Research Article

Conversion of Membrane Lipids to Jasmonates as a Key Pathway to Develop Somatic Embryos in *Arabidopsis thaliana*

Azadeh Khadem, Nasrin Moshtaghi*, Abdolreza Bagheri

[1] Department of Biotechnology and Plant Breeding, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran

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Abstract

Somatic embryogenesis encompasses the same set of various developmental processes similar to zygotic embryogenesis. The conversion of somatic cells to embryos also requires stages of differentiation and reprogramming of cells. Since somatic embryogenesis is a complex process, a comprehensive investigation is required to identify the effective gene networks and their interactions with environmental factors. As part of this study, bioinformatics tools and molecular studies were used to gain a better understanding of *Arabidopsis thaliana* somatic embryogenesis. The enriched pathways of somatic embryogenesis and their core-enriched genes were identified using gene set enrichment analysis. The results indicated that significant interaction between hormones helps to induce and develop somatic embryos. The gene ontology (including biological process, molecular function and cellular compartment) of core-enriched genes revealed that lipid storage and metabolism as well as stress response are the active biological pathways during somatic embryogenesis. In the protein-protein interaction network, TIR1/AFBs as auxin receptors exhibited the greatest number of interactions and proteins involved in lipid storage and metabolism acted as mediators between auxin receptors and ethylene perception. Also, Kyoto encyclopedia of genes and genomes analysis indicated that the metabolism of membrane lipids during somatic embryogenesis of *Arabidopsis* is primarily related to the biosynthesis of jasmonates and their derivatives. This process is initiated by Lypooxygenase proteins in the chloroplast, while Acyl-CoA oxidase 1 (ACX1) and Oxophytodienoate reductase 3 (OPR3) proceed this process in the peroxisome. The qRT-PCR analysis also confirmed the role of these genes during somatic embryogenesis, as the activity of these genes decreased at the beginning of 2,4-D treatment, but it increased during somatic embryogenesis. According to these results, jasmonates play an important role during somatic embryogenesis by mediating auxin signaling and stress response.

***Keywords*:** Bioinformatics, Hormone perception, Somatic embryo, Stress-related pathways

Introduction

During embryo formation, plant cells undergo a complex process for determining the cell fate and development (Lau et al., 2012). In order to germinate an embryo and grow as a seedling, all of the stages must be progressed precisely (de Vries & Weijers, 2017; Miransari & Smith, 2014). Plant cells also have totipotency features enabling the formation of embryos from a single somatic cell (Radoeva & Weijers, 2014). The somatic embryos have a similar structure to zygotic embryos (Loyola-Vargas & Ochoa-Alejo, 2016; Winkelmann, 1996), so it can be expected that the same cell fate and other developmental processes would occur during the formation of somatic embryos. These processes are influenced by environmental inducers and alter gene activity for somatic embryogenesis (Yang & Zhang, 2010). In order to understand how somatic embryos are induced from somatic tissues, the relationship between environmental inducers and genetic pathways of differentiation needs to be understood. Plant hormones play a crucial role in interacting with and adapting to their environment (Alazem & Lin, 2015). Plants control a wide range of physiological processes in cells by changing the amount of hormone production, transmission, and accumulation. Hormones have been commonly used for induction of somatic embryogenesis (Víctor M. Jiménez & Thomas, 2005). The competitive efficiency of somatic cells in embryogenesis can be predicted by examining the extent of hormone biosynthesis and perception (Jiménez, 2001). Therefore, it is important to identify accurately the role of hormones in exploring the network of genetic and environmental inducers of somatic embryogenesis. It has been difficult to identify all of the hormonal pathways, due to their wide range of effects. Also, most researches have focused on a

* Corresponding author's e-mail address: moshtaghi@um.ac.ir

single or small number of effective factors. As a result of these restrictions, some controversial results have been reported. Therefore, it is difficult to realize somatic embryogenesis precisely. In this regard, high throughput methods such as transcriptome analysis have contributed to obtain a comprehensive view about the physiological processes (Ward et al., 2012). Further, targeted analysis of high throughput data in a specific pathway such as somatic embryogenesis helps to identify other effective environmental factors involved in somatic embryogenesis which have been neglected. Through optimizing the environmental conditions, we will be able to drive the plant cells towards a desired goal, and enhance the performance of cells during a specific pathway such as somatic embryogenesis. With progress of bioinformatics, these methods reveal more information about the plant responses to the environment. In somatic embryogenesis of *Arabidopsis*, transcriptome analysis has also provided useful information. Fold-change investigations performed on high throughput data have clarified some effective pathways in somatic embryogenesis (Gliwicka et al., 2013; Wickramasuriya & Dunwell, 2015) but some several effective genes with less expression are neglected in the fold-change studies. Further information can still be obtained using more comprehensive and precise analysis such as gene set enrichment analysis (Subramanian et al., 2005). Therefore, based on data obtained from RNA sequencing of somatic embryogenesis in a previous report (Wickramasuriya & Dunwell, 2015), this study aimed to determine the activity of plant hormones and their mechanism of action during somatic embryogenesis. For this purpose, comprehensive investigation of the available data was done by GSEA, and then the core-enriched genes were studied more precisely through identifying protein-protein interactions. Finally, the expression of some enriched genes involved in conversion of membrane lipids to jasmonates pathway were studied during somatic embryogenesis.

Materials and Methods

In silico analysis

To perform the bioinformatics analysis, the log2 FPKM values of RNA high throughput sequencing at different stages of somatic embryogenesis of *Arabidopsis* were used (Wickramasuriya & Dunwell, 2015). GSEA analysis was done through the signal-to-noise criteria via 1000 permutations using GSEA 4.3 software (Subramanian et al., 2005). Different stages of somatic embryogenesis and *Arabidopsis* leaves were considered as phenotype labels. For precise investigation of the pathways affecting somatic embryogenesis, gene ontology of core-enriched genes was determined in three groups of biological pathways, molecular function, and cellular component. Also, Kyoto encyclopedia of genes and genomes (KEGG) analysis was done to achieve the metabolic pathways affecting somatic embryogenesis. Gene ontology and KEGG analysis were performed by ClueGO plugin in Cytospscae 3.9.1 software (Bindea et al., 2009). Kappa score was considered equivalent to 0.5, and only laboratory evidence of the criterion was investigated. Protein-protein interactions of core-enriched genes were done through determining the protein network via String online software. The network type was full string network, and only the proteins whose link score was above 0.4 were examined in the protein network.

Somatic embryogenesis

Twelve days after pollination of *Arabidopsis thaliana* cv Columbia-0, the siliques were collected and disinfected by sodium hypochlorite 1%. Next, immature embryos were withdrawn from inside the embryo sacs and cultured in MS culture medium containing 1 mg/L of 2,4-D and 7 g/L of agar. Two weeks after culture, the explants were transferred to $^1/_2$ MS medium without 2,4-D to develop embryos and grow seedlings (Figure 1).

qRT-PCR analysis

Immature zygotic embryos were collected at four stages: the day of culture (control), third day (pre-somatic embryogenesis), seventh day (time of induction of somatic embryogenesis), and 10th day (post-somatic embryogenesis). It was considered that somatic embryogenesis started with the formation of callus in the regions around the shoot apical meristem (Figure 1). They were then frozen by liquid nitrogen and kept at -80°C. RNA extraction was performed by RiboEX solution according to the manufacturer's instructions. The concentration of RNAs was normalized after measuring OD using Epoch microplate spectrophotometer (Epoch, USA), and the first strand of cDNA was synthesized by cDNA synthesis kit (AddBio, Korea). To perform qRT-PCR analysis, the reaction mixture contained 1 µL of cDNA (500 $\mu g.ml^{-1}$), 1 µL of each specific primers of the studied genes (Table 1), 10 µL of qPCR master mix (Yekta Tajhiz Azma Co.), and 7 µL sterilized distilled water. qRT-PCR was done using Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) and the data was

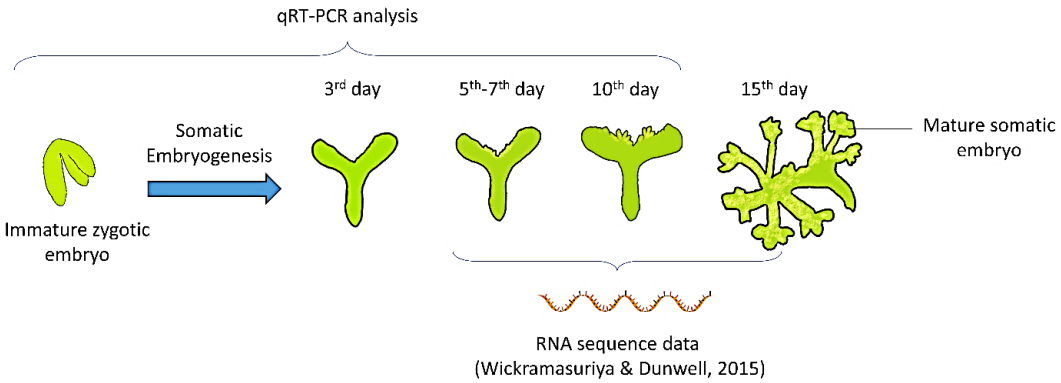

Figure 1. Stages of *Arabidopsis* somatic embryogenesis. This picture depicts the stages of somatic embryo development considered in this study.

Table 1. List of primers used in qRT-PCR analysis

Gene name	Locus ID	Sequence
β-TUBULIN	AT5G12250	Forward: 5'-TGGGAACTCTGCTCATATCT-3' Reverse: 5'-GAAAGGAATGAGGTTCACTG-3'
LOX1 (Lipoxygenase 1)	AT1G55020	Forward: 5'-TCTGTGTCTGACGAGGGTCGAATT-3' Reverse: 5'-ACTTTGCGGCAATACCTCTCTGG-3'
LOX6 (Lipoxygenase 6)	AT1G67560	Forward:5'-AGTGGAAGCGGAAGTGGAAG-3' Reverse: 5'-GAGGGCGTTGTTCTGCTAGT-3'
ACX1(Acyl-CoA oxidase 1)	AT4G16760	Forward:5'-ATCTTCGCAGAATCCCTGTGATA-3' Reverse: 5'-GCACCTAGCTTCAAGCACTTTACA-3'
OPR3 (Oxophytodienoate reductase 3)	AT2G06050	Forward: 5'-ACGGACCACTCCCGGCGGTTTTC-3' Reverse: 5'-CGTGAACTGCTTCCACAACTT-3'

analyzed with Bio-Rad CFX96 Manager and Excel software. *β-TUBULIN* was considered as the internal reference gene.

Results and Discussion

Considerable change of the activity of hormonal pathways during somatic embryogenesis

The GSEA results indicate that activity of hormones has changed mostly at the beginning and end of somatic embryogenesis. The activity of more hormonal pathways has decreased after five days of 2,4-D treatment, but it increased at the end of somatic embryogenesis. According to these data, response to auxin had considerable activity at the beginning of 2,4-D treatment, but it decreased after somatic embryo formation and during the growth of seedlings. During somatic embryogenesis, jasmonates showed the greatest change of activity, followed by gibberellin and salicylic acid (Figure S1). It is also expected from investigating different stages of somatic embryogenesis. The induction of embryonic cells in immature *Arabidopsis* embryos began on the fifth day after treatment with 2,4-D by starting formation of embryonic callus in shoot apical meristem, and on the seventh day, the entire embryo induction region around the shoot apical meristem began generating embryos. With the conversion of somatic embryos into seedlings, the activity of genes affecting differentiation and growth pathways decreased on the 15th day of 2,4-D treatment. These data indicated that somatic embryogenesis was characterized by a significant interaction between hormones. It has also been found that plant growth regulators play an important role in somatic embryogenesis, as knocking down of key genes of these pathways or using inhibitors of hormone perception causes impaired somatic embryogenesis (Bai et al., 2013; Chen & Chang, 2003; Nowak et al., 2015). Therefore, they should be examined more closely in terms of how they affect somatic embryogenesis.

Activity of biological processes under the influence of hormone perception

The gene ontology of core-enriched genes was examined in three groups of biological pathway, molecular function and cellular component. The results generally indicated that most of the proteins involved in somatic embryogenesis had either enzymatic or binding activity. Furthermore, cellular component of the genes showed that plastids, which are mostly composed of chloroplast and storage plastids, had a greater role than other intracellular organelles during somatic embryogenesis. The notable point on the 5th day compared to the control has been seed maturation as one of the active biological processes. This process associated with storage of lipids had considerable activity in the initiation of somatic embryogenesis. It suggested that after five days of the 2,4-D treatment, embryonic cells have formed, and the embryos have tended to mature. In this way, the lipid storage pathway became activated as a prerequisite for growth and maturity of the somatic embryo. Furthermore, the major activity reduction on 5th day is associated with pathways related to photosynthesis (Figure 2). It suggested that photosynthesis and lipid storage may be related to each other during the induction and growth of somatic embryos. During the maturation of zygotic embryos, lipid storage depends on light and oxygen which is released from photosynthesis. For growth and survival, zygotic embryos accumulate lipids as a source of energy when intracellular oxygen is low (Rolletschek et al., 2005). Somatic embryos are structurally and physiologically similar to zygotic embryos (Leljak-Levanić et al., 2015), therefore it is possible that they also store lipids to provide energy for growth. In addition, stress response was identified as another effective biological process during somatic embryogenesis. The response to stress decreased during the first 10 days of somatic embryogenesis. After that, in spite of removal of 2,4-D from the culture medium, the activity of genes involved in the stress response was increased. In previous studies, 2,4-D induces stress responses in plant cells, and these responses together with auxin signaling of 2,4-D affect the growth and development of somatic embryos (Philipsen & Offringa, 2017; Karami & Saidi, 2010). The results suggested that 2,4-D may indirectly affect stress induction during the maturation of somatic embryos of *Arabidopsis* by activating other physiological processes within the cells.

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Close relationship between plant hormones and the lipid and storage proteins in the PPI network

In order to identify the relationship of biological processes involved in somatic embryogenesis, the protein-protein interaction network of core-enriched genes was determined. The PPI network was centered on genes associated with auxin signaling and transmission. Transport inhibitor response 1/Auxin signaling F-BOXs (TIR1/AFB) receptors, which possess the highest level of protein communication, bind to auxin and activate downstream pathways through ubiquitination of Auxin/Indole-3-acetic acid (AUX/IAA) proteins (Salehin et al., 2015). Close to the PPI center was another protein cluster, which is related to transcription factors associated with ethylene such as *Ethylene overproducer 1* (*ETO1*), *Ethylene response 2* (*ETR2),* and *Ethylene insensitive 4* (*EIN4)* (Figure 3). Therefore, ethylene might play a key role during somatic embryogenesis, and its downstream pathways interact closely with auxin signaling. The genes associated with lipids and storage proteins are located between these two protein clusters (Figure 3). It suggested that in addition to auxin, other plant hormones also affect lipid storage and stress induction. It has been reported that when plant cells are exposed to stress, they convert membrane lipids to produce signaling lipids such as phosphatidic acid and free fatty acids (Hou et al., 2016).

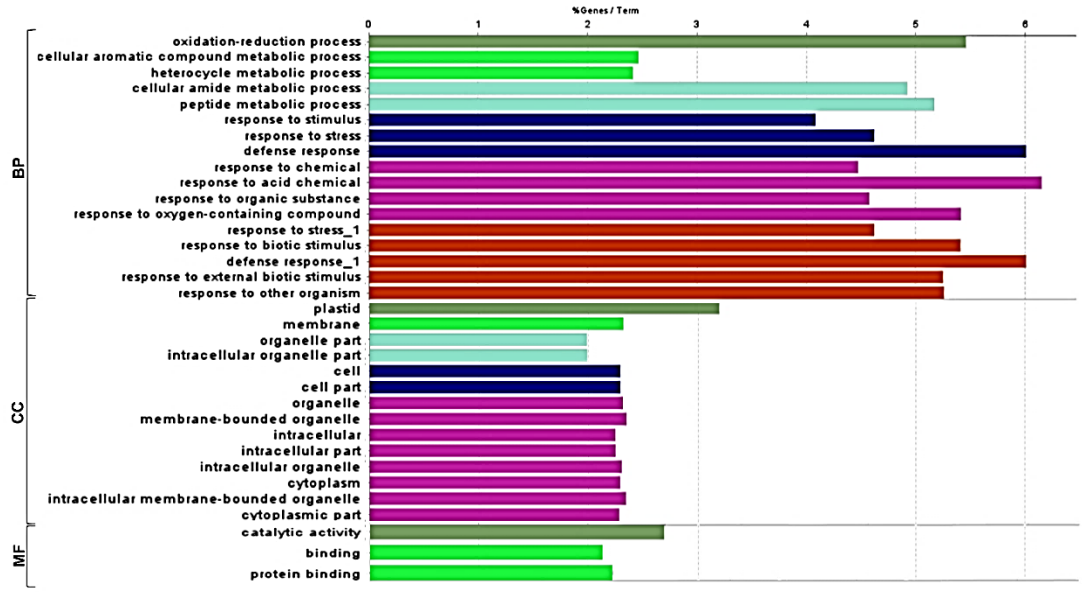

Figure 2. Gene ontology of core-enriched genes of *Arabidopsis* somatic embryogenesis. In each aspect of gene ontology, each color represents a specific gene ontology term.

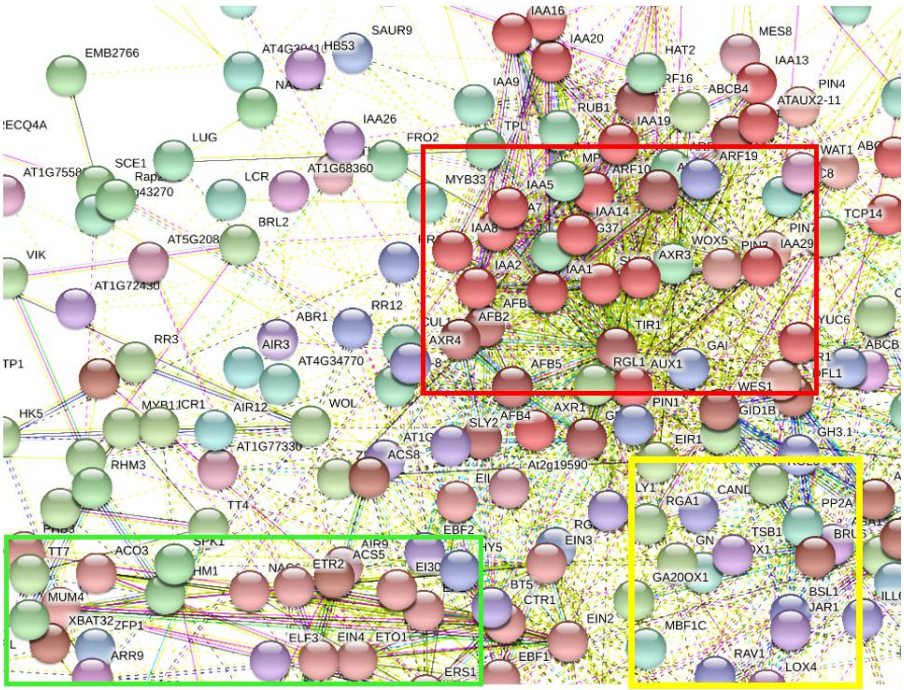

Figure 3. The central region of protein-protein interaction network of core-enriched genes. The red and green rectangles respectively represent auxin and ethylene perception. The genes associated with lipids and storage proteins are located in the yellow square.

It activates lipid-dependent signaling pathways, which results in induction of stress response (Okazaki & Saito, 2014). It has been found that any defects in function of proteins that are involved in metabolism of phospholipids impaired some biological processes such as embryogenesis and response to stress (Hou et al., 2016). Therefore, genes involved in lipid biosynthesis and metabolism may function as mediators between hormone signaling and stress induction during somatic embryogenesis.

Lipid metabolism as an active metabolic pathway during somatic embryogenesis

The KEGG analysis of metabolic pathways involved in somatic embryogenesis revealed that out

of eight active metabolic pathways in somatic embryogenesis. In this concept, four of them were involved in lipid metabolism and biosynthesis, and three of them were involved in hormone biosynthesis and signaling. The allocation of half of the active metabolic pathways to lipids in somatic embryogenesis indicated that their production and accumulation were essential for somatic embryogenesis. Also, more than half of the genes related to linoleic acid metabolism, including the *Lypooxygenase* (*LOX*) gene family, are involved in somatic embryogenesis (Table 2). Plant cells synthesize long chain fatty acids from linoleic acid and α-linolenic acid which are unsaturated fatty acids found in the cell membrane (Alché, 2019). This process is carried out by *LOX* gene family. It can be concluded that this gene family influenced the accumulation and conversion of lipids in plants. Also, these genes cooperate with *Oxophytodienoate reductase 3* (*OPR3*) and *Acyl-CoA oxidase 1* (*ACX1*) to convert α-linolenic acid to jasmonates (Zhai et al., 2017). It indicated that stress induction by 2,4-D treatment occurs gradually through lipid biosynthesis and metabolism. Since these genes play a significant role in somatic embryogenesis, changes in their activity should also be investigated during somatic embryogenesis.

Activity of the genes responsible for conversion of membrane lipids to jasmonates during somatic embryogenesis

Based on the results of bioinformatics analysis, the expression of *LOX1*, *LOX6*, *ACX1*, and *OPR3* genes was examined during the somatic embryogenesis. *LOX1* and *LOX6* are involved in the initial steps of α-linolenic acid conversion to jasmonates in the chloroplast, while *ACX1* and *OPR3* coordinate the oxidation process to produce the final compound of jasmonates in the peroxisome (Ali & Baek, 2020; Wasternack & Kombrink, 2010). The results of qRT-PCR analysis indicated that the expression of all examined genes decreased considerably after the treatment of immature embryos with 2,4-D, but increased during somatic embryogenesis. When somatic embryogenesis is induced by 2,4-D treatment, *ACX1* and *OPR3* reached their maximum expression. After that, their activity gradually decreased. However, the ascending trend of the expression of *LOX1* and *LOX6* genes persisted during the growth of seedlings (Figure 4). The results suggested that membrane lipids convert to jasmonates during somatic embryogenesis and activate downstream pathways of stress induction. Stress is one of the factors affecting reprogramming of plant cells and production of somatic embryos (Zavattieri et al., 2010). Induction of stress improved efficiency of production of somatic embryos and in some cases, induced somatic embryogenesis alone (Fehér, 2015; Ikeda-Iwai et al., 2003). Also, jasmonates has been reported as the intermediate hormone between stress and plant regeneration. Mira et al (2016) reported that jasmonates increase the expression of *JAZ1* and decrease the expression of *MYC2* which results in accumulation of IAA in plant cells. These processes have eventually resulted in successful somatic embryogenesis of *Arabidopsis* (Mira et al., 2016).

Table 2. Metabolic pathways under the influence of core-enriched genes of Arabidopsis somatic embryogenesis.

Gene Ontology ID	Gene Ontology Term	% Associated Genes	Number of Genes	Associated Genes
04016	MAPK signaling pathway	14.62	19	*ACS6, ATMPK8, EBF1, EBF2, ...*
04075	Plant hormone signal transduction	26.74	73	*ARR11, ERF1, IAA2, JAZ1, ...*
00591	Linoleic acid metabolism	55.56	5	*LOX1, LOX2, LOX3, LOX4, LOX6*
00592	alpha-Linolenic acid metabolism	41.67	15	*ACX1,LOX2, LOX3, OPR3, ...*
00901	Indole alkaloid biosynthesis	40.00	4	*ACL, MES1, MES3, MES9*
00904	Diterpenoid biosynthesis	50.00	11.00	*GA20OX2, GA20OX3, GA2OX2, ...*
00380	Tryptophan metabolism	32.65	16.00	*NIT1, SOT16, TAA1, YUC1, ...*
00966	Glucosinolate biosynthesis	26.09	6.00	*CYP79B2, CYP79B3, IMD1, ...*

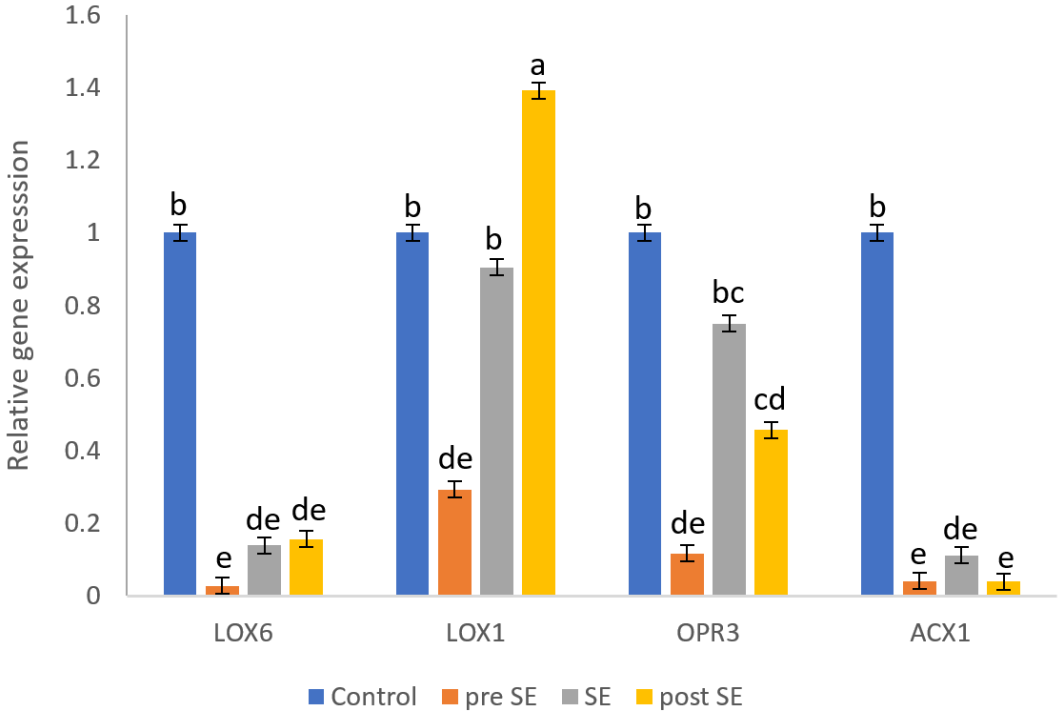

Figure 4. Relative gene expression level of some core-enriched genes involved in conversion of membrane lipids to jasmonates. *LOX6: Lypooxygenase 6*, *LOX1: Lypooxygenase 1*, *OPR3: Oxophytodienoate reductase 3*, *ACX1: Acyl-CoA oxidase 1*. Values with same letters are not significantly different at P<0.05.

Conclusion

In this experiment, it was found that somatic embryogenesis is the result of the interaction of various biological pathways involved in differentiation and development. Based on the results of this study, genes related to biosynthesis and metabolism of lipids were associated with hormones and stress signaling. In bioinformatics analysis, *LOX1*, *LOX6*, *ACX1*, and *OPR3*, which are involved in biosynthesis of jasmonates, were identified as the key genes in somatic embryogenesis of *Arabidopsis*. qRT-PCR analysis also confirmed the bioinformatic results as the expression of these genes changed during somatic embryogenesis. These results suggested that jasmonates function as one of the effective factors in somatic embryogenesis and in cooperation with other hormones, they activate downstream pathways such as stress induction. The process ultimately results in the induction and development of *Arabidopsis* somatic embryos.

A number of environmental stress factors, such as mechanical damage and pathogen attack, induce jasmonates. Therefore, the results of this study can be used in further studies to improve the efficiency of somatic embryogenesis by applying environmental stimuli or genetically modifying genes involved in jasmonates production and their downstream pathway.

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Competing interests

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

Abbreviations:

GSEA: Gene Set Enrichment Analysis
PPI: Protein-Protein Interaction
KEGG: Kyoto Encyclopedia of Genes and Genomes
GO: Gene Ontology

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