSCHEL (WUS) are involved in the induction of SE [17]. The specification of stem cell fate and the construction of shoot apical meristems (SAMs) are vital for plant regeneration [18]. WUS specifies the fate of stem cells and functions as the master of SAM [19]. Cytokinin is the regulator of SAM, and this response is mediated by B-type Arabidopsis response regulators (B-type ARRs) [11,20]. It has been reported that four B-type ARRs (ARR1, ARR2, ARR10, and ARR12) play essential roles in the construction of SAM and shoot regeneration [20,21]. Moreover, epigenetic reprogramming substantially regulates the transition of cell fate, formation of callus, induction of SE, and establishment of SAM [22–26]. An inhibitor of histone deacetylases can induce SE through the de-repression of genes related to SE [27,28]. Histone H3 lysine 27 trimethylation (H3K27me3) modification engages in cross-talk with hormones to regulate the establishment of embryogenic competence in Arabidopsis thaliana [15]. In addition, ROS homeostasis is involved in the de novo initiation of shoots and its closely related to the ability of plants to regenerate [29]. Peroxidases are always enhanced in SE and are used as the markers of SE capacities [30,31].

Even though much process has been made in understanding the underlying mechanisms of SE, our understanding of the molecular networks of SE is still limited, particularly in tree peony. An efficient protocol for shoot regeneration via SE was established in this study using the zygotic embryo as an explant. Based on this protocol, samples in five key stages, including the zygotic embryo explant (S0), callus formation (S1), embryonic callus (S2), somatic embryos (S3), and the regenerated shoots (S4) were collected for analysis by transcription sequencing. The transcriptome dynamics reveal a regulatory network that underlie hormones, chromatin modification, and ROS homeostasis to jointly induce SE. Our results provide a valuable reference for the molecular mechanisms that underly SE.

2. Results
2.1. Morphological and Histological Analysis of the Entire Plant Regeneration Process

An efficient system for callus induction, SE, shoot development, and root induction was established in this study (Figure 1a–h). The results showed that MS media supplemented with 3.0 mg L⁻¹ 6-BA and 1.0 mg L⁻¹ NAA could successfully induce embryonic callus and somatic embryos in tree peony (Peonnia ostii 'Fengdan'), WPM medium supplemented with 1.0 mg L⁻¹ 6-BA and 0.5 mg L⁻¹ GA₃ (SI3) enhanced shoot development from somatic embryos, and WPM medium supplemented with 3.0 mg L⁻¹ 6-BA, 2.0 mg L⁻¹ IBA, and 1.0 mg L⁻¹ acetylsalicylic acid in concert with a 15-day period of cold treatment, accelerated the induction of roots. The callus was initiated after 2–4 weeks of culture (Figure 1b). The induction ratio of non-embryonic callus was 97.92% (Figure 1i). Smooth and light-yellow embryonic calli were obtained after 2 months of culture (Figure 1c). The induction ratio of embryonic callus was 57.88%. The somatic embryos, including different stages from the globular to cotyledonary stages, were formed after 3 months of culture with
a newly constructed SAM (Figure 1d). Subsequently, the somatic embryos were transferred to four types of shoot induction medium for shoot development. Shoots were derived from those embryos and grew large after an additional 1–2 months of culture (Figure 1e–g). The regenerated shoots developed well in the SI3 medium, which resulted in the highest shoot regeneration ratio (98.2%), followed by SI1 with an induction ratio of 75% (Figure 1j). SI4 had the largest number of regenerated shoots per explant (8.75), followed by SI3 medium (6.10) and then SI2 medium (4.50) (Figure 1k). The regenerated shoots were cultured in R1-3 medium for root induction. The roots had regenerated after 2 months of culture (Figure 1h). The root induction ratio was significantly enhanced in R3 medium with an induction ratio of 86.11% (Figure 1l). The number of roots per explant was the highest in R3 medium (3.87) (Figure 1m).

Figure 1. Whole process of plant regeneration via somatic embryogenesis and the related indexes. (a) Zygotic embryo explant (S0), (b) Non-embryonic callus (S1), (c) Embryonic callus (S2), (d) Somatic embryos (S3), (e–g) The regenerated shoots (S4), (h) The regenerated roots, (i) Induction ratio of callus and embryonic callus, (j) Shoot regeneration ratio in different treatments, (k) Number of shoots per explant in different treatments, (l) Root induction ratio in different treatments, (m) Number of roots per explant in different treatments. Scale bar of (a–e) is 500 µm and scale bar of (f–h) is 1 cm. Different lowercase letters in (i–m) indicate significant differences among treatments (Duncan’s test at $p < 0.05$ after analysis of variance). The medium in (a–d) is WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 3.0 mg L$^{-1}$ 6-BA and 1.0 mg L$^{-1}$ NAA; the medium for shoot development in (e–g) is SI1–4, SI1 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 0.5 mg L$^{-1}$ 6-BA + 0.5 mg L$^{-1}$ GA$_3$); SI2 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 0.5 mg L$^{-1}$ 6-BA + 1.0 mg L$^{-1}$ GA$_3$); SI3 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 1.0 mg L$^{-1}$ 6-BA + 0.5 mg L$^{-1}$ GA$_3$); SI4 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 1.0 mg L$^{-1}$ 6-BA + 1.0 mg L$^{-1}$ GA$_3$); and the medium for root induction in (h) is RI1-3, RI1 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 3.0 mg L$^{-1}$ 6-BA + 3.0 mg L$^{-1}$ IBA + 2.0 mg L$^{-1}$ caffeic acid), RI2 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 3.0 mg L$^{-1}$ 6-BA + 3.0 mg L$^{-1}$ IBA + 1.0 mg L$^{-1}$ acetylsalicylic acid), RI3 (WPM + 4.0 mg L$^{-1}$ phytagel + 30 g L$^{-1}$ sucrose + 3.0 mg L$^{-1}$ 6-BA + 2.0 mg L$^{-1}$ IBA + 1.0 mg L$^{-1}$ acetylsalicylic acid).
We observed the morphology of the whole SE process using a stereomicroscope and found that there were four key stages involved in the whole process (Figure 2), including S1, the formation of non-embryonic callus; S2, the transition of cell fate to embryonic cell and the induction of embryonic callus; S3, the formation of somatic embryos and the de novo construction of SAM; and S4, the regenerated shoots from the somatic embryos. Observation by stereomicroscope and SEM showed that the somatic cells acquired pluripotency and formed calli after 2–4 weeks of culture (Figure 2a). These non-embryonic calli were soft, uneven, and white. The cells were disorganized. After 1–2 months of culture, certain sites of the normal callus gradually became smooth, and the cells became better organized (Figure 2b). By this time, the cell fate had changed from normal callus cells to embryonic cells. After 3 months of culture, somatic embryos in different stages were observed, and SAM was established (Figure 2c–e). Cells in the somatic embryos were all highly organized. Their surfaces were very smooth and glossy. After another 1–2 months of culture, the regenerated shoots had developed well in the SI medium (Figure 2f).

![Figure 2. Scanning electron microscope (SEM) observation of shoot regeneration process. (a) Non-embryonic callus (S1), (b) Embryonic callus (S2), (c–e). Somatic embryos (S3) with established SAMs, (f) The regenerated shoots (S4). Green arrows indicated the established SAMs, red arrows marked somatic embryos, and yellow arrow means regenerated shoots.]

2.2. Illumina Sequencing, De Novo Transcriptome Assembly, Functional Annotation, and Classification

Samples including the zygotic embryo explants at S0, the calli or embryos at S1–3, and the regenerated shoots at S4 were collected for transcriptomic analyses. In absence of reference genome, reads were filtered and used for the de novo transcriptome assembly. 32.1 Gb nucleotides were obtained by Illumina sequencing. The resulting transcriptome was 219.1 Mb size. After the removal of ambiguous reads, adapter sequences, and the low-quality reads, a total of 131,496 unigenes were assembled in tree peony with average sequence length of 948 bp, N50 length of 1666 bp, and GC percentage of 40.04% (Table 1).

BUSCO (Benchmarking Universal Single-Copy Orthologs) assessment results showed that the transcriptome was well assembled and the integrity was good (Figure S1a). The distribution of the unigene length is shown in Figure S1b. A total of 131,496 unigenes were identified (Table S1). The number of unigenes decreased as the size of sequences increased. The unigenes with more than 3000 bp nucleosides comprised 4.56%, unigenes greater than 1000 bp comprised 32.39%, and unigenes greater than 500 bp comprised 51.52%. In addition, 46,659 coding sequences (CDS) were predicted. The greatest numbers of CDS were 300–400 bp. The rates of CDS that were larger than 3000 bp, 1000 bp, and 500 bp were
3.41%, 42.99%, and 83.89%, respectively. A comparison of the unigenes was annotated with the homologous sequences of other plant species, and the best match among these nucleotide sequences was *Vitis vinifera* (19.05%) (Figure S1c).

### Table 1. Summary of Illumina transcriptome sequencing for different periods of somatic embryogenesis.

| Sample | Total Number | Total Length (bp) | Mean Length (bp) | N50 | GC (%) |
|--------|--------------|------------------|-----------------|-----|--------|
| S0     | 61,989       | 63,967,148       | 1031            | 1673| 40.83  |
| S1     | 50,923       | 50,805,120       | 997             | 1588| 41.34  |
| S2     | 83,500       | 68,001,019       | 814             | 1417| 40.26  |
| S3     | 57,926       | 56,509,588       | 975             | 1540| 41.23  |
| S4     | 69,093       | 61,369,650       | 888             | 1473| 40.84  |
| All-Unigene | 131,496     | 124,701,759     | 948             | 1666| 40.04  |

About 43.91% of all assembled unigenes were annotated by the NCBI non-redundant protein sequences (NR) database, 37.10% were annotated by NCBI non-redundant nucleotide sequences (NT) database; 31.98% were annotated by Swiss-Prot; 34.29% were annotated by Kyoto Encyclopedia of Genes and Genomes (KEGG); 34.10% were annotated using Eukaryotic Orthologous Groups of proteins/Clusters of Orthologous Groups of proteins (KOG); 23.08% were annotated in the prediction of protein structure domain database (Pfam); and 33.23% were annotated by Gene Ontology (GO) (Table 2). A total of 27,822 unigenes (21.15%) were annotated by all five public databases including NR, Swiss-Prot, KEGG, KOG, and Pfam, and more than 40% of all assembled unigenes were annotated by at least one database (Figure S2). Unigenes from NR database were also annotated by KEGG, Swiss-Prot, Pfam, or KOG with the proportion of 34.07%, 31.80%, 30.04%, 33.92%, respectively. A total of 37,345 (28.40%) were annotated by both KEGG and Swiss-Prot, 33,667 (25.60%) were annotated by both KEGG and Pfam, 39,876 (30.32%) were annotated by both KEGG and KOG, 32,266 (24.54%) were annotated by both Swiss-Prot and Pfam, 37,820 (28.76%) were annotated by both Swiss-Prot and KOG, and 33,858 (25.75%) unigenes were annotated by both Pfam and KOG (Figure S2). In addition, 31.89% genes from NR database were also annotated by NT (Figure S2). A total of 33,793 (25.70%) unigenes were annotated by both NT and Pfam, and 33,891 (25.77%) unigenes were annotated by both NT and Swiss-Prot (Figure S2).

### Table 2. The annotations of tree peony unigenes against the public databases.

| Values       | Total | NR      | NT      | Swiss-Prot | KEGG    | KOG     | Pfam    | GO      | Overall |
|--------------|-------|---------|---------|------------|---------|---------|---------|---------|---------|
| Unigene number | 131,496 | 57,737  | 48,779  | 42,056     | 45,084  | 44,846  | 41,037  | 43,696  | 66,261  |
| Percentage %   | 100%   | 43.91%  | 37.10%  | 31.98%     | 34.29%  | 34.10%  | 31.21%  | 33.23%  | 50.39%  |

KEGG is an important database used for understanding the gene functions of various biological systems. A total of 43,587 unigenes were assigned to KEGG pathways, divided into 5 main categories with 19 subcategories: ‘metabolism’ (61.04%) was dominant, followed by ‘genetic information processing’ (24.11%), ‘environmental information processing’ (6.44%), ‘cellular processes’ (4.57%), and ‘organismal systems’ (3.84%) (Figure 3a). ‘Metabolism’ had 11 sub-categories, among which, ‘global and overview maps’ with 10,033 unigenes (37.71%) accounted for the largest, followed by ‘carbohydrate metabolism’ (17.44%). ‘Genetic information processing’ included 4152 unigenes (39.51%) assigned to ‘translation’, 3118 (29.67%) assigned to ‘folding, sorting and degradation’, 2471 (23.52%) assigned to ‘transcription’, and 767 (7.30%) assigned to ‘replication and repair’. The results indicated that ‘metabolism’ and ‘genetic information processing’ were closely related to SE.
The KOG functional annotation of tree peony unigenes showed that a total of 44,846 unigenes were classified into 25 KOG functional groups, among which the top 8 categories were ‘general function prediction only’ (25.10%), ‘signal transduction mechanisms’ (10.58%), ‘function unknown’ (8.43%), ‘posttranslational modification, protein turnover’ (8.21%), ‘transcription’ (5.84%), ‘translation, ribosomal structure and biogenesis’ (4.21%), ‘carbohydrate transport and metabolism’ (3.91%), and ‘RNA processing and modification’ (3.88%) (Figure 3b). Additionally, 910 unigenes in ‘cytoskeleton’ accounted for 2.03%, 661 unigenes in ‘chromatin structure and dynamics’ occupied 1.42%, and 635 unigenes in ‘cell cycle control, cell division, chromosome partitioning’ took up 1.42%.

Figure 3. Functional analysis of the genes identified. (a) KEGG pathways distribution. (b) Gene Ontology (GO) assignments. (c) KOG functional classification. (d) Transcription factor (TF) categories of tree peony DEGs.
Gene ontology (GO) is used as a convenient tool for gene annotation as well as gene identification. Here, 231,222 unigenes annotated by GO were divided into three main categories: ‘biological process’ (40.03%), ‘cellular component’ (30.00%), and ‘molecular function’ (29.97%). Among the biological processes, ‘cellular process’ (34.82%) and ‘metabolic process’ (26.94%) were dominant, followed by ‘biological regulation’ (8.22%), ‘regulation of biological process’ (7.40%), ‘response to stimulus’ (6.26%), and ‘localization’ (5.34%) (Figure 3c). ‘Cellular process’ included ‘cellular anatomical entity’ (57.55%), ‘intracellular’ (33.17%), and ‘protein-containing complex’ (9.27%). ‘Binding’ (47.46%) and ‘catalytic activity’ (41.29%) were dominant in ‘molecular function’. These results obtained from KEGG, GO, and KOG analysis could be helpful for clarifying the mechanisms underlying SE in tree peony.

Among 1939 transcription factors (TFs), 250 were substantially up-regulated in embryonic callus (S2) and somatic embryos (S3) compared with those of non-embryonic callus (S1), including 56 ARFs, 32 MYB, 18 bHLH, 13 ARR-B, 13 AP2-EREBP, 10 GRAS, 9 NAC, 8 ABJ3VP1, 8 SBP, 8 Trihelix, 6 GRF, 6 OFP, 6 WRKY, 5 G2-like, 4 HSF, 4 bZIP, 4 C2C2-GATA, 4 C2H2, 4 C3H, 4 FRA1, 4 MADS, and 23 other TFs (Figure 3d), indicating that these TFs may be closely related with SE.

2.3. Analysis of DEGs

A total of 31,455, 29,562, 30,372, 20,561, 24,039, 15,734, 18,826, 16,128, and 21,358 DEGs were found in S0-vs.-S2, S0-vs.-S3, S0-vs.-S4, S1-vs.-S0, S1-vs.-S2, S1-vs.-S3, S1-vs.-S4, S2-vs.-S3, S2-vs.-S4, and S3-vs.-S4, respectively (Figure 4a and Table S2). S0-vs.-S2 and S0-vs.-S3 shared 20,978 unigenes, while S0-vs.-S1, S0-vs.-S2, and S0-vs.-S3 shared 16,802 unigenes (Figure S3). The identification of the regulatory pathways of SE was based on the analysis of DEGs in S1-vs.-S2 and S1-vs.-S3. An additional analysis showed that 13,580 and 14,676 unigenes were up-regulated in S1-vs.-S2 and S1-vs.-S3, respectively, while 6981 and 9363 unigenes were down-regulated in these two groups, respectively (Figure 4c). ‘Plant hormone signal transduction’ were primarily enriched in either S1-vs.-S3 or S1-vs.-S2 group (Figures 4d and S4a). The top regulatory pathways in the overlap part of S1-vs.-S2 and S1-vs.-S3 were ‘plant hormone signal transduction,’ ‘carbon metabolism,’ and ‘phenylpropanoid biosynthesis’ (Figure S4c). GO analysis showed that ‘nucleus’ and ‘DNA binding’ were the most enriched in the S1-vs.-S3 group (Figure S4e), while ‘plasma membrane’ and ‘oxidoreductase activity’ were enriched in the S1-vs.-S2 group and in the overlap part (Figure S4b,d). Taken together, 224 DEGs related to SE, cell division, cell fate specification (Table 3), and the regulatory pathways (Table S3), including hormones, epigenetic reprogramming, and stress responses, were identified based on the additional analysis of DEGs.
Figure 4. The number of DEGs in 10 groups and KEGG and GO enrichment analysis of the DEGs in S1-vs-S2 and S1-vs-S3. (a) Number of DEGs in 10 groups. (b) Venn diagram showing overlap between S1-vs-S2 and S1-vs-S3. (c) The number of up-regulated and down-regulated DEGs in S1-vs-S2 and S1-vs-S3. (d) KEGG enrichment of the up-regulated DEGs in S1-vs-S3. (e) GO enrichment of the up-regulated DEGs in S1-vs-S3.

Table 3. Identification of the candidate genes annotated to somatic embryogenesis.

| No.Unigene ID | Gene Name            | Homology Species & GeneBank | CDS Length of Homology Species (bp) |
|---------------|----------------------|-----------------------------|------------------------------------|
| SAM construction and somatic embryogenesis           |                      |                             |                                    |
| 1             | Unigene17677_All     | WUS              | Durio zibethinus, XM_02896431.1 | 1167                              |
| 2             | CL13560.Contig2_All  | KNAT6            | Vitis vinifera, XM_002263277.3   | 957                               |
| 3             | Unigene15414_All     | LET6             | Vitis riparia, XM_034844888.1    | 969                               |
| 4             | Unigene22766_All     | CUC2             | Vitis vinifera, XM_010645872.2   | 1092                              |
| 5             | CL13521.Contig1_All  | CUC2             | Vitis vinifera, XM_010645872.2   | 1167                              |
| 6             | CL12019.Contig1_All  | ATH1             | Vitis riparia, XM_034850593.1    | 6672                              |
| 7             | CL1776.Contig3_All   | ATH1             | Vitis vinifera, XM_010650028.2   | 1773                              |
| 8             | CL14012.Contig2_All  | ATBBI5           | Vitis riparia, XM_034842502.1    | 2538                              |
| 9             | CL1733.Contig3_All   | MAIN             | Papaver somnifera, XM_026565731.1| 1881                              |
| 10            | CL9670.Contig1_All   | LEU              | Vitis riparia, XM_034843924.1    | 2601                              |
| 11            | CL5605.Contig4_All   | REV              | Vitis riparia, XM_034832108.1    | 2535                              |
| 12            | CL5605.Contig5_All   | REV              | Vitis riparia, XM_034832108.1    | 2535                              |
| 13            | Unigene28422_All     | WOX9             | Theobroma cacao, XM_018122711.2  | 1131                              |
| 14            | CL2926.Contig4_All   | WRY2             | Theobroma cacao, XM_034822984.1  | 2241                              |
| 15            | Unigene25839_All     | WOX11            | Theobroma cacao, XM_034822984.1  | 2241                              |
| 16            | Unigene1508_All      | WOX4             | Theobroma cacao, XM_018122711.2  | 1131                              |
| 17            | Unigene5906_All      | BBM              | Theobroma cacao, XM_034822984.1  | 2241                              |
| 18            | Unigene5605_All      | DCAF1            | Theobroma cacao, XM_034822984.1  | 2241                              |

Table 3: Identification of candidate genes annotated to somatic embryogenesis.
Table 3. Cont.

| No.| Unigene ID       | Gene Name               | Gene Name               | Sequence Length (bp) | Homology Species & GeneBank Number | CDS Length of Homology Species (bp) |
|----|------------------|-------------------------|-------------------------|----------------------|-----------------------------------|-----------------------------------|
| 19 | CL1628.Contig3_All| ATML1                   | Homeobox-leucine zipper protein MERISTEM L1 | 2844                 | Vitis vinifera, XM_002266652.3 | 2181                             |
| 20 | CL13336.Contig1_All| SERK2                   | Somatic embryogenesis receptor kinase 2 | 2395                 | Olae europaea var. sylvestris, XM_022994562.1 | 1884                             |
| 21 | CL13336.Contig2_All| SERK1                   | Somatic embryogenesis receptor kinase 1 | 3775                 | Citrus unshiu, AB115767.1 | 1866                             |
| 22 | CL13336.Contig3_All| SERK4                   | Somatic embryogenesis receptor kinase 4-like | 1969                 | Durio zibethinus, XM_002881312.1 | 2049                             |
| 23 | CL4277.Contig6_All| SERK1                   | Somatic embryogenesis receptor kinase 1 | 2465                 | Citrus unshiu, AB115767.1 | 1866                             |
| 24 | CL11415.Contig4_All| AR31                    | B3 domain-containing transcription factor AR31-like | 3013                 | Pistacia vera, XM_031422459.1 | 2751                             |
| 25 | Unigene27385_All | AR31                    | B3 domain-containing transcription factor AR31-like | 2293                 | Pistacia vera, XM_031422459.1 | 2751                             |
| 26 | Unigene66623_All | AR31                    | B3 domain-containing transcription factor AR31-like | 1431                 | Durio zibethinus, XM_022896099.1 | 879                              |
| 27 | CL13462.Contig5_All| SCR                    | SCARECROW                | 2869                 | Carica papaya, XM_022041537.1 | 2367                             |
| 28 | Unigene4284_All  | SCR                    | SCARECROW                | 872                  | Carica papaya, XM_022041537.1 | 2367                             |
| 29 | CL13774.Contig1_All| SHR                  | SHORT-ROOT               | 2515                 | Vitis riparia, XM_034836085.1 | 1485                             |
| 30 | CL5245.Contig6_All| CDC2                   | Cell division control protein 2 homolog C | 1193                 | Prunus avium, XM_019733344.1 | 915                              |
| 31 | CL5245.Contig6_All| CDC2                   | Cell division control protein 6 homolog-like isoform X1 | 1797                 | Tripterygium wilfordii, XM_038827262.1 | 1686                             |
| 32 | CL9293.Contig4_All| CYCA1-1                | Cyclin-A1-1-like         | 1861                 | Actinidia chinensis var. chinensis, NKKQ10000011.1 | 1485                             |
| 33 | Unigene7587_All  | CYCA1-1                | Cyclin-A1-1-like         | 1404                 | Jatropha curcas, XM_01221795.3 | 1467                             |
| 34 | CL2318.Contig2_All| CYCD1-1               | Cyclin-D1-1-like         | 1741                 | Vitis riparia, XM_034818905.1 | 1068                             |
| 35 | Unigene14153_All| CYCD4-2               | Cyclin-D4-2-like         | 2040                 | Camellia sinensis, XM_028263128.1 | 1791                             |
| 36 | CL16644.Contig1_All| MYB3R-4               | Transcription factor MYB3R-4-like isoform X2 | 3910                 | Vitis riparia, XM_019221250.1 | 3483                             |
| 37 | CL9005.Contig2_All| SCC3                   | Sister-chromatid cohesion protein 3 isoform X2 | 3638                 | Vitis riparia, XM_01922189.1 | 2265                             |
| 38 | CL5482.Contig5_All| CDK3                   | Cyclin-dependent kinase C-2 isoform X1 | 1532                 | Vitis riparia, XM_034818851.1 | 1098                             |
| 39 | CL12373.Contig2_All| CYCA3-1               | Cyclin-A3-1              | 1470                 | Vitis riparia, XM_034818851.1 | 1098                             |
| 40 | CL8129.Contig1_All| CYCA3-1               | Cyclin-A3-1              | 1754                 | Vitis riparia, XM_002283116.3 | 1362                             |
| 41 | CL12075.Contig1_All| G2MOR                | G2/mitotic-specific cyclin S13-7 | 2396                 | Vitis riparia, XM_034836315.1 | 1794                             |
| 42 | CL14446.Contig3_All| GRF1                   | Growth-regulating factor 1 | 1822                 | Vitis riparia, XM_034839265.1 | 1092                             |
| 43 | CL14446.Contig3_All| GRF1                   | Growth-regulating factor 1-like | 1574                 | Vitis riparia, XM_034839265.1 | 1092                             |
| 44 | CL11999.Contig3_All| GRF3                   | Growth-regulating factor 3 | 1261                 | Vitis riparia, XM_034839265.1 | 1155                             |
| 45 | Unigene21950_All | GRF3                   | Growth-regulating factor 3 | 1660                 | Vitis riparia, XM_03485923.1 | 1749                             |
| 46 | CL1368.Contig5_All| GRF4                   | Growth-regulating factor 4 isoform X2 | 1775                 | Vitis riparia, XM_034847823.1 | 1578                             |
| 47 | CL13597.Contig2_All| GRF4                   | Growth-regulating factor 3 | 978                  | Thedorea cassa, XM_007052051.2 | 639                              |
| 48 | Unigene8884_All  | YAB1                   | Axial regulator YABBY 1  | 1180                 | Vitis riparia, XM_022825929.4 | 558                              |
| 49 | Unigene5866_All  | YAB1                   | Axial regulator YABBY 5  | 1486                 | Nelumbo nucifera, XM_010250312.2 | 3093                             |
| 50 | CL1629.Contig7_All| RBR                   | Ratinoblastoma-related protein-like | 2766                 | Ricinus communis, XM_002529942.4 | 3063                             |
| 51 | Unigene30474_All| RBR                   | Ratinoblastoma-related protein-like isoform X2 | 1086                 | Vitis riparia, XM_002277501.3 | 1038                             |
| 52 | Unigene18914_All| WIP2                  | Zinc finger protein WIP2 | 1378                 | Hersmania umbratica, XM_01422800.1 | 1035                             |
| 53 | CL9842.Contig2_All| WIP2                  | Zinc finger protein WIP2-like | 8164                 | Vitis riparia, XM_034830903.1 | 8283                             |

2.4. Identification and Analysis of the Profile of Expression of Putative Decisive TFs Associated with SE

Decisive TFs that were annotated to SE, including WUSCHEL-related homebox 9 (WOX9, Unigene28422_All), WOX11 (Unigene25819_All), WRKY transcription factor 2 (WRKY2, CL2926.Contig4_All), WOX4 (Unigene1508_All), SERKs (SERK2, CL13336.Contig1_All; SERK1, CL13336.Contig2_All and CL4277.Contig6_All; SERK4, CL3929, Contig2_All), BBM (Unigene9596_All), AR13 (CL11145.Contig4_All and Unigene27385_All), FLUS3 (Unigene66623_All), DDB1- and CUL4-associated factor homolog 1 (DCAF1, Unigene5205_All), and homeobox-leucine zipper protein MERISTEM L1 (ATML1, Unigene 10553_All and Unigene10553_All), were identified. The levels of expression of WOX9 (Unigene28422_All), WRKY2 (CL2926.Contig4_All), and WOX11 (Unigene25819_All) were notably high in the zygotic embryo explant (S0), but they decreased substantially in S1, increased from S1 to S3, and then decreased in S4 (Figure 5a). The level of expression was the highest in the zygotic embryo explant (S0), followed by the somatic embryos in S3. The level of expression of WOX4 (Unigene1508_All) increased from S0 to S3 and...
decreased in S4 with a significantly higher level in S3 than in the other stages. The levels of expression of three SERKs (SERK2, CL13336.Contig1_All; SERK1, CL13336.Contig2_All; and SERK4, CL3929.Contig2_All) were enhanced in all processes (S1–S4) of SE, particularly in S3. They increased from S0 to S3 and decreased in S4 with a peak in the S3 of SE. The level of expression of another SERK1 (CL4277.Contig6_All) decreased slightly in S1 and S2, increased dramatically in S3, and then decreased in S4 with the highest level in S3. The level of expression of BBM (Unigene9596_All) was notably high in the zygotic embryo explant (S0). There was no expression in S1, and the level of expression increased steadily in S2, climbed dramatically in S3, and then was substantially reduced greatly in S4. The levels of expression of ABI3 (CL11145.Contig4_All and Unigene27385_All) and FUS3 (Unigene66623_All) were the highest in the zygotic embryo explant (S0). They decreased dramatically in S1, increased steadily in S2 and S3, and finally decreased again in S4. The level of expression of DCAFI (Unigene5205_All) was the highest in zygotic embryo explant. It decreased in S1, increased substantially in S2, and then decreased again in the latter two stages. The level of expression of ATML1 (Unigene10553_All and Unigene10553_All) increased from S1 to S3 and then decreased in S4 with its greatest level in S3.

Figure 5. Identification and analysis of the DEGs annotated to somatic embryogenesis. (a) Putative decisive DEGs annotated to SE. (b) Important DEGs involved in cell division and expansion. (c) DEGs related with cell fate specification and shoot apical meristem (SAM) construction.

2.5. Identification and Analysis of the Profiles of Expression of Putative DEGs Annotated to Cell Division, Cell Fate Determination, and SAM Construction in SE

A total of 32 DEGs were annotated to cell division. Three DEGs were involved in asymmetric cell division, including SCARECROW (SCR, CL13462.Contig5_All and Unigene4284_All) and SHORT-ROOT (SHR, CL13774.Contig1_All). The levels of expression were all relatively high in S0, decreased in S1, increased from S1 to S3, and then decreased in S4 (Figure 5b). The level of expression of SCR was the highest in S0, followed by S3, while the level of expression of SHR was the highest in S3. There were 12 DEGs involved in cell cycle division, and cell division control protein (CDC2, CL529.Contig1_All; CDC6, CL5245.Contig6_All), CYCA1-1 (CL2953.Contig4_All), CYCA2-4 (Unigene7857_All), Cyclin-D1-1 (CYCD1-1, CL2318.Contig2_All), CYCD4-2 (Unigene22536_All), MYB3R-1 (CL11664.Contig1_All), sister-chromatid cohesion protein 3 (SCC3, CL3905.Contig3_All), Cyclin-dependent kinase G-2 (CDKG-2, CL5482.Contig5_All), cyclin-A3-1 (CYCA3-1, CL12537.Contig2_All and CL8129.Contig1_All), and G2/mitotic-specific cyclin S13-6 (CCNB1-2, CL12075.Contig1_All). All the DEGs related to cell division were substantially enhanced in S3. Six DEGs were associated with cell expansion, including growth-regulating factor 1 (GRF1, CL14446.Contig3_All and CL4841.Contig1_All), GRF3
Five DEGs were involved in the specification of cell fates, including YABBY1 (YAB1, Unigene8884_All and Unigene5866_All), Retinoblastoma-related protein (RBR, CL1629.Contig7_All, and Unigene30474_All) and Zinc finger protein (WIP2, Unigene18914_All and CL9942.Contig2_All), and NO VEIN (NOV, CL7208.Contig5_All). The level of expression of YAB1 decreased in S1 and increased consistently from S1 to S3 before decreasing in S4 (Figure 5c). The level of expression of one YAB1 (Unigene8884_All) was the highest in S3 of SE, while that of the other YAB1 (Unigene5866_All) was the highest in zygotic embryo explant (S0), followed by the S3 of SE. The level of expression of RBR (CL1629.Contig7_All and Unigene30474_All) also decreased from S0 to S1, increased from S1 to S3, and then decreased in S4 with its highest level in S3 of SE. The level of expression of WIP2 (Unigene18914_All and CL9942.Contig2_All) increased from S0 to S3 and decreased in S4 with a peak in S3 of SE. The level of expression of NOV increased from S0 to S2 and then decreased in the latter two stages with its highest level in S2, followed by the S3 of SE.

Ten DEGs were related to meristem construction, including WUS (Unigene17677_All), homeobox protein knotted-1-like LET6 (Unigene15414_All), Homebox protein knotted-1-like 6 (KNAT6, CL13560.Contig2_All), CUC2 (Unigene22766_All and CL13321.Contig1_All), Homebox protein ATH1 (CL1776.Contig3_All), MAINTENANCE OF MERISTEMS (MAIN, CL1733.Contig3_All), Transcriptional corepressor LEUNIG (LEU, Unigene64502_All and CL9670.Contig1_All), and REVOLUTA (REV, CL5605.Contig5_All). All the DEGs involved in de novo meristem construction were significantly enhanced in the S3 stage (Figure 6c). The levels of expression of WUS (Unigene17677_All), KNAT6 (CL13560.Contig2_All), one CUC2 (CL13321.Contig1_All), ATH1 (CL1776.Contig3_All) and REV (CL5605.Contig5_All) increased from the S0 to S3 stages and decreased in S4 with their highest level in S3. The levels of expression of LET6 (Unigene15414_All) and another CUC2 (Unigene22766_All) were notably high in the zygotic embryo explant (S0), and there was no expression in the normal callus in S1. It increased substantially from S1 to S3 and then decreased in S4 with a peak in S3. The level of expression of LEU (CL9670.Contig1_All) decreased in S1, increased in S2, and decreased in S3 and S4. There was no expression of MAIN (CL1733.Contig3_All) in S1. However, it increased dramatically to its highest level in S2 and then decreased from S2 to S4.

**Figure 6.** Identification and analysis of the DEGs annotated to the synthesis and signal pathways of hormones. (a) Auxin pathway. (b) Cytokinin pathway. (c) Pathways of other hormones, such as abscisic acid (ABA), gibberellic acid (GA), and ethylene.
2.6. Identification and Analysis of the Profiles of Expression of Important DEGs Associated with Hormone Synthesis and the Signaling Pathway

A total of 85 DEGs annotated to hormone pathways including 38 DEGs annotated to the auxin pathway, 25 DEGs annotated to the cytokinin pathway, 10 DEGs annotated to the abscisic acid (ABA) pathway, 2 DEGs annotated to the gibberellin (GA) pathway, and 10 DEGs annotated to the ethylene pathway (Figure 6). Three DEGs were identified that were involved in the synthesis of auxin. The levels of expression of two *indole-3-acetic acid-amido (IAA)* synthetase GH3.10 (Unigene16861_All) and GH3.6 (Unigene10922_All) increased from S0 to S3 and decreased in S4 with a peak in S3 (Figure 6a). The level of expression of *indole-3-pyruvate monooxygenase YUC* (CL9460.Contig1_All) increased dramatically in S1, decreased in S2 and S3, and then increased in S4 with a relatively high level in S1 and S4. Nine DEGs were involved in auxin transporter or distribution. The levels of expression of the auxin efflux carrier component (PINs, CL9657.Contig2_All and Unigene10530_All) and one *BIG GRAIN 1* (BG1, Unigene9560_All) increased from S0 to S3 and decreased in S4 with a peak in S3. The level of expression of the other BG1 (Unigene8559_All) decreased in S1, increased from S1 to S3, and then decreased in S4 with a peak in S3. The level of expression of one auxin transporter-like protein 3 (LAX3, CL2840.Contig1_All) increased from S0 to S2 and decreased in S3–4, and the other LAX3 was highly enhanced in S3 to levels significantly higher than those of the other stages. The level of expression of Co-chaperone protein p23-1 (P23-1, CL9062.Contig2_All) decreased in S1, increased to the highest level in S2, and then decreased in S3–4. There were six auxin receptors, including five AUXIN SIGNALING F-BOX 2 (AFB2) and one TRANSPORT INHIBITOR RESPONSE 1 (TIR1). Four AFB2 (Unigene27194_All, Unigene27196_All, Unigene40406_All, and Unigene72333_All) and TIR1 (CL10648.Contig4_All) were all enhanced in S2. They decreased first in S1, and substantially increased in S2, and then decreased in the latter two stages. The level of expression of another AFB2 (Unigene21382_All) increased from S0 to S3 and decreased in S4 with a peak in S3. There were 22 auxin-responsive proteins (ARFs). Most of these ARFs were enhanced in S3. The level of expression of one ARF4 (CL11425.Contig2_All) and ARF5 (CL565.Contig2_All) increased from S0 to S3 and then decreased in S4 with a peak in S3. The levels of expression of ARF3 (CL8431.Contig9_All), ARF2 (Unigene29603_All), ARF2B (CL7022.Contig2_All), two ARF4 (Unigene7938_All and CL3267.Contig2_All), and ARF9 (CL2228.Contig4_All) decreased in S1, increased from S1 to S3, and then decreased in S4. The level of expression of one ARF18 (Unigene377_All) increased in S1, decreased in S2 and S3, and then increased in S4 with its highest level in S1, while the level of expression of the other ARF18 (Unigene378_All) decreased slightly in S1, increased from S1 to S3, and then decreased in S4. Moreover, the level of expression of all IAAs was enhanced in S3 with a significantly higher level than that of the other stages. Three auxin-induced proteins were identified. The levels of expression of AUX6b (Unigene27633_All), AUX15 (Unigene6177_All), and AUX22B (CL14076.Contig2_All) were the highest in S3 with a similar trend.

Seven DEGs for cytokinin synthesis were identified. The level of expression of tRNA *dimethylallyltransferase 2* (IPT2, CL3091.Contig2_All) was enhanced in S1, decreased in S2 and S3, and then increased in S4 with a significantly higher level in S1 than that of the other stages (Figure 6b). The level of expression of *adenylate isopentenyltransferase 5 (IPT5)*, Unigene8674_All increased from S0 to S2, decreased in S3, and then increased in S4 with a peak in S2. The level of expression of *cytokinin riboside 5′-monophosphate phosphoribohydrolase (LOG1)*, Unigene10839_All was also the highest in S2. The levels of expression of one *LOG* (Unigene2390_All) and LOG3 (CL11378.Contig1_All) increased from S0 to S3 and decreased in S3 with a peak in S3. The level of expression of LOG5 (CL13131.Contig1_All) decreased in S1, increased from S1 to S3, and then decreased in S4 with a peak in S3. The level of expression of the other *LOG* (Unigene5319_All) increased from S0 to S3, decreased in S2, and increased to its highest level in S3. A total of 14 cytokinin signal transduction genes were identified. The levels of expression of two *histidine kinase 3* (AHK3, CL2238.Contig4_All), *AHP2* (CL11912.Contig2_All), two-component response
regulator (ARR1, CL12697.Contig2_All), and PRR37 (CL14817.Contig17_All) decreased in S1, increased dramatically in S2, and then decreased in the latter two stages with a peak in S2. The levels of expression of APROP2 (CL660.Contig4_All), APROP7 (CL10511.Contig4_All), and one AHK4 (Unigene39624_All) increased from S0 to S2 and decreased in S3 and S4 with their highest levels in S2, followed by S3. The levels of expression of the other AHK4 (CL15136.Contig2_All), APROP1 (Unigene13676_All), and ARR12 (CL5885.Contig2_All) increased from S0 to S3 and decreased in S4 with a peak in S3. The levels of expression of APROP1 (CL660.Contig3_All) was also the most enhanced in S3. The level of expression of another histidine-containing phosphotransfer protein 1 (AHP1, CL1552.Contig1_All) increased substantially in S1 and decreased in the following three stages with the highest level in S1. The levels of expression of ORR21 (CL3407.Contig3_All), ARR9 (CL5520.Contig1_All), and one purine permease 1 (PUP1, CL6432.Contig1_All) were first enhanced in S1, then reduced in S2 and S3, and increased in S4 with a relatively high level in S1 and S4.

Seven DEGs were related to ethylene synthesis, including, ACO4 (CL240.Contig1_All), ACO (CL6909.Contig1_All), and PIF4 (CL6123.Contig5_All). The levels of expression of 1-aminocyclopropane-1-carboxylate oxidase 1 (ACO1, CL3712.Contig4_All) and ACO4 (CL240.Contig1_All) were enhanced in S1; ACO (CL6909.Contig2_All) were enhanced in S2, while PIF4 (CL6123.Contig5_All) was enhanced in S3 of SE (Figure 6c). Three DEGs were involved in signal pathway of ethylene. The levels of expression of ethylene-responsive transcription factors (ERF, Unigene11940_All) and ethylene-insensitive protein3 (EIN3, CL2223.Contig3_All) were enhanced in S1, and the other ERF (CL9359.Contig3_All) was enhanced in S2 of SE. Therefore, most DEGs that annotated to the ethylene synthesis and signaling pathway were enhanced during the early stages (S1-S2) of SE. Moreover, two DEGs annotated to gibberellin receptor GID1c (CL1374.Contig1_All and CL1913.Contig1_All) were identified, and their levels of expression were highly enhanced during the whole process of SE (Figure 7a). Ten DEGs were annotated to ABA pathway. The levels of expression of DEAD-box ATP-dependent RNA helicase 3 (RH3, CL86.Contig2_All) decreased first and then increased with a relatively high level in S0 and S2–4 (Figure 7b). The levels of expression of ABSCISIC ACID-INSENSITIVE 5-like proteins (DPBF3, CL3285.Contig3_All) and one abscisic acid receptor PYR1 (Unigene11665_All) shared a similar trend with a peak in S3. ABF4 (CL4510.Contig1_All) and abscisic acid receptor PYL3 (Unigene6464_All) were enhanced in S2. The levels of expression of PYL4 (Unigene1227_All and Unigene24912_All) and another ABF4 (CL11123.Contig2_All) were enhanced in S1 and S4. The levels of expression of RNA-binding protein ARP1 (CL12028.Contig5_All) was the highest in S4, while that of PYL9 (Unigene1617_All) was much higher in both S3 and S4. Thus, the levels of expression of the DEGs that annotated to the ABA synthesis and signaling pathway were enhanced during the developmental process of somatic embryos.
2.7. Analysis of the Profiles of Expression of Important DEGs Annotated to Epigenetic Modifications

A total of 27 DEGs were classified to epigenetic reprogramming. Most of epigenetic reprogramming DEGs, including SYD (CL10174.Contig2_All), HAT1 (Unigene1152_All), HAT3.1 (CL5455.Contig2_All), TOP2 (CL11525.Contig2_All), TOP68 (CL3272.Contig2_All), MCM2 (Unigene27426_All), PKL (CL2977.Contig5_All), ASHRI (Unigene26959_All and Unigene26961_All; ATX2, CL11554.Contig2_All; ATXR6, Unigene75853_All; ATXR7, CL4760.Contig1_All; Contig1_All; CLF, CL14179.Contig1_All; SUVH4, CL3208.Contig6_All, Unigene16769_All, and Unigene5039_All), JMJD7 (CL1008_All), DDM1 (Unigene16899_All), HAM1 (CL9658.Contig5_All), and one HAC1 (CL3517.Contig5_All) were enhanced in S3 of SE process (Figure 7a). The levels of expression of the other two HAC1 (Unigene10525_All and Unigene19127_All) increased from S0 to S2 and then decreased in S3 and S4 with a peak in S2. The levels of expression of the negative regulators JMJD4 (Unigene10554_All), BRM (CL12991.Contig1_All) and HDA9 (CL9589.Contig4_All) were substantially greatly reduced in S3.

2.8. Identification and Analysis of the Profiles of Important DEGs Associated with Stress Responses

A total of 32 DEGs were annotated to antioxidant molecules and enzymes, including one D-galacturionate reductase (GALUR, CL1193.Contig2_All), one L-galactose dehydrogenase (LGALDH, Unigene5397_All), two monodehydroascorbate reductase (MDAR5, CL10437.Contig1_All and CL4597.Contig1_All), four L-ascorbate oxidase (AAO, CL12764.Contig1_All, Unigene27570_All, CL15166.Contig2_All, and Unigene10841_All), one methylsterol monooxygenase 1-1 (SMO1-1, Unigene25370_All), one cationic peroxidase 1 (PNC1, CL2502.Contig3_All and Unigene28418_All), 16 peroxidases (PER11, Unigene24833_All; PER12, CL10036.Contig1_All; PER13, Unigene25803_All; PER17, Unigene19698_All; PER2, CL15223.Contig2_All; PER20, CL1309.Contig2_All; PER21, Unigene14786_All; PER3, Unigene73031_All; PER4, CL9105.Contig1_All and Unigene886_All; PER42, Unigene25358_All; PER43, CL12453.Contig2_All; PER45, Unigene77066_All; PER47, Unigene22864_All; PER64, CL11055.Contig1_All; PER73, Unigene28208_All), one peroxidise P7 (PERP7, Unigene7518_All), two glutathione peroxidase 2 (GPX2, CL2142.Contig2_All; GPX5, CL14121.Contig2_All), one transmembrane

![Heatmaps](image-url)
ascorbate ferrireductase 1 (CYB561A, CL15597.Contig2_All), and one Thioredoxin M3 (GAT1, Unigene25004_All). A total of 16 DEGs annotated to stress responses were enhanced in S1, and 15 DEGs were the highest in S2 and S3 with a trend of first increasing and then decreasing (Figure 7b).

2.9. Validation of the Expression of Important Candidate DEGs by qRT-PCR

To confirm the accuracy of the high-throughput sequencing results, nine important DEGs involved in SE were analyzed by qRT-PCR. The results showed that all nine DEGs were generally consistent with the RNA-Seq datasets. The levels of expression of BBM, FUS3, WRKY2, WOX9, and WOX11 were the highest in the zygotic embryo explant (S0), decreased substantially in S1 and gradually increased from S1 to S3, with the highest level in S3 of the SE. The levels of expression of SERK, CUC, WUS, and WOX4 increased from S0 to S3 with a significantly higher level in S3 than in the other stages (Figure 8).

Figure 8. Validation of the expression of nine important candidate DEGs by qRT-PCR. Different lowercase letters indicate significant differences among different stages (Duncan’s test at *p* < 0.05 after analysis of variance). The broken line represents the expression patterns detected by transcriptome analysis. There were three SERKs and two CUC detected by transcriptome analysis. The patterns of expression were shown in different colors.
3. Discussion

Plant regeneration via SE could highly increase the speed of propagation and thereby shorten the breeding period. Plant regeneration from tree peony zygotic embryos has been studied in recent decades, and some progress has been made. However, the regenerative capacity was very low and depended substantially on the genotypes [13]. The inner molecular mechanisms were still unclear. In this study, we established an effective protocol for a plant regeneration system in tree peony via SE using the zygotic embryo as the explant. Based on this efficient plant regeneration system, we analyzed the molecular dynamics during SE. The important genes and probable pathways that regulate shoot regeneration via SE were identified in tree peony. KEGG and GO analyses demonstrated that the DEGs annotated to hormone pathways, particularly the cytokinin and auxin pathways; epigenetic modifications, stress responses, cell division, cell fate determination, meristem construction, and SE-specific genes were significantly enriched, which is consistent with previous studies [32,33] indicating that SE and the enhanced regenerative capacity are associated with the related processes described above. Accordingly, the most frequently represented TFs during tree peony somatic embryogenesis were ARFs, MYB, bHLH, ARR-B, AP2-EREBP, NAC, GRAS, ABI3VP1, WRKY, and bZIP, which were substantially up-regulated in embryonic callus (S2) and somatic embryos (S3) compared with that of the non-embryonic callus (S1), indicating that these TFs are closely related to embryonic transition and somatic embryo formation. Similar TFs have also been detected in maize through transcriptome analysis [34]. ARFs are important auxin response factors, and B-type ARRs are important cytokinin response regulators. Both play vital roles in SE [19,35,36] and demonstrate the important roles of auxin and cytokinin in the regulation of SE. It has also been reported that auxin and stress often work in concert to modulate SE [37,38]. MYB, bHLH, NAC (including CUC2), WRKY, bZIP, GRAS, and ABI3VP1 (such as ABI3, FUS3, LEC2) are involved in stress responses and may crosstalk with hormones to regulate SE [34,39–42]. In addition, hormones, particularly auxin and cytokinin, could alter epigenetic modifications and induce SE [9]. Therefore, hormones, particularly auxin and cytokinin, could highly function in concert with stress responses and epigenetic modifications to control the induction of SE and development of somatic embryos. Based on the expression patterns in our study and according to the prior research [43–47], we proposed a model of a molecular regulatory network during tree peony SE (Figure 9).

Figure 9. A model of a molecular regulatory network during tree peony somatic embryogenesis. Black arrows indicate positive regulation. Black lines without arrows mean either positive or negative regulation. Genes marked red related to epigenetic modification accelerate somatic embryogenesis, while genes marked in blue inhibit somatic embryogenesis.
3.1. Establishment of an Efficient Plant Regeneration System via SE in Tree Peony

SE is a powerful tool that is widely used for commercial plant propagation and breeding by genetic engineering. This study showed that MS medium supplemented with 3.0 mg L\(^{-1}\) 6-BA and 1.0 mg L\(^{-1}\) NAA could successfully induce embryonic callus and SE in tree peony. WPM medium supplemented with 1.0 mg L\(^{-1}\) 6-BA and 0.5 mg L\(^{-1}\) GA\(_3\) (SI3) enhanced the development of shoots from somatic embryos, and WPM medium supplemented with 3.0 mg L\(^{-1}\) BA, 2.0 mg L\(^{-1}\) IBA, and 1.0 mg L\(^{-1}\) acetylsalicylic acid in concert with a 15-day period of cold treatment accelerated the induction of roots. The whole success induction ratio from S0 to S4 was 55.56%. Progress has also been made on plant regeneration from zygotic embryo explants of tree peony in our previous research and that of Du et al. (2020) with an embryo induction ratio of 48% [10,12]. Therefore, the induction of embryonic callus and the development of somatic embryos was much enhanced in this study compared with the previous studies. Based on the established plant regeneration system, the underlying molecular mechanisms of SE were further analyzed in more detail in tree peony using transcriptome sequencing techniques.

3.2. Analysis of the Putative Decisive TFs Annotated to SE

WOX9 is important in the initiation of SE, and its overexpression leads to an increase in embryogenic capacity in Medicago truncatula [48]. WRKY2 regulates the patterns of division of the basal cells during the early stage of embryogenesis by activating the expression of WOX8 and WOX9 [44]. WOX11 promotes cell fate specification during embryogenesis [49]. WOX4 accelerates embryo development and germination during SE in grape [50]. In this study, the levels of expression of WOX9, WRKY2, and WOX11 were the highest in zygotic embryo explants (S0). They were reduced in S1 and highly enhanced in the somatic embryos (S3). WOX9 is also highly expressed in the somatic embryos of Norway spruce (Picea abies) and M. truncatula [51,52]. The level of expression of WOX4 was enhanced in all stages (S1–S4) of SE, and it was the highest in somatic embryos (S3). Similar results were observed in V. vinifera [50]. SERK plays a key role in embryogenic competence acquisition and SE [53]. The level of expression of SERK was enhanced in all processes (S1–S4) of SE, particularly in the somatic embryo formation (S3). SERK1 and SERK2 are highly expressed during embryogenic formation and the developmental stages in A. thaliana and Oryza sativa, respectively [54,55]. The LEC1-ABSCISIC ACID INSENSITIVE 3 (ABI3)-FUS3-LEC2 (LAFL) complex and BBM are master regulators of SE, and BBM activates the LAFL network [56]. The ectopic expression of BBM triggers a conversion from vegetative to embryonic growth [57]. In this study, high expressions of BBM, AB13 and FUS3 were identified in zygotic embryo explants (S0) and in somatic embryos (S3). In conifers, the levels of expression of SERK and BBM increase during the later development of SE [58]. DCAF1 is essential for plant embryogenesis [39], while ATML1 plays an important role in embryonic pattern formation [60]. The levels of expression of DCAF1 were enhanced in embryonic callus (S2), while the level of expression of ATML1 was the highest in somatic embryo (S3). The high levels of expression of the above DEGs in the somatic embryos (S3) indicates their decisive roles in SE.

3.3. Identification and Analysis of the Profiles of Expression of Putative DEGs Annotated to Cell Division, Cell Fate Determination and De Novo Meristem Construction Involved in the Process of SE

Plant regeneration is accompanied by the establishment of new stem cells and meristems, which often require the reactivation of potential cell division [61]. Asymmetric cell divisions are central to the establishment of SAM and root apical meristem (RAM), determination of cell fate, and development of embryos [62]. In embryogenesis, asymmetric cell division forms a spherical proembryo, and the SAM is generated from the four most apical cells [63]. SCR and SHR are involved in asymmetric cell division [64,65]. We found that SCR and SHR were enhanced in the somatic embryo formation process (S3). Thus, all the DEGs related to cell division and expansion were significantly enhanced in this
stage. Similar results were found in alfalfa (*Medicago sativa*) [66]. These results showed that there were active asymmetric divisions and normal cell division during somatic embryo formation (S3), which substantially contributes to the establishment of SAM.

The de novo construction of SAM is essential for shoot regeneration by either organogenesis or SE, and the acquisition of stem cell identity is extremely important for the de novo construction of SAM [18]. SAM is always established during somatic embryo formation in embryogenesis [63]. *YABs* are involved in the determination of abaxial cell fate and regulate the initiation of embryonic SAM development during embryogenesis [45]. *RBR* plays a central role in controlling the cell fate establishment and meristem cell differentiation during the process of plant regeneration [67,68]. *NOV* is essential for the determination of cell fate during embryogenesis [69], and *WIP2* is involved in determination of stem cell fate within embryonic meristems [46,70]. In this study, the levels of expression of *YABs*, *RBR*, and *WIP2* were enhanced in somatic embryo formation process (S3), and the level of expression of *NOV* was the highest in embryonic callus (S2). *WUS*, a transcription factor expressed in the organizing center of SAM, determines the fate of stem cells. It interacts with *CLV* to produce a *WUS/CLV* self-regulatory loop that is critical for the maintenance of stem cell identity in SAM during SE [71]. *Class I KNOX* genes, including *KNAT1* and *KNAT6*, are required for the initiation and maintenance of SAM during embryonic development [72,73]. *CUC1* and *CUC2* are involved in regulating the formation of SAM during embryogenesis by activating *STM* and *KNAT6* [72]. Other important TFs, including *ATH1*, *ATHBs*, *REV*, *MAIN*, and *LEU*, are also involved in the regulation of the initiation, maintenance, and development of SAM [74–77]. In this study, the levels of expression of *WUS*, *KNAT6*, *CUC2*, *ATH1*, *ATHB15*, and *REV* were all substantially enhanced in the somatic embryos (S3). The level of expression of *MAIN* was the highest in embryonic callus (S2), followed by somatic embryos (S3). One *LEU* (Unigene64502_All) was expressed at the highest level in somatic embryos (S3), while the other *LEU* (CL9670.Contig1_All) was enhanced in embryonic callus (S2). Similarly, a high level of expression of *WUS* was also detected in the embryonic callus and somatic embryos in *A. thaliana* [47]. The levels of expression of *KNAT6* and *CUC* are elevated during embryogenesis, so that SAM is established [72]. Therefore, most of the DEGs related to the construction of SAM were enhanced in somatic embryo formation process (S3), proving that SAM was established during the process of somatic embryo formation (S3), and the DEGs described were important participants in this process. In addition, the trends of expression of nine decisive DEGs in SE detected by qRT-PCR were generally consistent with the RNA-Seq datasets. Therefore, the most decisive and most frequently represented genes, including *SERK*, *WOX9*, *WOX11*, *WOX4*, *WRKY2*, *BBM*, *FLS3*, *CUC*, and *WUS*, were characterized as the molecular markers for tree peony SE.

3.4. Identification and Analysis of the Profiles of Expression of the Important DEGs Annotated to Hormone Pathways and Their Roles in the Regulation of SE

Plant growth regulators play vital roles in SE [78]. Auxin promotes the acquisition of cell totipotency and induces SE by altering the accessibility of chromatin [15]. Auxin biosynthesis maintains embryo identity and growth during SE [79]. We found that *YUC* was enhanced in S1 and S4, while the IAA synthetase *GHs* were increased in S3 of the SE process, indicating the important role of auxin synthesis in the induction of callus, the construction of SAM, and the formation and development of embryos in tree peony. The establishment of auxin gradients correlates with the expression of *WUS* and activates the polar localization of *PIN1*, which gives rise to the formation of SAM during SE [47,80]. *PINs*, *LAXs*, and *BG1* regulate polar auxin transport [81] and play a regulatory role in SE [82]. In this study, the levels of expression of *PINs* and *BG1* were enhanced in S3. The levels of expression of two *LAX3* were enhanced in S2 and S3, respectively. The *PIN1* is substantially enhanced during the specific place of *A. thaliana*, which is critical for the regulation of WUS during SE [47]. The results showed that polar auxin transport genes function concomitantly with the auxin synthesis genes to regulate the establishment of auxin gradients in stages S2–S3 of SE in tree peony. Auxin synthesis, transport, and signal pathway genes are also
up-regulated and have been identified in maize (Zea mays) during SE [83]. AFB2, one auxin receptor, is involved in auxin-regulated embryogenesis [84]. The levels of expression of AFB2 and the other DEGs associated with auxin receptors were enhanced in the embryonic callus (S2), indicating that a receptor perceived auxin as a signal during this stage. ARFs and IAA s play key roles in regulating auxin-responsive transcription [85]. In Arabidopsis, multiple arf mutants displayed SE defects [35,36]. IAA9 has been shown to be upregulated during the initiation of SE [86]. Most of the DEGs of ARFs and all those of the IAA s were enhanced in somatic embryos (S3), which was similar to the results observed in rubber tree (Hevea brasiliensis) [87], proving again that auxin has a critical role in the regulation of SE.

The determination of cell fate and the de novo construction of SAM are primarily regulated by auxin and cytokinin [88,89]. It has been reported that a deficiency of cytokinin reduced the size and activity of SAM [90]. In this study, cytokinin synthesis IPT2 was enhanced in S1; IPT5 and one LOG1 were enhanced in S2; while the other four LOGs were enhanced in S3, indicating that cytokinin plays important roles in the process of SE. AHKs are cytokinin receptors, and AHPs act as signaling shuttles between the nucleus and cytoplasm [21]. The cytokinin response is mediated by B-type ARRs as positive regulators in the two-component cytokinin signaling pathway, whereas type-A ARRs function as negative regulators of the downstream responses [91]. In A. thaliana, B-type ARRs (ARR1, ARR2, ARR10, and ARR12) play essential roles in the de novo construction of SAM [19]. In addition, cytokinin can modulate the accessibility of chromatin and the expression of WUS through the B-type ARRs [11,21]. The genes involved in cytokinin biosynthesis and signal transduction pathways increase remarkably in expression during SE in cotton (Gossypium spp.) [92]. The levels of expression of ARRs are enhanced during the process of SAM establishment and maintenance in A. thaliana [21]. The levels of expression of the DEGs annotated to cytokinin signaling were also elevated during the process of SE. The level of expression of AHP1 was the highest in S1. AHK3, ARR1, APRR2, APRR7, and PRR37 were expressed at their highest levels in S2. APRR1 and ARR12 were the most enhanced in S3, which indicates the important roles of these cytokinin signaling genes in the SE of tree peony.

Ethylene, GA, and ABA are also involved in the induction or development of the somatic embryos [78]. Ethylene modulates the induction of somatic embryos and the development of globular embryos [93], which is markedly connected to hormonal crosstalk (with auxin and cytokinin in particular), as well as stress responses [94]. Most of the DEGs that were annotated to the ethylene synthesis and signaling pathway were enhanced during the early stages (S1–S2) of SE, indicating their roles in the induction of SE. GA3 enhances the level of expression of a KNOTTED-like homeobox gene (KNOX) and stimulates the formation and germination of somatic embryos [95]. We also found that the level of expression of GLD1c was substantially enhanced during the whole process of SE. ABA induces SE and is essential for the acquisition of embryogenic competence [96,97]. In this study, the ABA biosynthetic gene was expressed at its highest levels in S2 with a relatively high level in S2–S4. Thus, the levels of expression of DEGs annotated to the ABA signal pathway were enhanced in the late stages of SE. ABA biosynthesis, receptors and signaling response genes were also up-regulated during the SE of A. thaliana [98]. Therefore, ethylene, GA, and ABA also play important roles in the induction and development of SE in tree peony.

3.5. Identification and Analysis of the Profiles of Expression of the Important DEGs Annotated to the Regulation of Epigenetic Modifications and Their Roles in the Regulation of SE

Epigenetic modifications include DNA methylation and histone modifications, such as acetylation, methylation, and phosphorylation, that orchestrate the structure and accessibility of chromatin and regulate global reprogramming of the cell transcriptome [99]. In recent years, epigenetic modifications have emerged as critical factors for the control of the transition of cell fate, callus formation, and SE through extensive transcriptional reprogramming [15,100]. DNA methylation is one of the most studied epigenetic mechanisms owning to its essential role in gene expression and SE [101]. The balance between
hypermethylation and hypomethylation is key to the success of SE [102,103]. DDM1 is required to maintain the DNA methylation status and promote chromatin remodeling [104]. The level of expression of DDM1 was up-regulated during SE, particularly in S3.

Histone modification is one of the most important epigenetic modifications, and it plays a key role in the regulation of gene expression [105]. Histone acetyltransferases (HATs) promote the open state of chromatin [106], while histone deacetylases (HDACs) trigger the condensation of chromatin [107]. These two antagonistically acting enzymes work in concert to control SE by regulating gene expression [99,108]. Histone deacetylase inhibitors have been reported to accelerate the expression of embryogenesis-related genes and increase embryogenic potential [109]. Histone acetyltransferase1 (HAC1) acetylates histones, thus providing a specific tag for transcriptional activation and promoting the expression of WUS and SAM organization [110]. Histone lysine methyltransferases (HKMTs) are extremely important enzymes that modify chromatins, which transcriptionally activate or repress genes by regulating their chromosomal states [111]. H3K4, H3K36, and H3K79 methylations are involved in active transcription, while H3K9, H4K20, and H3K27 methylations are associated with gene silencing [112]. Class III HKMTs, including ATX1-5, regulate the establishment of shoot identity via H3K4 trimethylation and demethylation, while ATXR7 may accelerate the demethylation of H3K36 [25]. Moreover, SPLAYED (SYD), a catalytic component of the chromatin structure-remodeling complex, controls the fate of stem cells via the regulation of promoter of WUS in the SAM [113]. The levels of expression of histone modification genes are highly elevated in the SE of rubber tree (Hevea brasiliensis) [87]. This study also showed that most of the epigenetic genes (78.6%) were highly enhanced in S3 of the SE process. The level of expression of HAC1 peaked in S2, while the level of expression of negatively regulated genes, including JM14, BRM, and HDA9, were highly reduced in S3, indicating that epigenetic modifications, particularly those involved in histone modifications, played crucial roles in the regulation of SE.

Moreover, it has been widely reported that hormones, particularly auxin and cytokinin, could modify the levels of epigenetic modifications, which causes an extensive reprogramming of the transcriptome and finally contributes to SE [9]. Exogenous hormones such as auxin can modify the levels of DNA methylation in embryogenic cells, which regulates the expression of genes involved in SE, such as BBM1, WUS, and LEC [78]. The removal of H3K27me3 at the WUS locus is a prerequisite for its induction by the cytokinin response factors B-type ARRs [19]. In this study, the results also showed that the expression of part of the DEGs involved in auxin and cytokinin pathways share similar trends with epigenetic modification genes, and part of them peaked earlier than the genes for epigenetic modification, indicating that the effect of auxin and cytokinin precedes epigenetic modification or functions at the same time.

3.6. Analysis of the Important DEGs Annotated to Stress Responses and Their Relationship with SE

The ROS produced during stress condition is known to function as a signal that regulates plant growth and development [114]. Moreover, stress induces the remodeling of fate of plant cells and induces SE in concert with hormone-regulated pathways [115]. It has been reported that cellular stress conditions prompt vegetative cells to acquire embryogenic competence through cellular reprogramming [53]. A high content of catalase and ascorbate peroxidase has a stimulatory effect on SE [30]. Cationic peroxidase is required for SE in asparagus (Asparagus officinalis) [116]. The activities of peroxidase are always substantially enhanced in embryonic callus compared with non-embryonic calli. Therefore, peroxidases are used as markers of the capacities of SE in different species [31,117]. In addition, the ROS homeostasis in stress conditions often engages in crosstalk with the auxin pathways to induce SE [37]. In this study, 32 DEGs were annotated to antioxidant molecules and enzymes, and all of them were up-regulated in SE. This was similar to the trends of expression of the DEGs involved in auxin synthesis and signaling pathways. Peroxidase transcripts were also expressed differently at different stages of SE in wheat (Triticum
aestivum) and oil palm (Elaeis guineensis) [118,119]. These results indicate that stress and auxin may work in concert to accelerate SE.

4. Materials and Methods

4.1. Plant Materials and Culture Conditions

P. ostii ‘Fengdan’ plants were cultured in the resource garden of the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China. Uniformly sized full and glossy seeds were collected in September 2020 and washed three times in tap water with a few drops of detergent for 10 min, and then immersed in sterilized water for 24 h. The seeds were further disinfected using 3% sodium hypochlorite (NaClO) for 5 min, 75% ethanol for 3 min, and finally rinsed five times with sterilized water. Zygotic embryos were picked out from the sterilized seeds and cultured on Murashige and Skoog medium (MS) supplemented with 3.0 mg L\(^{-1}\) 6-benzylaminopurine (6-BA), 1.0 mg L\(^{-1}\) 1-naphthylacetic acid (NAA), 4.0 mg L\(^{-1}\) Phytagel, and 30 g L\(^{-1}\) sucrose under dark and poikilothermic (22 °C for 16 h and 18 °C for 8 h) conditions for 3 months to induce the callus and somatic embryos. Those regenerated somatic embryos were then cultured on shoot induction medium, including SI1 [(woody plant medium) WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 0.5 mg L\(^{-1}\) 6-BA + 0.5 mg L\(^{-1}\) GA\(_3\)], SI2 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 0.5 mg L\(^{-1}\) 6-BA + 1.0 mg L\(^{-1}\) GA\(_3\)), SI3 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 0.5 mg L\(^{-1}\) 6-BA + 0.5 mg L\(^{-1}\) GA\(_3\)), SI4 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 1.0 mg L\(^{-1}\) 6-BA + 1.0 mg L\(^{-1}\) GA\(_3\)) for shoot development. When the shoots had grown to 3–4 cm, each was separated and cultured on root induction medium, RI1 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 3.0 mg L\(^{-1}\) IBA + 2.0 mg L\(^{-1}\) caffeic acid), RI2 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 3.0 mg L\(^{-1}\) 6-BA + 3.0 mg L\(^{-1}\) IBA + 1.0 mg L\(^{-1}\) acetylsalicylic acid), RI3 (WPM + 4.0 mg L\(^{-1}\) phytagel + 30 g L\(^{-1}\) sucrose + 3.0 mg L\(^{-1}\) 6-BA + 2.0 mg L\(^{-1}\) IBA + 1.0 mg L\(^{-1}\) acetylsalicylic acid) at 4 °C for 15 days, followed by a poikilothermic (22 °C for 16 h and 18 °C for 8 h) condition for 45 days to induce roots. The ratio of explants with callus induction, embryonic callus induction, shoot induction, and root induction were recorded after 14 days, 2 months, 4 months, and 6 months of culture, respectively. The number of shoots per explant was also determined after 4 months of culture. Samples at different culture stages including S0 (zygotic embryo explant, ZE), S1 (non-embryonic callus, after 1 month of culture), S2 (embryogenic callus, after 2 months of culture), S3 (somatic embryos, after 3 months of culture), and S4 (the regenerated shoots after 4 months of culture) were also collected for histological and transcriptomic analyses. The former samples were stored in 2.5% glutaraldehyde and incubated at 4 °C for 8 h, while the latter samples were immediately frozen in liquid nitrogen and stored at −80 °C until use.

4.2. Morphology and Histological Analysis of Whole Plant Regeneration Process

All the induction stages were recorded and photographed using a Leica Stereo Microscope (Leica LED2500, Wetzlar, Germany). For scanning electron microscopy (SEM) observation, the samples in 2.0% glutaraldehyde were washed with 0.1 M phosphate buffer (PBS, pH 7.0) and fixed by 1% osmic acid (OsO\(_4\)) for 2 h at 4 °C. Subsequently, they were dehydrated in a series of ethanol (30%, 50%, 70%, 90%, and 100%) and then dried, gold coated, and photographed with a SU-8010 (Hitachi, Tokyo, Japan) scanning electron microscope.

4.3. RNA-Seq, cDNA Library Construction, and Sequence Assembly and Annotation

Total RNA from samples at five different stages (S0–S4) was extracted using Trizol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. Two embryos, calli or shoots were used in each sample. After digestion with RNase-free recombinant DNase I, the quality and quantity of the total RNA were determined by NanoDrop spectrophotometer (Thermo Scientific™, Waltham, MA, USA). High-quality RNA from the five samples were used for cDNA library construction and BGISEQ-500 RNA-Seq. The cDNA Library preparation was performed following the Illumina manufacturer’s
instructions. The double-stranded cDNA of the above five samples was sequenced using an Illumina HiSeq™ 4000 (4 × 100 bp read length) platform at the Beijing Genomics Institute Company, Shenzhen, China. All transcription sequencing data raw files has been deposited at the NCBI Sequence Read Archive under the BioProject ID PRJNA864612.

The quality of raw reads from each library was assessed using the FASTQC program and Trimomatic software (0.39) to filter the reads and remove adapter sequences. The clean reads were then assembled de novo using Trinity to obtain reference transcriptome unigenes for annotation and DGE analysis. The quality of the assembled transcripts was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO) database (https://busco.ezlab.org/, accessed on 1 August 2021). The longest open reading frame (ORF) per transcript contig was identified using TransDecoder. The sequence similarities and ORFs were predicted and identified by BLASTP, ORF finder, and TransDecoder software (v.5.1) (Created by Broad Institute, MA, USA). The Unigenes were annotated and functionally classified by conducting a BLAST search against seven databases, including NR (NCBI non-redundant protein sequences), NT (NCBI non-redundant nucleotide sequences), Pfam (the prediction of protein structure domain), Kyoto Encyclopedia of Genes and Genomes (KEGG), Eukaryotic Orthologous Groups of proteins/Clusters of Orthologous Groups of proteins (KOG/COG), KEGG Orthology (KO), and Gene Ontology (GO).

4.4. Analysis of Differentially Expressed Genes, and GO and KEGG Enrichment

The differentially expressed genes (DEGs) were analyzed using DESeq2. The false discovery rate (FDR) is used as a correction in many applied multiple testing problems. The parameters were set at $p$-adjust of $<0.05$, false discovery rate (FDR) $\leq 0.001$, and $\log_2$(Fold change) $\geq 2$ with fragments per kilobase million (FPKM) of $>1$ in at least one sample. GO and KEGG enrichment analyses were performed using Fisher’s exact test for the elucidation of the biological functions of the genes. The heatmap of DEGs was constructed using Heml software (Version 1.0) (Created by Huazhong University of Science and Technology, Wuhan, China).

4.5. Validation of Gene Expression by Quantitative Real-Time PCR (qRT-PCR)

Important DEGs related to SE were selected to check their relative gene expression by qRT-PCR. RNA was extracted from the samples using a total RNA extraction kit (Tiangen, Beijing, China) according to the manufacturer’s protocol. The quality and quantity of RNA were assessed with 1.2% agarose electrophoresis and a NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA), respectively. Primers were designed using Premier 5.0 software (Created by Premier company, Toronto, Canada). The first-strand cDNA synthesis was performed using a FastQuant RT Kit (Tiangen, Beijing, China) following the manufacturer’s instructions. The relative expression of the candidate genes was calculated using a double standard curve according to the CFX96 Real-Time system (Bio-Rad, Hercules, CA, USA) by normalizing to the reference gene ACTIN according to Wang et al. (2020). All qRT-PCR reactions were performed in triplicate with at least three biological replicates. The primer information used in this experiment are shown in Table S4.

4.6. Data Analysis and Statistics

The experimental assays used to obtain all results were repeated at least three times. Results were presented as means $\pm$ the standard error (SE) and analyzed using one-way ANOVA, followed by Duncan’s multiple-range test. Statistical analysis was performed with SPSS 22.0 (SPSS Institute, IBM, Endicott, NY, USA).

5. Conclusions

A highly efficient regeneration system of tree peony via SE was established. Based on this regeneration system, the transcriptomes of five SE stages were analyzed. Totals of 32,176, 20,561, and 24,039 DEGs were identified in pairwise comparisons of S0-vs.-S1, S1-vs.-S2, and S1-vs.-S3, respectively. Functional characterizations of the DEGs based
on GO and KEGG analyses are presented. A total of 224 DEGs were identified for their potential associations with SE and the regulatory pathways, including 26 decisive DEGs in SE and SAM construction, 21 DEGs annotated to cell division and expansion, seven DEGs annotated to cell fate determination, 37 DEGs annotated to auxin signaling pathway, 24 DEGs annotated to cytokinin signal pathway, 10 DEGs annotated to the ABA signaling pathway, 2 DEGs annotated to the GA signaling pathway, and 10 DEGs annotated to stress responses. The genes involved in these processes were discussed in this study, which helps to elucidate their roles in SE.

The temporal program for gene expression by qRT-PCR during SE was also analyzed, and the results confirmed the patterns of gene expression of the transcriptomes. Taken together, cell division, particularly asymmetric cell division was enhanced during SE. Moreover, the determination of cell fate and the genes related to meristem formation played central roles in the construction of SAM during somatic embryo formation. The hormone signaling pathways work in concert with epigenetic modifications and stress responses to regulate the induction of SE and the development of somatic embryos. SERK, WOX9, BBM, FUS3, CUC, and WUS were characterized as molecular markers for SE in tree peony. This study improves our understanding of the molecular mechanisms that underlie SE.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/ijms231810595/s1](https://www.mdpi.com/article/10.3390/ijms231810595/s1).

**Author Contributions:** Conceptualization and Methodology, X.Z. and X.R.; Resources, X.R. and X.Z.; Data Curation, H.C., C.L., X.R., T.T.A., C.Y., F.W. and S.W.; Writing—Original Draft Preparation, H.C. and C.L.; Writing—Review and Editing, X.R.; Visualization and Supervision, X.R.; Project Administration, X.R.; Funding Acquisition, X.R. and X.Z. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was supported by the Beijing Municipal Natural Science Foundation (No. 6214045), the National Natural Science Foundation of China (No. 32102415), and the China Agriculture Research System (CARS-21).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We thank MogoEdit (http://www.mogoedit.com, accessed on 25 July 2022) for its linguistic assistance during the preparation of this manuscript.

**Conflicts of Interest:** The authors declare that they have no competing interests.

**References**

1. Wang, Z.; Zhu, C.; Liu, S.; He, C.; Chen, F.; Xiao, P. Comprehensive metabolic profile analysis of the root bark of different species of tree peonies (Paeonia Sect. Moutan). *Phytochemistry* **2019**, *163*, 118–125. [CrossRef] [PubMed]

2. Zhang, L.; Chen, Z.; Gong, W.; Zou, Y.; Xu, F.; Chen, L.; Huang, H. Paeonol ameliorates diabetic renal fibrosis through promoting the activation of the Nrf2/ARE pathway via up-regulating Sirt1. *Front. Pharmacol.* **2018**, *9*, 512. [CrossRef] [PubMed]

3. Liu, N.; Feng, X.; Wang, W.; Zhao, X.; Li, X. Paeonol protects against TNF-alpha-induced proliferation and cytokine release of rheumatoid arthritis fibroblast-like synoviocytes by upregulating FOXO3 through inhibition of miR-155 expression. *Inflamm. Res.* **2017**, *66*, 603–610. [CrossRef]

4. Zhao, B.; Shi, Q.J.; Zhang, Z.Z.; Wang, S.Y.; Wang, X.; Wang, H. Protective effects of paeonol on subacute/chronic brain injury during cerebral ischemia in rats. *Exp. Ther. Med.* **2018**, *15*, 3836–3846. [CrossRef]

5. Zhang, L.; Ma, C.; Gu, R.; Zhang, M.; Wang, X.; Yang, L.; Liu, Y.; Zhou, Y.; He, S.; Zhu, D. Paeonol regulates hypoxia-induced proliferation of pulmonary artery smooth muscle cells via EKR 1/2 signalling. *Eur. J. Pharmacol.* **2018**, *834*, 257–265. [CrossRef] [PubMed]

6. Wang, S.C.; Sun, H.L.; Hsu, Y.H.; Liu, S.H.; Lii, C.K.; Tsai, C.H.; Liu, K.L.; Huang, C.S.; Li, C.C. α-Linolenic acid inhibits the migration of human triple-negative breast cancer cells by attenuating Twist1 expression and suppressing Twist1-mediated epithelial-mesenchymal transition. *Biochem. Pharmacol.* **2020**, *180*, 114152. [CrossRef] [PubMed]
7. Wang, X.; Qiu, S.; Wang, X.; Amuti, A.; Dang, L. Developing peony (Paeonia suffruticosa Andr.) seed oil products based on \( \alpha \)-Linolenic acid by microemulsification. *Ind. Crops Prod.* **2021**, *161*, 113211. [CrossRef]

8. Wen, S.; Cheng, F.; Zhong, Y.; Wang, X.; Li, L.; Zhang, Y.; Qiu, J. Efficient protocols for the micropropagation of tree peony (*Paeonia suffruticosa* ‘Jin Pao Hong’, *P. suffruticosa* ‘Wu Long Peng Sheng’, and *P. ×lemoinei* ‘High Noon’) and application of arbuscular mycorrhizal fungi to improve plantlet establishment. *Sci. Hortic.* **2016**, *201*, 10–17. [CrossRef]

9. Wójcikowska, B.; Wójcik, A.M.; Gaj, M.D. Epigenetic regulation of auxin-induced somatic embryogenesis in plants. *Int. J. Mol. Sci.* **2020**, *21*, 2307. [CrossRef]

10. Du, Y.M.; Cheng, F.Y.; Zhong, Y. Induction of direct somatic embryogenesis and shoot organogenesis and histological study in tree peony (*Paeonia sect. Moutan*). *Plant Cell Tiss. Org.* **2020**, *141*, 557–570. [CrossRef]

11. Wu, L.Y.; Shang, G.D.; Wang, F.X.; Gao, J.; Wan, M.C.; Xu, Z.G.; Wang, J.W. Dynamic chromatin state profiling reveals regulatory roles of auxin and cytokinin in shoot regeneration. *Dev. Cell* **2022**, *57*, 526–542.e7. [CrossRef] [PubMed]

12. Ren, X.X.; Liu, Y.; Jeong, B.R. Enhanced somatic embryo induction of a tree peony, *Paeonia ostii* ‘Fengdan’, by a combination of 6-benzylaminopurine (BA) and 1-naphthylacetic acid (NAA). *Plants* **2020**, *9*, 3. [CrossRef] [PubMed]

13. Wu, S.; Chen, L.; Tian, R. Micropropagation of tree peony (*Paeonia sect. Moutan*): A review. *Plant Cell Tiss. Org.* **2019**, *141*, 1–14. [CrossRef]

14. Perez-Garcia, P.; Moreno-Risueno, M.A. Stem cells and plant regeneration. *Annu. Rev. Genet.* **2018**, *52*, 485–510. [CrossRef] [PubMed]

15. Wang, F.; Shang, G.; Wu, L.; Xu, Z.; Zhao, X.; Wang, J. Chromatin accessibility dynamics and a hierarchical transcriptional regulatory network structure for plant somatic embryogenesis. *Dev. Cell* **2020**, *54*, 742–757. [CrossRef]

16. Soriano, M.; Li, H.; Jacquard, C.; Angenent, G.C.; Krochko, J.; Offringa, R.; Boutillier, K. Plasticity in cell division patterns and auxin transport dependency during in vitro embryogenesis in *Brassica napus*. *Plant Cell* **2014**, *26*, 2568–2581. [CrossRef]

17. Méndez-Hernández, H.A.; Ledezma-Rodríguez, M.; Avilez-Montalvo, R.N.; Juárez-Gómez, Y.L.; Skeete, A.; Aviléz-Montalvo, J.; De-la-Peña, C.; Loyola-Vargas, V.M. Signaling overview of plant somatic embryogenesis. *Front. Plant Sci.* **2019**, *10*, 77. [CrossRef]

18. Oulbi, S.; Kohaich, K.; Baaziz, M.; Belkoura, I.; Loutfi, K. Peroxidase enzyme fractions as markers of somatic embryogenesis. *Int. J. Mol. Sci.* **2018**, *19*, 3218. [CrossRef] [PubMed]

19. Shim, S.; Lee, H.G.; Park, O.S.; Shin, H.; Lee, K.; Lee, H.; Huh, J.H.; Seo, P.J. Dynamic changes in DNA methylation occur in TE regions and affect cell proliferation during leaf-to-callus transition in Arabidopsis. *Sci. Hortic.* **2018**, *221*, 258–267. [CrossRef]

20. Mozgová, I.; Muñoz-Viana, R.; Hennig, L. PRC2 represses hormone-induced somatic embryogenesis in vegetative tissue of *Arabidopsis thaliana*. *PLoS Genet.* **2017**, *13*, e1006562. [CrossRef]

21. Wójcikowska, B.; Botor, M.; Mórnończyk, J.; Wójcik, A.M.; Nodzyński, T.; Karcz, J.; Gaj, M.D. Trichostatin a triggers an embryogenic transition in Arabidopsis embryos via an auxin-related pathway. *Front. Plant Sci.* **2018**, *9*, 1353. [CrossRef]

22. Zhang, H.; Zhang, T.T.; Liu, H.; Shi, Y.; Wang, M.; Bie, X.M.; Li, X.G.; Zhang, X.S. Thioredoxin-mediated ROS homeostasis explains natural variation in plant regeneration. *Plant Physiol.* **2018**, *176*, 2231–2240. [CrossRef]

23. Bahmankar, M.; Mortazavian, S.M.M.; Tohidfar, M.; Sadat Noori, S.A.; Izadi Darbandi, A.; Corrado, G.; Rao, R. Chemical compositions, somatic embryogenesis, and somaclonal variation in cumin. *BioMed Res. Int.* **2017**, *2017*, 7283806. [CrossRef]

24. Oulbi, S.; Khoaicha, K.; Baaziz, M.; Belkoura, I.; Loutfi, K. Peroxidase enzyme fractions as markers of somatic embryogenesis capacities in olive (*Olea europaea* L.). *Plants* **2021**, *10*, 901. [CrossRef] [PubMed]

25. Chen, M.; Zhang, J.; Zhou, Y.; Li, S.; Fan, X.; Yang, L.; Guan, Y.; Zhang, Y. Transcriptome analysis of *Lilium Oriental* × *Trumpet* hybrid roots reveals auxin-related genes and stress-related genes involved in picloram-induced somatic embryogenesis induction. *J. Hortic. Sci. Biotechnol.* **2019**, *94*, 317–330. [CrossRef]

26. Chen, Y.; Xu, X.; Liu, Z.; Zhang, Z.; XuHan, X.; Lin, Y.; Lai, Z. Global scale transcriptome analysis reveals differentially expressed genes involve in early somatic embryogenesis in *Dimocarpus longan* Lour. *BMC Genom.* **2020**, *21*, 4. [CrossRef]

27. Ding, M.; Dong, H.; Xue, Y.; Su, S.; Wu, Y.; Li, S.; Liu, H.; Li, H.; Han, J.; Shan, X.; et al. Transcriptomic analysis reveals somatic embryogenesis-associated signaling pathways and gene expression regulation in maize (*Zea mays* L.). *Plant Mol. Biol.* **2020**, *104*, 647–663. [CrossRef] [PubMed]

28. Su, Y.H.; Liu, Y.B.; Zhou, C.; Li, X.M.; Zhang, X.S. The microRNA167 controls somatic embryogenesis in *Arabidopsis* through regulating its target genes ARF6 and ARF8. *Plant Cell Tiss. Org.* **2016**, *124*, 405–417. [CrossRef]
36. Wojciekowska, B.; Gaj, M.D. Expression profiling of AUXIN RESPONSE FACTOR genes during somatic embryogenesis induction in Arabidopsis. Plant Cell Rep. 2017, 36, 843–858. [CrossRef]

37. Zhou, T.; Yang, X.; Guo, K.; Deng, J.; Xu, J.; Gao, W.; Lindsey, K.; Zhang, X. ROS homeostasis regulates somatic embryogenesis via the regulation of auxin signaling in cotton. Mol. Cell. Proteom. 2016, 15, 2108–2124. [CrossRef]

38. Thibaud-Nissen, F.; Shealy, R.T.; Khanna, A.; Vodkin, L.O. Clustering of microarray data reveals transcript patterns associated with somatic embryogenesis in soybean. Plant Physiol. 2003, 132, 118–136. [CrossRef]

39. Yang, X.; Zhang, X.; Yuan, D.; Jin, F.; Zhang, Y.; Xu, J. Transcript profiling reveals complex auxin signalling pathway and transcription regulation involved in dedifferentiation and redifferentiation during somatic embryogenesis in cotton. BMC Plant Biol. 2012, 12, 110. [CrossRef]

40. Gliwicka, M.; Nowak, K.; Balazadeh, S.; Mueller-Roeber, B.; Gaj, M.D. Extensive modulation of the transcription factor transcriptionome during somatic embryogenesis in Arabidopsis thaliana. PLoS ONE 2013, 8, e69261. [CrossRef]

41. Zhang, B.; Liu, J.; Yang, Z.E.; Chen, E.Y.; Zhang, C.J.; Zhang, X.Y.; Li, F.G. Genome-wide analysis of GRAS transcription factor gene family in Gossypium hirsutum L. BMC Genom. 2018, 19, 348. [CrossRef] [PubMed]

42. Nuruuzzaman, M.; Sharoni, A.M.; Kikuchi, S. Roles of NAC transcription factors in the regulation of biotic and abiotic stress responses in plants. Front. Microbiol. 2013, 4, 248. [CrossRef] [PubMed]

43. Long, Y.; Yang, Y.; Pan, G.; Shen, Y. New insights into tissue culture plant-regeneration mechanisms. Front. Plant Sci. 2022, 13, 926752. [CrossRef] [PubMed]

44. Ueda, M.; Zhang, Z.; Laux, T. Transcriptional activation of WUSCHEL-related homeodomain transcription factors during somatic embryogenesis in Arabidopsis thaliana. Plant Physiol. 2002, 124, 2761–2770. [CrossRef] [PubMed]

45. Crawford, B.C.; Sewell, J.; Golembeski, G.; Roshan, C.; Long, J.A.; Yanofsky, M.F. Plant development. Genetic control of distal stem cell fate within root and embryonic meristems. Science 2015, 347, 655–659. [CrossRef]

46. Su, Y.H.; Zhao, X.Y.; Liu, Y.B.; Zhang, C.L.; O’Neill, S.D.; Zhang, X.S. Auxin-induced WUS expression is essential for embryonic stem cell renewal during somatic embryogenesis in Arabidopsis. Plant J. 2009, 59, 448–460. [CrossRef]

47. Tvorogova, V.E.; Fedorova, Y.A.; Potsenkovskaya, E.A.; Kudriashov, A.A.; Efremova, E.P.; Kvitkovskaya, V.A.; Wolabu, T.W.; Zhang, F.; Tadege, M.; Lutova, L.A. The WUSCHEL-related homeobox transcription factor MtWOX9-1 stimulates somatic embryogenesis in Medicago truncatula. Plant Cell Tiss. Org. 2019, 138, 517–527. [CrossRef]

48. Wei, X.; Geng, M.; Li, J.; Duan, H.; Li, F.; Ge, X. GhWOX11 and GhWOX12 promote cell fate specification during embryogenesis. Ind. Crops Prod. 2022, 184, 115031. [CrossRef] [PubMed]

49. Gambino, G.; Minuto, M.; Boccacci, P.; Perrone, I.; Vallania, R.; Gribaudo, I. Characterization of expression dynamics of WOX8/9 links zygothe polarity to embryo development. Dev. Cell 2011, 20, 264–270. [CrossRef] [PubMed]

50. Kumaran, M.K.; Bowman, J.L.; Sundaresan, V. YABBY polarity genes mediate the repression of KNOX homeobox genes in Arabidopsis. Plant Cell 2002, 14, 2761–2770. [CrossRef] [PubMed]

51. Palovaara, J.; Hallberg, H.; Stasolla, C.; Hakman, I. Comparative expression pattern analysis of WUSCHEL-related homebox 2 (WOX2) and WOX8/9 in developing seeds and somatic embryos of the gymnosperm Picea abies. New Phytol. 2010, 188, 122–135. [CrossRef] [PubMed]

52. Kurdyukov, S.; Song, Y.; Sheahan, M.B.; Rose, R.J. Transcriptional regulation of early embryo development in the model legume Medicago truncatula. Plant Cell Rep. 2014, 33, 349–362. [CrossRef] [PubMed]

53. Gulzar, B.; Mujib, A.; Malik, M.Q.; Sayeed, R.; Mamgain, J.; Ejaz, B. Genes, proteins and other networks regulating somatic embryogenesis in plants. Front. Plant Sci. 2014, 5, 31. [CrossRef] [PubMed]

54. Hecht, V.; Vielle-Calzada, J.P.; Hartog, M.V.; Schmidt, E.D.; Boutilier, K.; Grossniklaus, U.; de Vries, S.C. The Arabidopsis Somatic Embryogenesis Receptor Kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol. 2001, 127, 803–816. [CrossRef]

55. Singla, B.; Khurana, J.P.; Khurana, P. Structural characterization and expression analysis of the SERK/ERL gene family in rice (Oryza sativa). Int. J. Plant Genomics 2009, 2009, 539402. [CrossRef]

56. Horstman, A.; Li, M.; Heidmann, I.; Weemen, M.; Chen, B.; Muino, J.M.; Angenent, G.C.; Boutilier, K. The BABY BOOM transcription factor activates the LEC1-ABI3-FUS3-LEC2 network to induce somatic embryogenesis. Plant Physiol. 2017, 175, 848–857. [CrossRef]

57. Boultier, K.; Offringa, R.; Sharma, V.K.; Kieft, H.; Ouellet, T.; Zhang, L.; Hattori, J.; Liu, C.M.; van Lammeren, A.A.; Miki, B.L.; et al. Ectopic expression of BABY BOOM triggers a conversion from vegetative to embryogenic growth. Plant Cell 2002, 14, 1737–1749. [CrossRef]

58. Rupps, A.; Raschke, J.; Rummel, M.; Linke, B.; Zoglauer, K. Identification of putative homologs of Larix decidua to BABYBOOM (BBM), LEAFY COTYLEDON1 (LEC1), WUSCHEL-related HOMEobox2 (WOX2) and SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK) during somatic embryogenesis. Plant Cell 2016, 243, 473–488. [CrossRef]

59. Zhang, Y.; Feng, S.; Chen, F.; Chen, H.; Wang, J.; McCall, C.; Xiong, Y.; Deng, X.W. Arabidopsis DDB1-CUL4 associated factor1 forms a nuclear E3 ubiquitin ligase with DDB1 and CUL4 that is involved in multiple plant developmental processes. Plant Cell 2008, 20, 1437–1455. [CrossRef]

60. Lu, P.; Porat, R.; Nadeau, J.A.; O’Neill, S.D. Identification of a meristem L1 layer-specific gene in Arabidopsis that is expressed during embryonic pattern formation and defines a new class of homeobox genes. Plant Cell 1996, 8, 2155–2168. [CrossRef]
61. Ishida, S.; Suzuki, H.; Iwaki, A.; Kawamura, S.; Yamaoka, S.; Kojima, M.; Takebayashi, Y.; Yamaguchi, K.; Shigenobu, S.; Sakakibara, H.; et al. Diminished auxin signaling triggers cellular reprogramming by inducing a regeneration factor in the Liverwort Marchantia polymorpha. *Plant Physiol.* 2022, 63, 384–400. [CrossRef] [PubMed]

62. Alejo-Vinogradova, M.T.; Ornelas-Ayala, D.; Vega-León, R.; Garay-Arroyo, A.; García-Ponce, B.; Álvarez-Buylla, E.R.; Sanchez, M.P. Unraveling the role of epigenetic regulation in asymmetric cell division during plant development. *J. Exp. Bot.* 2022, 73, 38–49. [CrossRef] [PubMed]

63. De Smet, I.; Beeckman, T. Asymmetric cell division in land plants and algae: The driving force for differentiation. *Nat. Rev. Mol. Cell Biol.* 2011, 12, 177–188. [CrossRef] [PubMed]

64. Heidstra, R.; Welch, D.; Scheres, B. Mosaic analyses using marked activation and deletion clones dissect *Arabidopsis* SCARECROW action in asymmetric cell division. *Genes Dev.* 2004, 18, 1964–1969. [CrossRef] [PubMed]

65. Levesque, M.P.; Vernoux, T.; Busch, W.; Cui, H.; Wang, J.Y.; Blilou, I.; Hassan, H.; Nakajima, K.; Matsumoto, N.; Lohmann, J.U.; et al. Whole-genome analysis of the SHORT-ROOT developmental pathway in *Arabidopsis*. *PLoS Biol.* 2006, 4, e143. [CrossRef] [PubMed]

66. Pasternak, T.P.; Prinsen, E.; Ayaydin, F.; Miskolczi, P.; Potters, G.; Asard, H.; Van Onckelen, H.A.; Dudits, D.; Fehér, A. The Role of auxin, pH, and stress in the activation of embryogenic cell division in leaf protoplast-derived cells of alfalfa. *Plant Physiol.* 2002, 129, 1807–1819. [CrossRef]

67. Wyrzykowski, J.; Schordert, M.; Pien, S.; Gruissem, W.; Fleming, A.J. Induction of differentiation in the shoot apical meristem by transient overexpression of a retinoblastoma-related protein. *Plant Physiol.* 2006, 141, 1338–1348. [CrossRef]

68. Borghi, L.; Gutzat, R.; Füttener, J.; Laizet, Y.; Morin, H.; Traas, J.; Pautot, V. Identification of genes required for embryo development in *Arabidopsis*. *Plant Physiol.* 2004, 135, 1206–1220. [CrossRef] [PubMed]

69. Balkunde, R.; Kitagawa, M.; Xu, X.M.; Wang, J.; Jackson, D. SHOOT MERISTEMLESS trafficking controls axillary meristem formation, meristem size and organ boundaries in *Arabidopsis*. *Plant J.* 2017, 90, 435–446. [CrossRef] [PubMed]

70. Shao, M.; Nolle, C.; Werr, N. Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on heterodimerization with BLH proteins expressed in discrete sub-domains of the shoot apical meristem of *Arabidopsis thaliana*. *Nucleic Acids Res.* 2006, 34, 1281–1292. [CrossRef] [PubMed]

71. Zuo, J.; Niu, Q.; Frugis, G.; Chua, N. The WUSCHEL gene promotes vegetative-to-embryonic transition in *Arabidopsis*. *Plant J.* 2002, 30, 349–359. [CrossRef] [PubMed]

72. Belles-Boix, E.; Hamant, O.; Witiak, S.M.; Morin, H.; Traas, J.; Pautot, V. *KNAT6*: An *Arabidopsis* homeobox gene involved in meristem activity and organ separation. *Plant Cell* 2006, 18, 1900–1907. [CrossRef] [PubMed]

73. Balkunde, R.; Kitagawa, M.; Xu, X.M.; Wang, J.; Jackson, D. SHOOT MERISTEMLESS trafficking controls auxillary meristem formation, meristem size and organ boundaries in *Arabidopsis*. *Plant J.* 2017, 90, 435–446. [CrossRef] [PubMed]

74. Cole, M.; Nolle, C.; Werr, N. Nuclear import of the transcription factor SHOOT MERISTEMLESS depends on heterodimerization with BLH proteins expressed in discrete sub-domains of the shoot apical meristem of *Arabidopsis thaliana*. *Nucleic Acids Res.* 2006, 34, 1281–1292. [CrossRef] [PubMed]

75. Green, K.A.; Prigge, M.J.; Katszman, R.B.; Clark, S.E. CORONA, a member of the class III homeodomain leucine zipper gene family in *Arabidopsis*, regulates stem cell specification and organogenesis. *Plant Cell* 2005, 17, 691–704. [CrossRef]

76. Wenig, U.; Meyer, S.; stadler, R.; Fischer, S.; Werner, D.; Lauter, A.; Melzer, M.; Hoth, S.; Weingartner, M.; Sauer, N. Identification of MAIN, a factor involved in genome stability in the meristems of *Arabidopsis thaliana*. *Plant Cell* 2005, 17, 495–483. [CrossRef] [PubMed]

77. Otsuga, D.; DeGuzman, B.; Prigge, M.J.; Drews, G.N.; Clark, S.E. REVOLUTA regulates meristem initiation at lateral positions. *Plant J.* 2001, 25, 223–236. [CrossRef] [PubMed]

78. De-la-Peña, C.; Nic-Can, G.I.; Galaz-Ávalos, R.M.; Avillez-Montalvo, R.; Loyola-Vargas, V.M. The role of chromatin modifications in somatic embryogenesis in plants. *Front. Plant Sci.* 2015, 6, 635. [CrossRef] [PubMed]

79. Li, M.; Wrobel-Marek, J.; Heidmann, I.; Horstman, A.; Chien, B.; Reis, R.; Angenent, G.C.; Boutilier, K. Auxin biosynthesis maintains embryo identity and growth during BABY BOOM-induced somatic embryogenesis. *Plant Physiol.* 2012, 188, 1095–1110. [CrossRef]

80. Sun, Y.H.; Zhang, X.S. Auxin gradients trigger de novo formation of stem cells during somatic embryogenesis. *Plant Signal Behav.* 2009, 4, 574–576. [CrossRef]

81. Liu, L.; Tong, H.; Xiao, Y.; Che, R.; Xu, F.; Hu, B.; Liang, C.; Chu, J.; Li, J.; Chu, C. Activation of Big Grain1 significantly improves grain size by regulating auxin transport in rice. *Proc. Natl. Acad. Sci. USA* 2015, 112, 11102–11107. [CrossRef]

82. Song, S.; Wang, Z.; Ren, Y.; Sun, H. Full-length transcriptome analysis of the ABCB, PIN/PIN-LIKES, and AUX/LAX families involved in somatic embryogenesis of *Lilium pumilum* DC. *Fisch. Int. J. Mol. Sci.* 2020, 21, 453. [CrossRef]

83. Loyola-Vargas, V.; Gorgona-Castillo, E.; Quintana-Escobar, A.; Nic Can, G.; Maria, R.; Avalos, G. Transcriptome analysis of the induction of somatic embryogenesis in *Coffea canephora* and the participation of ARF and Aux/IAA genes. *PeerJ* 2019, 7, e7752. [CrossRef]

84. Cui, X.; Guo, Z.; Song, L.; Wang, Y.; Cheng, Y. NCP1/ATMOB1a plays key roles in auxin-mediated *Arabidopsis* development. *PLoS Genet.* 2016, 12, e1005923. [CrossRef]

85. Wang, S.; Hagen, G.; Guilfoyle, T.J. ARF-Aux/IAA interactions through domain III/IV are not strictly required for auxin-responsive gene expression. *Plant Signal Behav.* 2013, 8, e24526. [CrossRef]

86. Ooi, S.; Choo, C.N.; Ishak, Z.; Ong-Abdullah, M. A candidate auxin-responsive expression marker gene, *EglIAA9*, for somatic embryogenesis in oil palm (*Elaeis guineensis* Jacq.). *Plant Cell Tiss. Org.* 2012, 110, 201–212. [CrossRef]
87. Wang, Y.; Li, H.L.; Zhou, Y.K.; Guo, D.; Zhu, J.H.; Peng, S.Q. Transcriptomes analysis reveals novel insight into the molecular mechanisms of somatic embryogenesis in Hevea brasiliensis. BMC Genom. 2021, 22, 183. [CrossRef]
88. Cheng, Z.J.; Wang, L.; Sun, W.; Zhang, Y.; Zhou, C.; Su, Y.H.; Li, W.; Sun, T.T.; Zhao, X.Y.; Li, X.G.; et al. Pattern of auxin and cytokinin responses for shoot meristem induction results from the regulation of cytokinin biosynthesis by AUXIN RESPONSE FACTOR3. Plant Physiol. 2013, 161, 240–251. [CrossRef]
89. Wu, M.; Su, R.; Li, X.; Ellis, T.; Lai, Y.; Wang, X. Engineering of regulated stochastic cell fate determination. Proc. Natl. Acad. Sci. USA 2013, 110, 10610–10615. [CrossRef]
90. Hwang, I.; Sheen, J. Two-component circuitry in Arabidopsis cytokinins signal transduction. Nature 2001, 413, 383–389. [CrossRef]
91. Argyros, R.D.; Mathews, D.E.; Chiang, Y.H.; Palmer, C.M.; Thibault, D.M.; Etheridge, N.; Argyros, D.A.; Mason, M.G.; Kieber, J.J.; Schaller, G.E. Type B response regulators of Arabidopsis play key roles in cytokinin signaling and plant development. Plant Cell 2008, 20, 2102–2116. [CrossRef] [PubMed]
92. Xu, Z.; Zhang, C.; Zhang, X.; Liu, C.; Wu, Z.; Yang, Z.; Zhou, K.; Yang, X.; Li, F. Transcriptome profiling reveals auxin and cytokinin regulating somatic embryogenesis in different sister lines of cotton cultivar CCR124. J. Integr. Plant Biol. 2013, 55, 631–642. [CrossRef] [PubMed]
93. Ptak, A.; Taichy, A.E.; Wyzgolik, G.; Henry, M.; Laurain-Mattar, D. Effects of ethylene on somatic embryogenesis and galanthamine content in Leucojum aestivum l. cultures. Plant Cell Tiss. Org. 2010, 102, 61–67. [CrossRef]
94. Neves, M.; Correia, S.; Cavaleiro, C.; Canhoto, J. Modulation of organogenesis and somatic embryogenesis by ethylene: An Overview. Plants 2021, 10, 1208. [CrossRef]
95. Montero-Cortés, M.; Sáenz, L.; Córdova, I.; Quiroz, A.; Verdeil, J.L.; Oropesa, C. GA3 stimulates the formation and germination of somatic embryos and the expression of a KNOTTED-like homeobox gene of Cocos nucifera (L.). Plant Cell Rep. 2010, 29, 1049–1059. [CrossRef]
96. Kikuchi, A.; Sanuki, N.; Higashi, K.; Koshiba, T.; Kamada, H. Abscisic acid and stress treatment are essential for the acquisition of embryogenic competence by carrot somatic cells. Planta 2006, 223, 637–645. [CrossRef]
97. Fernandez, S.C.; Gamage, C.K. Abscisic acid induced somatic embryogenesis in immature embryo explants of coconut (Cocos nucifera L.). Plant Sci. 2000, 151, 193–198. [CrossRef]
98. Chen, B.; Fiers, M.; Dekkers, B.J.W.; Maas, L.; van Esse, G.W.; Angenent, G.C.; Zhao, Y.; Boutillier, K. ABA signalling promotes cell totipotency in the shoot apex of germinating Linum. J. Exp. Bot. 2021, 72, 6418–6436. [CrossRef]
99. Morończyk, J.; Brąszewska, A.; Wójcikowska, B.; Chwiałkowska, K.; Nowak, K.; Wójcik, A.M.; Kwaśniewski, M.; Gaj, M.D. Insights into the histone acetylation-mediated regulation of the transcription factor genes that control the embryogenic transition in the somatic cells of Arabidopsis. Cells 2022, 11, 863. [CrossRef]
100. Birnbaum, K.D.; Roudier, F. Epigenetic memory and cell fate reprogramming in plants. Regeneration 2017, 4, 15–20. [CrossRef]
101. Osorio-Montalvo, P.; Sáenz-Carbonell, L.; De-la-Peña, C. 5-Azacytidine: A promoter of epigenetic changes in the quest to improve plant somatic embryogenesis. Int. J. Mol. Sci. 2018, 19, 3182. [CrossRef]
102. Grzybikowska, D.; Nowak, K.; Gaj, M.D. Hypermethylation of auxin-responsive motifs in the promoters of the transcription factor genes accompanies the somatic embryogenesis induction in Arabidopsis. Int. J. Mol. Sci. 2020, 21, 6849. [CrossRef]
103. Chakrabarty, D.; Yu, K.W.; Paek, K.Y. Detection of DNA methylation changes during somatic embryogenesis of Siberian ginseng (Eleuterococcus senticosus). Plant Sci. 2003, 165, 61–68. [CrossRef]
104. Brzeski, J.; Jerzmanowski, A. Deficient in DNA methylation 1 (DDM1) defines a novel family of chromatin-remodeling factors. J. Biol. Chem. 2003, 278, 823–828. [CrossRef]
105. Berr, A.; Shafiq, S.; Shen, W. Histone modifications in transcriptional activation during plant development. Biochim. Biophys. Acta (BBA)—Gene Regul. Mech. 2011, 1809, 567–576. [CrossRef]
106. Lee, K.K.; Workman, J.L. Histone acetyltransferase complexes: One size doesn’t fit all. Nat. Rev. Mol. Cell Biol. 2007, 8, 284–295. [CrossRef]
107. Park, S.Y.; Kim, J.S. A short guide to histone deacetylases including recent progress on class II enzymes. Exp. Mol. Med. 2020, 52, 204–212. [CrossRef]
108. Kumar, V.; Thakur, J.K.; Prasad, M. Histone acetylation dynamics regulating plant development and stress responses. Cell Mol. Life Sci. 2021, 78, 4467–4486. [CrossRef]
109. Martinez, O.; Arjones, V.; González, M.V.; Rey, M. Histone deacetylase inhibitors increase the embryogenic potential and alter the expression of embryogenesis-related and HDAC-encoding genes in grapevine (Vitis vinifera L., cv. Mencia). Plants 2021, 10, 1164. [CrossRef]
110. Bull, T.; Michelmore, R. Molecular determinants of in vitro plant regeneration: Prospects for enhanced manipulation of lettuce (Lactuca sativa L.). Front. Plant Sci. 2022, 13, 888425. [CrossRef] [PubMed]
111. Pontvianne, F.; Blevins, T.; Pikaard, C.S. Arabidopsis histone lysine methyltransferases. Adv. Bot. Res. 2010, 53, 1–22. [CrossRef] [PubMed]
112. Earley, K.W.; Shook, M.S.; Brower-Toland, B.; Hicks, L.; Pikaard, C.S. In vitro specificities of Arabidopsis co-activator histone acetyltransferases: Implications for histone hyperacetylation in gene activation. Plant J. 2007, 52, 615–626. [CrossRef] [PubMed]
113. Kwon, C.S.; Chen, C.; Wagner, D. WUSCHEL is a primary target for transcriptional regulation by SPL10D in dynamic control of stem cell fate in Arabidopsis. Genes Dev. 2005, 19, 992–1003. [CrossRef] [PubMed]
114. Kocsy, G.; Tari, I.; Vanková, R.; Zechmann, B.; Gulyáš, Z.; Poór, P.; Galiba, G. Redox control of plant growth and development. *Plant Sci.* **2013**, *211*, 77–91. [CrossRef]

115. Fehér, A. Somatic embryogenesis—Stress-induced remodeling of plant cell fate. *Biochim. Biophys. Acta* **2015**, *1849*, 385–402. [CrossRef]

116. Takeda, H.; Kotake, T.; Nakagawa, N.; Sakurai, N.; Nevin, D.J. Expression and function of cell wall-bound cationic peroxidase in asparagus somatic embryogenesis. *Plant Physiol.* **2003**, *131*, 1765–1774. [CrossRef]

117. Gallego, P.; Martin, L.; Blazquez, A.; Guerra, H.; Villalobos, N. Involvement of peroxidase activity in developing somatic embryos of *Medicago arborea* L. Identification of an isozyme peroxidase as biochemical marker of somatic embryogenesis. *J. Plant Physiol.* **2014**, *171*, 78–84. [CrossRef]

118. Singla, B.; Tyagi, A.K.; Khurana, J.P.; Khurana, P. Analysis of expression profile of selected genes expressed during auxin-induced somatic embryogenesis in leaf base system of wheat (*Triticum aestivum*) and their possible interactions. *Plant Mol. Biol.* **2007**, *65*, 677–692. [CrossRef]

119. Lin, H.C.; Morcillo, F.; Dussert, S.; Tranchant-Dubreuil, C.; Tregear, J.W.; Tranbarger, T.J. Transcriptome analysis during somatic embryogenesis of the tropical monocot *Elaeis guineensis*: Evidence for conserved gene functions in early development. *Plant Mol. Biol.* **2009**, *70*, 173–192. [CrossRef]