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ORIGINAL ARTICLE

Regulatory networks of hormone-involved transcription factors and their downstream pathways during somatic embryogenesis of *Arabidopsis thaliana*

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Abstract
Somatic embryogenesis (SE) depends on a variety of developmental pathways that are influenced by several environmental factors. Therefore, it is important to understand the relationship between environmental and genetic factors by identifying the gene networks involved in SE through gene set enrichment analysis (GSEA). For determination of SE effective transcription factors, upstream sequences of core-enriched genes were analyzed. The results indicated that response to hormones is one of the biological pathways activated by the enriched TFs at all stages of somatic embryogenesis and about half of the hormonal pathways were enriched. On the fifth day after 2,4-Dichlorophenoxyacetic acid (2,4-D) treatment, the activity of hormone-affecting genes reached its maximum. At this time, more transcription factors regulated the enriched genes compared to the other stages of somatic embryogenesis. MYBs, AT-HOOKs, and HSFs are the main families of transcription factors which affect core-enriched genes during SE. *CCA1*, *PRR7*, and *TOC1* and their related genes at the center of protein–protein interaction of SE-key transcription factors, involved in the regulation of the circadian clock. Gene expression analysis of *CCA1*, *PRR7*, and *TOC1* revealed that the genes involved in circadian clock reached their maximum activity when embryonic cells formed. Also, auxin response elements were identified at the upstream of SE-circadian clock transcription factors, indicating that they might mediate between auxin signaling and SE-related hormonal pathways as well as SE marker genes such as *AGL15*, *BBM*, and *LEC*s. Based on these results, it is possible that the cellular circadian rhythm activates various developmental pathways under the influence of auxin signal transduction and their interactions determine the induction of somatic embryogenesis. According to the results of this study, modifying pathways affected by SE-related transcription factors such as circadian rhythm may result in cell reprogramming and increase somatic embryogenesis efficiency.

Keywords *Arabidopsis thaliana* · Auxin response · Cell reprogramming · Circadian rhythm · Functional transcriptomics · Reproduction · 2,4-D

Abbreviations

ABA	Abscisic acid
*ARF*s	Auxin response factors
BR	Brassinosteroids
GA	Gibberellin
GO	Gene ontology
GSEA	Gene set enrichment analysis
IAA	Indole-3-acetic acid
JA	Jasmonates
KEGG	Kyoto encyclopedia of genes and genomes
PPI	Protein–protein interaction
SA	Salicylic acid
SE	Somatic embryogenesis
TF	Transcription factor
TFBS	Transcription factor binding site
2,4-D	2,4-Dichlorophenoxyacetic acid

Introduction

Somatic embryogenesis (SE) in plants consists of the formation of embryonic cells in somatic tissues and the differentiation of these cells into mature embryos. Several factors are effective during the induction of embryonic cells and their development. They activate various biological pathways in

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the plant cells by affecting the genes, and their total activity leads to reprogramming and proper development of the embryo. Plant hormones, especially auxins, are the important factors to induce somatic embryogenesis (Gaj 2004). Identification of the role of plant hormones during somatic embryogenesis requires the study of various physiological processes and genetic factors. In some reports, intracellular levels of abscisic acid (ABA), auxin, or salicylic acid (SA) determine the ability of tissue or even cells of a tissue to produce somatic embryos (Fraga et al. 2016; Jiménez and Bangerth 2001). Also some plant stress hormones or oxidative-derived substances such as jasmonates, reactive oxygen species and nitric oxide in cooperation with the other environmental factors modulate different stages of plant development including embryogenesis (Kamińska et al. 2021; Prajapati et al. 2022; Wang et al. 2022). Therefore, the previous reports points out the necessity for broad studies to obtain the functional relationship network of different factors affecting induction and development of somatic embryogenesis.

Transcription factors are the first genes affected by hormone signal transmission, determining how biological pathways develop during somatic embryogenesis. The transcription factor *ETHYLENE RESPONSE FACTOR 022* (*ERF022*) in association with *LEAFY COTYLEDON 2 (LEC2)*, plays a key role in the induction of somatic embryogenesis in *Arabidopsis* (Nowak et al. 2015). Application of ethylene or its precursor leads to increase of the expression of *YUCCA* (*YUC*) and *DR5* as the markers of auxin perception during somatic embryogenesis of *Arabidopsis* (Bai et al. 2013). On the other hand, the balance of ABA and gibberellins (GA) affects the induction of somatic embryos and their maturation with an impact on the activity of *FUSCA3* (*FUS3*) and the other genes of *LEC* transcription factors (Braybrook and Harada 2008; Stone et al. 2008). When some transcription factors such as *BABY BOOM* (*BBM*), *LEC2* and *AHL15* are overexpressed, hormones are no longer required to induce somatic embryogenesis (Karami et al. 2021; Wang et al. 2016; Zuo et al. 2002). Such studies show that transcription factors involved in somatic embryogenesis, especially those affected by hormone signaling, are closely related to each other. The overexpression of *BBM* transcription factor, for example, triggers somatic embryogenesis by activating *LEC1*, *LEC2*, *ABSCISIC ACID INSENSITIVE 3* (*ABI3)* and *FUS3* (Horstman et al. 2017). Therefore, identification of effective transcription factors in different biological processes can not only help to find more key genes during each process, but also leads to a better understanding of the pathways in a particular biological process.

The expression fold change of different genes in a biological process from transcriptome data is one way to identify different pathways. However, pathways with less expression differences but active in the biological process, may remain unknown so this method is limited in the comprehensive review of transcriptome data. In this regard, Gene Set Enrichment Analysis (GSEA) is an important approach to study transcriptome data comprehensively. GSEA uses common gene sets in different biological pathways or under the influence of common regulatory mechanisms as a criterion for evaluating transcriptome data and thus identifies pathways that are effective in a specific biological process such as somatic embryogenesis (Subramanian et al. 2005). Further study of these pathways will lead us to identification of key transcription factors in somatic embryogenesis. But the question is that which biological pathways are downstream of SE key transcription factors and whether these transcription factors and hormonal pathways play a role as target genes of auxin signaling during SE? In this study, a comprehensive study of transcriptome data in SE of *Arabidopsis* was performed using GSEA analysis and the relationships among potential transcription factors of hormonal pathways were investigated using further bioinformatics analyzes. Finally, it is predicted whether circadian rhythm transcription factors act under the influence of the auxin signaling pathway or are independently effective during somatic embryogenesis. This is the first report of GSEA study on Arabidopsis somatic embryogenesis and the first evidence of the role of circadian rhythm during induction and development of somatic embryos. As most different developmental events are established during formation of plant embryo, these findings help to get a new insight into developmental pathways during somatic embryogenesis and the whole plant life.

Materials and methods

GSEA analysis of transcription factors and genes affecting hormonal pathways

Existing RNA-seq data of *Arabidopsis* somatic embryogenesis (Wickramasuriya and Dunwell 2015) considering leaf tissue as control and three stages of SE (5, 10 and 15 days after embryo culture) were used as raw data in this study. Gene ID of *Arabidopsis* transcription factors was obtained from the Plant Transcription Factor Database (PlantTFBD) (Jin et al. 2015, 2017). Gene ontology information was also obtained from the TAIR database (Berardini et al. 2015) in order to compile a database of genes affecting hormonal pathways including biosynthesis, transmission, signaling, metabolism and response of each hormones consists of auxins, cytokinins, gibberellins, abscisic acid, ethylene, salicylic acid, brassinosteroids and jasmonates. The gene identifiers were used as reference gene set in GSEA analysis using GSEA software (Subramanian et al. 2005). Among the

genes in each pathways, core enriched genes were selected for the analysis of upstream regulatory sequences.

Identification of transcription factors targeting core-enriched genes and marker genes of SE

Upstream sequence analysis of the selected genes was performed separately by *Athamap* and *Pscan* online software (Ole Steffens et al. 2004; Zambelli et al. 2009). JASPAR 2020 (Fornes et al. 2020) was used as the database for transcription factor binding sites. Binding sites of all transcription factor families, including 33 families, were searched in 1000 bp upstream sequences of the genes. Among the genes in each pathways, core-enriched genes were selected for the analysis of upstream regulatory sequences.

Determination the biological process of candidate transcription factors and Kyoto encyclopedia of genes and genomes (KEGG) analysis

The *ClueGO* plugin from *Cytoscape* software was used to perform KEGG analysis and determine the gene ontology of potential transcription factors affecting somatic embryogenesis (Bindea et al. 2009; Kanehisa and Goto 2000; Paul Shannon et al. 2003). The network specificity was global and only biological pathways with p value less than 0.05 and containing at least five transcription factors were examined.

Protein–protein interaction (PPI) network

String online software (Szklarczyk et al. 2015) was used in order to obtain the PPI network between the candidate transcription factors and determine the key transcription factors. In preparing the PPI network, both physical and functional relationships of proteins were considered. Protein bindings of TFs up to a maximum of three protein mediators were considered and unbound proteins in the network were eliminated.

Somatic embryogenesis

Somatic embryogenesis was induced according to previous instruction (Gaj 2004). For this purpose, 12-day-old immature zygotic embryos were cultured on B5 medium supplemented with 5 µM 2,4-D, 0.2% (w/v) sucrose and 0.8% (w/v) agar. 14 days after embryo culture, the explants were transferred to MS medium without any hormones to allow the development of somatic embryos.

Gene expression analysis

qRT-PCR was performed to determine the expression fold of selected genes based on bioinformatics studies. Three biological and two technical replicates were used for gene expression analysis. Total RNAs were isolated in 3, 7 and 10 days after 2,4-D treatment using RiboEx RNA Extraction Solution (GeneAll biotechnology, Republic of Korea). cDNA was synthesized using Add Script cDNA Synthesis Kit (Add bio, South Korea). Real-time mixture reagents was prepared by ExcelTaq™ SYBR master mix (SMOBIO, Taiwan), 500 ng.µl^{-1} cDNA and gene-specific primers (Table 1) and the reaction was performed using Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Raw data were extracted by Bio-Rad CFX96 Manager software and the Relative gene expression level was calculated according to the $2^{-\Delta\Delta CT}$ method. Expression of *β-TUBULIN* as internal reference gene in the day of culture was used to normalize gene expression levels.

Statistical analysis

To perform GSEA analysis, the genes were ranked according to *Signal2noise* metric and the number of permutations was 1000. Finally, genetic pathways with p value less than 0.05 were selected in two compared phenotypic groups. qRT-PCR analysis was conducted with three biological replicates. The data of each biological replicates were obtained from the average of two technical replicates. Three plates contained 40 explants were considered as one technical replicate. Gene expression data were analyzed by one-way ANOVA (analysis of variance) followed by least significant difference (LSD) test at 0.05 probability level using JMP software (version 8.0.2, SAS Institute Inc., Cary, NC).

Results

Biological pathways under the influence of transcription factors involved in embryogenesis

The results revealed that SE transcription factors regulate several important biological pathways that are crucial to the development of somatic embryos. Development, response to

Table 1 List of primers used in RT-qPCR analysis

Gene name	Sequence
β-TUBULIN	F:5′-TGGGAACTCTGCTCATATCT-3′
	R:5′-GAAAGGAATGAGGTTCACTG-3′
CCA1	F:5′-TCTGTGTCTGACGAGGGTCGAATT-3′
	R:5′-ACTTTGCGGCAATACCTCTCTGG-3′
PRR7	F:5′-AGTGGAAGCGGAAGTGGAAG-3′
	R:5′-GAGGGCGTTGTTCTGCTAGT-3′
TOC1	F:5′-ATCTTCGCAGAATCCCTGTGATA-3′
	R:5′-GCACCTAGCTTCAAGCACTTTACA-3′

endogenous stimulus and metabolic regulation of RNA are among these pathways. It is interesting to note that response to hormones is one of the biological pathways activated by the enriched TFs at all stages of somatic embryogenesis, although some hormone response genes decreased in activity on the fifth day compared to the control treatment (yellow circles in Fig. S1). The results indicated that after receiving the auxin signal, other hormones play a role in inducing embryonic cells and their development into mature embryos, either independently or cooperatively. This issue underscores the importance of studying further hormonal pathways in detail.

Transcription factors affecting hormonal pathways during somatic embryogenesis of Arabidopsis

GSEA analysis showed that 16 of the 33 hormonal pathways were enriched during somatic embryogenesis of *Arabidopsis*. Figure S2 is the highest number of red dots as TFs significantly regulating genes, so it shows that the transcription factors regulating hormone-involved genes at the fifth day were significantly higher than those at other stages of somatic embryogenesis. These results indicate a large number of genes are involved in the induction of embryonic cells through hormonal pathways and also, several transcription factors regulate these genes. The highest enrichment was observed in genes responding to auxin, followed by genes responding to salicylic acid and jasmonates, but gibberellins (GA), brassinosteroides (BR) and ethylene (ET) were slightly enriched during somatic embryogenesis. It is noted that on the 15th day, auxin-related genes were negatively enriched. These data suggest that 2,4-D induces somatic embryogenesis and activates various downstream pathways, leading to the development of somatic embryos. Also, salicylic acid and jasmonate may contribute to this process (Fig. 1).

Some families of transcription factors were found to play a more effective role in the hormonal pathways involved in *Arabidopsis* somatic embryogenesis. Among 691 SE-hormonal transcription factors, MYBs have the largest proportion of SE-hormonal transcription factors which followed by AT-HOOKs (Fig. 2). Also, the most core-enriched genes involved in somatic embryogenesis were regulated by MYBs, followed by heat shock factors and AT-HOOKs. Based on the ratio of transcription factors to genes, HSF and AT-HOOK transcription factors regulate the greatest number of genes during somatic embryogenesis.

Identification of circadian rhythm transcription factors at the center of somatic embryogenesis PPI network

The protein–protein interaction network of SE-hormonal transcription factors helped to identify key regulators with more confidence. This also revealed more about their biological effects during somatic embryogenesis. It was found that the most of identified transcription factors are related to each other based on the PPI network. Functional annotation of the transcription factors was carried out through gene ontology and KEGG analysis

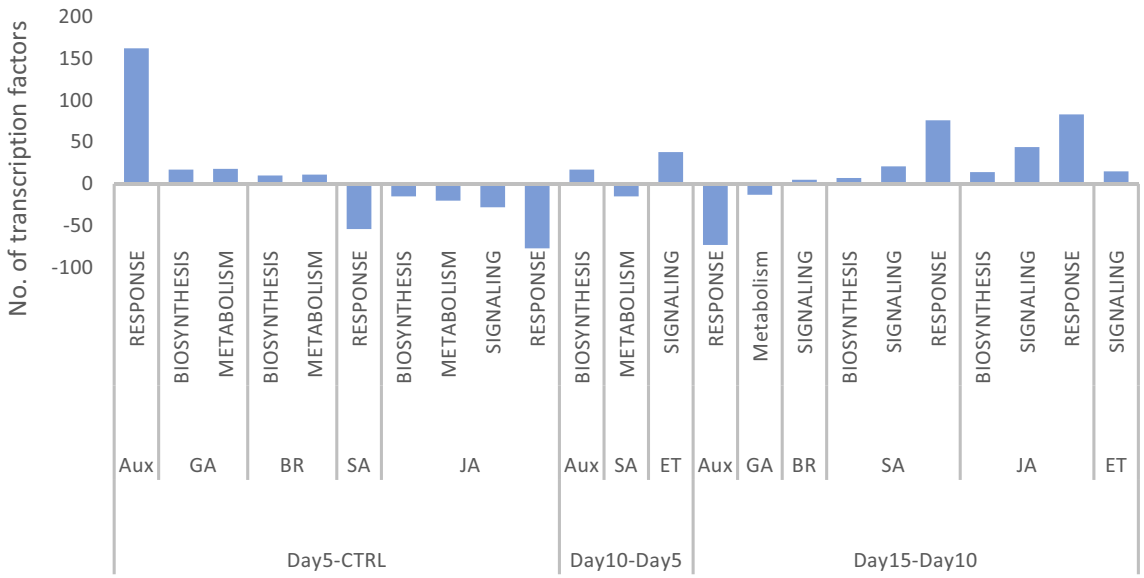

Fig. 1 Number of transcription factors regulating different hormone-involved genes during somatic embryogenesis of Arabidopsis. Positive and negative values, respectively, represent transcription factors regulating core-enriched genes with GSEA positive and negative normalized enrichment scores (*Aux* auxins, *GA* gibberellins, *BR* brassinosteroids, *SA* salicylic acid, *JA* jasmonates, *ET* ethylene)

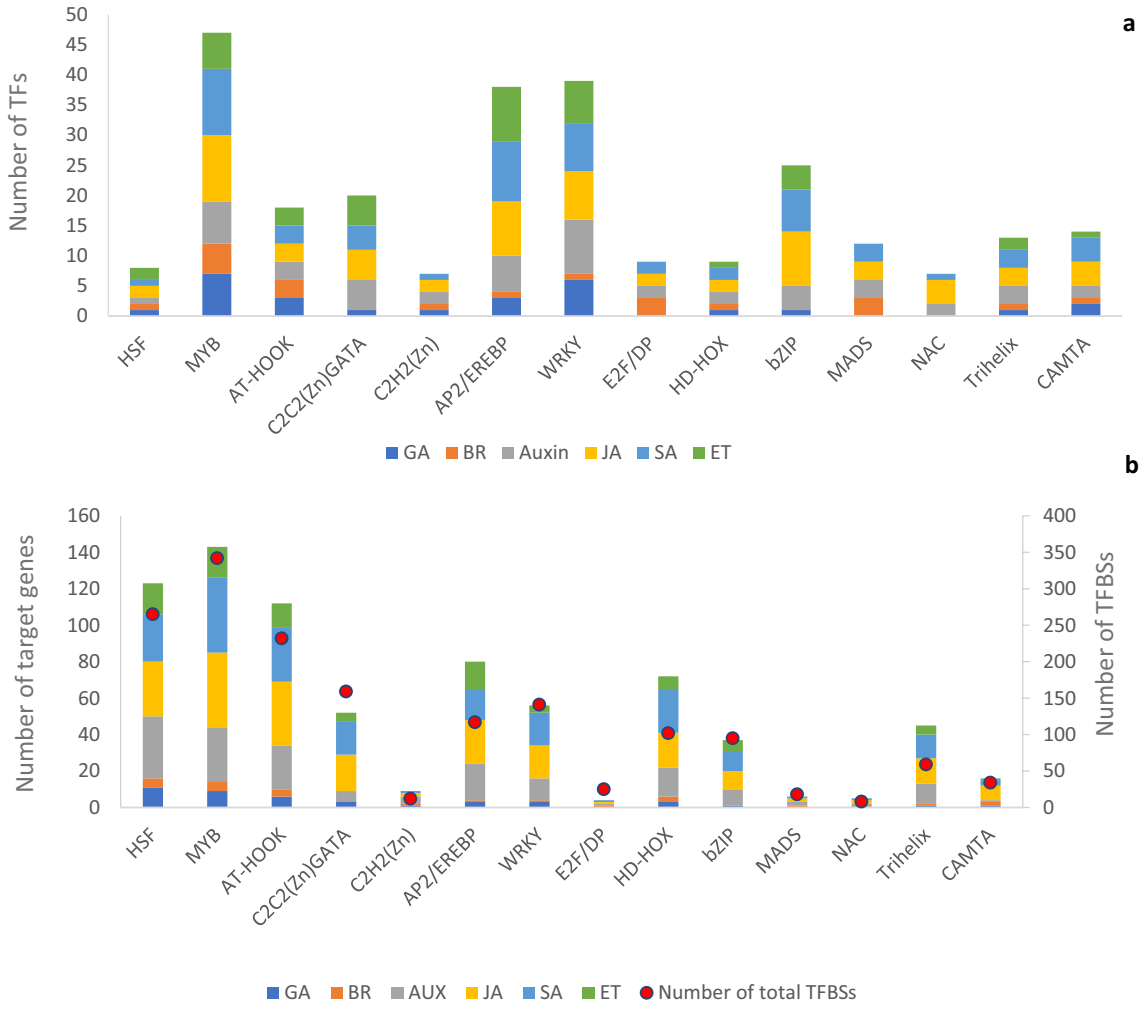

Fig. 2 The activity of transcription factors involved in hormonal pathways affecting somatic embryogenesis of Arabidopsis. **A** Number of effective transcription factors regulating SE core-enriched genes, **B** Number of their target genes and transcription factor binding sites in the upstream sequence of target genes (*Aux* auxins, *GA* gibberellins, *BR* brassinosteroids, *SA* salicylic acid, *JA* jasmonates, *ET* ethylene)

(Ma et al. 2019). *LATE ELONGATED HYPOCOTYL 1* (*LHY*), *TIMING OF CAB EXPRESSION 1* (*TOC1*), and *EARLY FLOWERING 3* (*ELF3*) were at the center of this PPI network and all of them belong to the MYB transcription factor family and play a key role in regulating plant circadian rhythm (Fig. 3). In addition, the association of the AT-HOOK and HSF transcription factor families in this PPI network suggests that these transcription factors may interact with the circadian rhythm of cells through regulating hormonal pathways, thereby playing a role in somatic embryogenesis. According to KEGG results, circadian rhythm was a common pathway among these transcription factors, as 12 genes or 33% of the transcription factors in the PPI network are involved (Table 2).

qRT-PCR analysis endorsed the activity of circadian rhythm pathway during somatic embryogenesis

In order to validate the results of bioinformatic studies, qRT-PCR analysis of three transcription factors at the center of PPI, *CCA1*, *PRR7* and *TOC1* was performed. It is interesting to note that gene expression of *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*), *PSEUDO-RESPONSE REGULATOR 7 (PRR7*) and *TOC1* at the center of PPI was significantly decreased 3 days after 2,4-D treatment but the highest expression of these genes was observed at 7th day. Therefore, activity of genes involved in circadian rhythm was firstly decreased due to in vitro conditions including 2,4-D but during somatic embryogenesis, it significantly increased

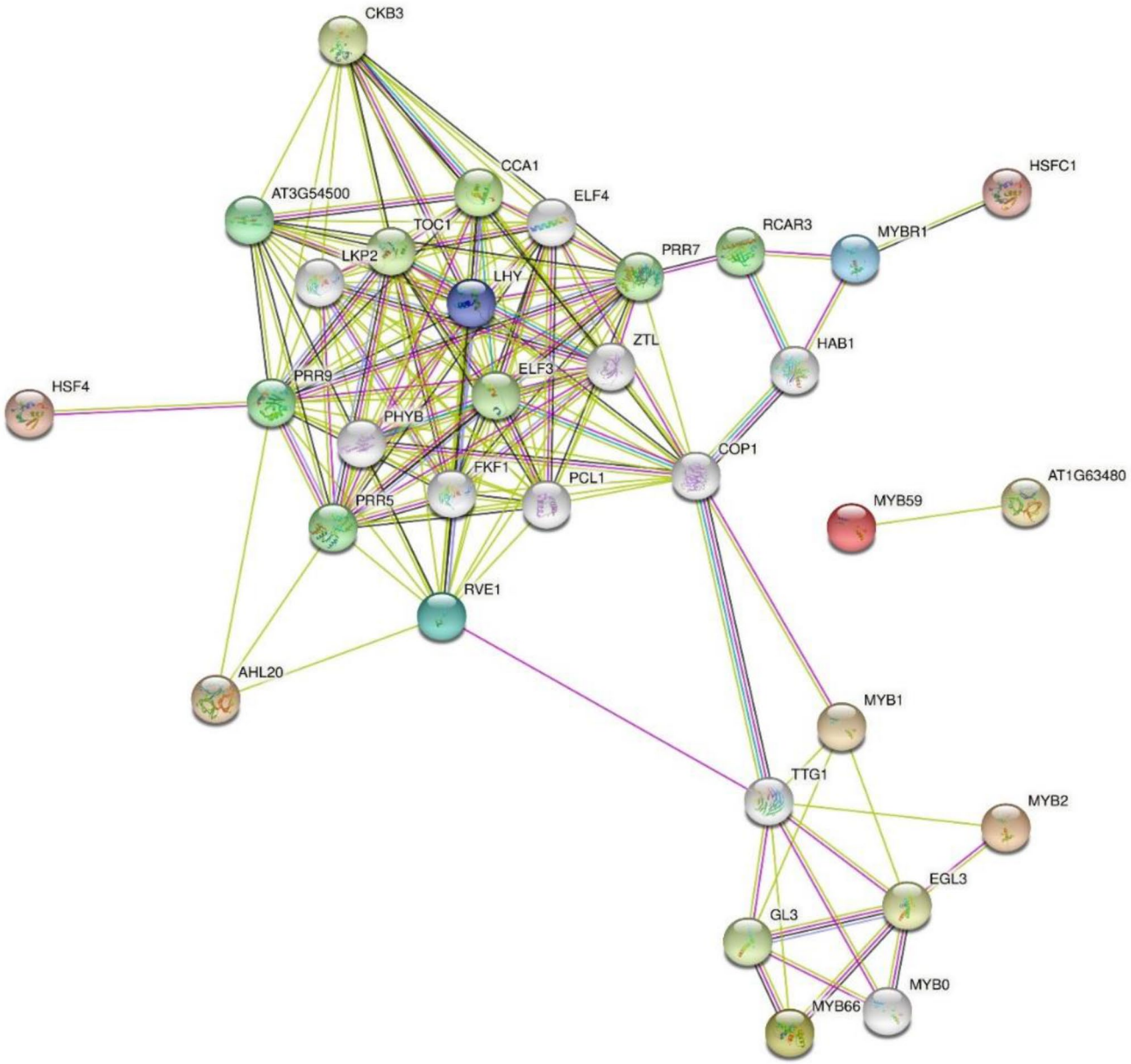

Fig. 3 Protein–protein interaction (PPI) network of key transcription factors regulating SE core-enriched genes using String. A main cluster composed of MYBs, HSFs and AHLs and a smaller connected cluster composed of MYBs are seen in the PPI network. Most proteins that are located in the main cluster involved in regulation of circadian rhythm

Table 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis results of MYBs, HSFs and AT-HOOKs which have at least one interaction in the protein–protein interaction network of Arabidopsis somatic embryogenesis (GO: Gene Ontology)

GOID	GO term	% Associated genes	No. genes	Associated genes found
KEGG:04712	Circadian rhythm	33.33	12.00	*CCA1*, *CKB3*, *COP1*, *ELF3*, *FKF1*, *LHY*, *PHYB*, *PRR5*, *PRR7*, *PRR9*, *TOC1*, *ZTL*

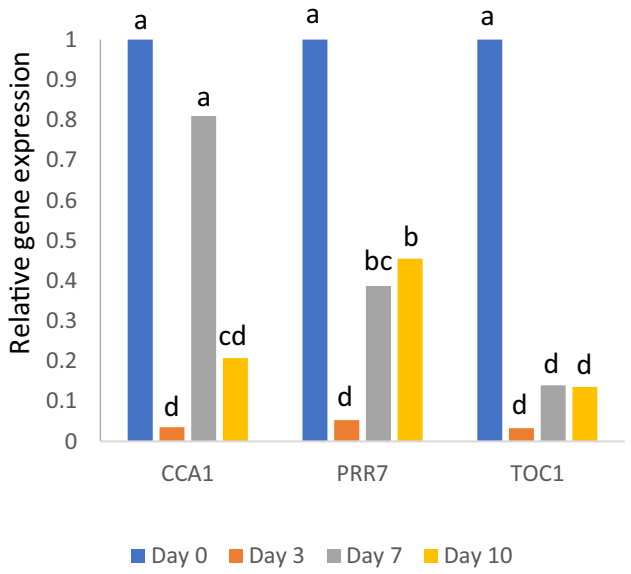

Fig. 4 Gene expression levels of circadian rhythm transcription factors during somatic embryogenesis of Arabidopsis

when embryogenic cells began to form. *CCA1* reached its maximum activity at the time of embryogenic cell formation and it decreased later but *PRR7* and *TOC1* had approximately the same level of activity during this time (Fig. 4).

Auxin signaling may regulates circadian rhythm transcription factors of somatic embryogenesis

Somatic embryogenesis in *Arabidopsis* occurs mainly by the application of auxins, particularly 2,4-D. As a consequence, transcription factors which play a role in somatic embryogenesis are likely to be directly or indirectly affected by auxin signaling. For this purpose, auxin response was investigated by exploring the binding sites of auxin response factors (*ARF*s) in the upstream sequence of SE key transcription factors. Interestingly, *ARF*s binding sites were identified in the upstream sequence of 11 transcription factors, including *CCA1*, *REVEILLE 1 (RVE1)*, and *LHY* (Table S1). These results suggest that the AT-HOOK, HSF, and MYB transcription factors associated with plant circadian rhythm not only play a key role as transcription factors in hormonal pathways during SE, but also they are directly influenced by auxin signaling. On the other hand, *LEC2* was identified as the target gene for transcription factors involved in circadian rhythm. It is one of the essential transcription factors to induce somatic embryogenesis. In previous studies, *ARF*s binding sites were also identified in the upstream sequence of *LEC1* and *LEC2*, which indicates that these transcription factors are directly regulated by auxin signaling (Wójcikowska and Gaj 2017). The findings indicate that auxin signaling affects somatic embryogenesis in different pathways such as circadian rhythm. However, it should be noted that the *ARF5*, *ARF6*, *ARF7*, *ARF8*, and *ARF19* activate the downstream response pathway to auxin, whereas the other *ARF*s inhibit it. In this study, both activator and inhibitor *ARF*s potentially affected SE-hormonal key transcription factors.

Discussion

Gene Set Enrichment Analysis revealed effective biological pathways during somatic embryogenesis that could not be detected with "Fold Change" studies and allows for a more comprehensive study than previously reported. In this study, during somatic embryogenesis, genes are found to perform a broad range of biological activities in response to hormones, particularly stress-related hormones including salicylic acid and jasmonates. Salicylic acid and jasmonates positively regulated plant physiological properties under stress conditions such as exposure to heavy metals but they protected plant cells from further damage at lower heavy metal treatments (Agnihotri and Seth 2019; Yadav et al 2022). Gupta and Seth (2021) have also reported that salicylic acid modulated chromium toxicity by improving antioxidant defense signaling. Therefore, some stress responses may be occurred during somatic embryogenesis but it should be alleviated at the most suitable level. It was also expected that gibberellins-related pathways were slightly enriched during somatic embryogenesis because different types of gibberellins have different biological functions in plant cells. In this regard, Igielski and Kepczynska (2017) have reported that among bioactive forms of gibberellins, only GA3 is supposed to increase embryogenic competence of *Medicago truncatula* cells in leaf explants. There is less evidence about effects of brassinosteroids (BR) on somatic embryogenesis. Guo et al. (2012) have demonstrated that BR signaling is dependent of the activity of *Somatic Embryogenesis Receptor Kinase*s (*SERK*s) but they improved somatic embryogenesis efficiency of *Coffea arabica* only in combination with cytokinins and they had no effect on embryogenic competence alone (Chone et al. 2018). A previous study on *Arabidopsis* SE transcriptome data has shown that genetic factors strongly influence hormone biosynthesis during somatic embryogenesis. Also, hormone biosynthesis and stress response are the two active biological pathways during maturation of *Arabidopsis* somatic embryos (Wickramasuriya and Dunwell 2015). It has been indicated that membrane lipids are converted to jasmonates during somatic embryogenesis and the activity of some effective genes in this pathway including *LIPOXYGENASE 1 (LOX1)* and *OXOPHYTODIENOATE-REDUCTASE 3* (*OPR3*) are increased during development of somatic embryos (Khadem et al. 2022). To induce somatic embryogenesis, plant cells

are treated with external auxin or with biological stress, and both methods increase levels of intracellular auxin (Korver et al. 2018; Shibasaki et al. 2009). In *Arabidopsis*, 2,4-D activates various transcription factors during SE via auxin signaling or stress-related pathways (Feher 2005; Wójcik et al. 2020), leading to the emergence of embryonic cells around the shoot apical meristem. 2,4-D also accumulates ROS compounds and stimulates stress hormones (Hansen and Grossmann 2000) resulting in the induction of somatic embryogenesis and its development (Nowak and Gaj 2016; Zavattieri et al. 2010). Also polyamines are suggested to mediate somatic embryogenesis through alterations in some stress-derived substances like nitric oxide in plant cells (Santa-Catarina et al 2007; Gupta et al. 2022). Approximately 50% of the TFs that are activated in downstream of 2,4-D are involved in stress-related pathways (Gliwicka et al. 2013). This suggests that stress-related hormones and intracellular auxins play a mutually beneficial role in SE induction and development. ABA promotes somatic embryogenesis by initiation of embryonic cells as competent cells of wheat have higher ABA levels (Jiménez 2005). Also Gretchen Hagen3 (GH3) is regulated by both IAA and ABA during somatic embryogenesis of *Arabidopsis thaliana* (Zhang et al. 2019). Somatic embryogenesis is influenced by some of the same biological pathways under the influence of different plant hormones.

After 2,4-D treatment, transcription factors activity is changed purposefully during somatic embryogenesis and it got reach to the highest level on the fifth day. In our study, embryonic cells were induced approximately seven days after 2,4-D treatment of immature zygotic embryos. Therefore, the activity of different genetic pathways is expected to change on the fifth day after 2,4-D treatment. Somatic embryogenesis was initiated by activating auxin-responsive transcription factors, but their activity decreased later. This indicates that auxin is necessary to induce SE, but further processes can go on without it. It has been shown that auxin is essential to induce embryonic cells, but its presence after SE induction makes deformed embryos due to its inhibitory effect on the differentiation of both shoot and root apical meristems (Horstman et al. 2017). Additionally, auxin is more biosynthesized at the early stage of zygotic embryogenesis (Robert et al. 2018). Therefore, formation of both zygotic and somatic embryos may demonstrate the same dynamic response to auxin.

During somatic embryogenesis, MYBs, HSFs, and AT-HOOKs had the greatest impact on core-enriched genes during somatic embryogenesis. Wickramasuriya and Dunwell (2015) reported that BHLHs followed by MYBs and AP2/EPEBPs were the top differentially expressed TFs during somatic embryogenesis of *Arabidopsis*. MYBs belong to a big family of transcription factors which some of them efficiently induce somatic embryogenesis. In *Arabidopsis*, *PGA37/MYB118* and its homolog, *MYB115* increase the efficiency of somatic embryogenesis by promoting cell reprogramming independent of *WUS* expression (Wang et al. 2009). Also, the activity of *AHL15* as a member of AT-HOOKs determines the plant cells fate in early stages of somatic embryogenesis (Karami et al. 2021). Because a number of genes in these transcription factor families have been proven to be involved in somatic embryogenesis, it is possible that more genes are involved in somatic embryogenesis, whose functions have not yet been identified.

Based on the results, circadian rhythm was the common pathway by which most of the transcription factors interact with each other to induce somatic embryogenesis. It also demonstrated that some circadian rhythm transcription factors mediate auxin signaling with some somatic embryogenesis genes, including *AGAMOUS-LIKE 15* (*AGL15*), *BBM*, *LEC1* and *LEC2*. In our experiment, gene expression analysis of *CCA1*, *PRR7* and *TOC1* also confirmed the activity of genes during somatic embryogenesis, as the expression of all the three genes was increased when embryonic cells formed. Therefore, circadian clock might has an essential role in induction and development of somatic embryogenesis. Some transcription factors involved in response to hormones, such as auxin response factors (*ARF*s), display a diurnal rhythm in their activity (Xue et al. 2020). Also, integrative role of plant hormones was also reported to regulate flower development via circadian rhythm pathways (Li et al. 2022). This suggests that the cellular circadian clock plays a significant role in regulating hormone activity in plant cells. Conversely, changes in the perception of hormones such as auxin, ethylene and cytokinin result in a change in the rhythmic accuracy of the cellular clock and its daily pattern (Robertson et al. 2009). Seed germination in *Arabidopsis* is also affected by the circadian clock. In the early stages of seed germination, *PRR5*, *PRR7*, and *PRR9* interact with *ABI5* and help this process to proceed properly (Yang et al. 2021). It has been indicated that auxin signaling in *Arabidopsis* is regulated by the cellular circadian clock, which is why most auxin responses occur at the end of the day (Penfield and Hall 2009; Xue et al. 2020). A disruption of *TOC1* activity has also prevented the response to GA and ABA (Penfield and Hall 2009). Therefore, the circadian clock might also affect the response to the other hormones during somatic embryogenesis. It is interesting to note that IAA did not significantly affect the rhythmic activity of *CCA1* and *TOC1*, but 2,4-D significantly reduced the rhythmic activity, since *CCA1* does not respond to changes in light conditions in the presence of 2,4-D (Covington and Harmer 2007). As regulators of circadian rhythm, CRYPTOCHROME 1 (CRY1) and PHYTOCHROME B (phyB) also directly interact with ARF6 and ARF8 proteins in a blue and red light-dependent manner (Mao et al. 2020). Both activating and inhibiting *ARF*s affected SE key transcription factors. This suggests

that self-regulation occurs in response to auxin signaling during somatic embryogenesis. It has also been found that the auxin signaling pathway can be self-regulated during *Arabidopsis* somatic embryogenesis by activating *AGL15*. *AGL15* inhibits the further activity of *TRANSPORT INHIBITOR RESPONSE 1/AUXIN F-BOX PROTEIN*s (*TIR1/AFB*s) as auxin receptors and increases the expression of *Auxin/Indole-3-Acetic Acid* (*AUX/IAA*) genes, which inhibit the activity of *ARF*s (Zheng et al. 2016). Therefore, the cooperation between plant hormones and cellular circadian rhythm is crucial for different physiological processes such as somatic embryogenesis.

Conclusion

The results of this study indicated that transcription factors modulated various biological pathways during somatic embryogenesis including development, response to endogenous stimulus and metabolic regulation of RNA. GSEA analysis of hormone-involved genes revealed that auxin, salicylic acid and jasmonates pathways had the most activity during somatic embryogenesis of *Arabidopsis*. Among SE effective transcription factors, MYBs, AT-HOOKs and HSFs regulated the highest number of genes involved in hormonal pathways. Also the most-interacted proteins at PPI network belonged to MYB transcription factors. Functional annotation and gene expression analysis revealed that SE hormonal key transcription factors collaborated to induce and develop somatic embryogenesis via circadian rhythm. Interestingly, *ARF*s binding sites were identified in the upstream sequence of 11 central transcription factors of PPI network. Therefore, these TFs might be the mediator of auxin signaling and some SE marker genes including *AGL15*, *BBM*, *LEC1* and *LEC2*. This study provides comprehensive information about effective genetic pathways and environmental clues involved in *Arabidopsis* somatic embryogenesis. Based on these results, two approaches can be considered in order to improve the efficiency of somatic embryogenesis: (1) modifying some key genes that control circadian rhythm in order to obtain a strong genetic background, and (2) altering some environmental conditions to force plant cells to reprogram their circadian clock in order to produce embryonic cells.

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Data availability All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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