DOI: https://doi.org/10.53040/cga12.45 UDC: 575.2:582.2/.3:582.998.2

TEMPORAL GENE EXPRESSION PATTERNS DURING MICROSPOROGENESIS IN SUNFLOWER

Angela PORT, 0000-0003-3994-8918
angela.port@usm.md

Center of Functional Genetics, Moldova State University,
Chisinau, Republic of Moldova

Abstract: *The objective of this study was to characterize the transcriptional variation of a selected set of genes in fertile sunflower (Helianthus annuus) anthers by quantifying their expression levels using Ral-time PCR across four stages of microsporogenesis. The results reveal that over 90% of the analyzed genes exhibit dynamic or oscillatory expression patterns, reflecting stage-specific regulatory responses. A moderate increase in gene expression variability during the tetrad and microspore stages, compared to the earlier phases of microsporogenesis, such as pachytene and meiotic division, was observed. The prevalence of dynamic expression highlights complex transcriptional regulation required for maintaining physiological homeostasis during microsporogenesis. These results contribute to the functional validation of candidate genes and offer insights into the genetic and physiological mechanisms underlying male fertility in sunflower.*

Keywords: candidate genes, gene expression dynamics, microsporogenesis, qPCR, sunflower.

INTRODUCTION

Microsporogenesis represents a critical phase in the formation of the male gametophyte, requiring extensive transcriptomic reprogramming to coordinate cellular differentiation and structural transitions. Although co-regulation of gene expression is a common feature of stress and developmental responses [1], it remains highly gene-specific and dependent on the physiological context.

Over the past years, numerous transcriptomic studies have been conducted in various crop species, including sunflower (*Helianthus annuus*), focusing on tissue-specific gene expression under diverse experimental conditions. These efforts have primarily explored plant responses to abiotic [2, 3] and biotic stressors [4, 5], as well as strategies aimed at improving fertility and yield performance [6, 7]. However, despite the growing interest in sunflower genomics, investigations of differential gene expression specifically focused on male reproductive development remain relatively limited compared to those conducted in other major crop species.

The objective of this study was to characterize the transcriptional variation of a selected set of genes across different stages of microsporogenesis in fertile sunflower anthers, in order to reveal stage-specific patterns in gene expression dynamics.

The analysis of candidate gene expression represents is an essential step in the experimental validation and functional annotation of genes, particularly in the case of species whose genome is partially functionally characterized, as is the case of Helianthus annuus. Also, expression data can significantly contribute to refining existing gene annotations and deepening our understanding of their biological roles.

MATERIALS AND METHODS

Plant materials. Anthers of sunflower plants from the inbred line SW501 (Magroselect ASP, Soroca, Republic of Moldova) were used as the biological material. For accurate selection of anthers corresponding to specific stages of microsporogenesis, florets and anthers were subjected to cytological and histological analysis, as described in the previous study [8].

RNA isolation and RT-qPCR analysis. Total RNA was isolated from anthers at four microsporogenesis stages: pachytene (P), meiotic divisions (D), tetrads (T), and young microspores (M), using TRIzol reagent (Invitrogen, Thermo Fisher Scientific), according to the manufacturer's protocol. cDNA synthesis was performed using RevertAid Reverse Transcriptase (Thermo Fisher Scientific) with arbitrary hexamer primers and oligo(dT)18 primers. Gene expression analysis was carried out with the Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific).

Relative gene expression levels were calculated using the $2^{\wedge-\Delta\Delta CT}$ method, with *actin* (GenBank: AF282624.1) as the reference gene. Expression differences (fold change, FC) between microsporogenesis stages were considered biologically relevant at $FC \geq 1.5$ and statistically significant at $p \leq 0.05$, according to ANOVA, and Bonferroni correction.

Gene expression analysis covered 108 genes, mostly candidates with putative functions, grouped into five functional categories based on Gene Ontology terms.

RESULTS AND DISCUSSIONS

The variation in gene expression levels in the anthers of fertile sunflower plants under normal physiological conditions reflects the internal dynamics of male reproductive development. Thus, heatmap analysis of relative expression values across different phases of microsporogenesis (pachytene, meiotic divisions, tetrads, and microspores) revealed genes with similar expression patterns, indicating the existence of co-regulated gene groups, which suggests their involvement in common biological processes. Additionally, the chromatic contrast distribution on the expression map reveals a certain degree of heterogeneity both among genes and between the phases of microsporogenesis. It is also observed that gene expression levels are higher for a larger number of genes in the microspore phase compared to the other three analyzed phases.

For a detailed characterization of transcript content variation in anthers

depending on the phases of microsporogenesis, boxplot representations of relative expression data and agglomerative hierarchical clustering analysis were used (Figure 1).

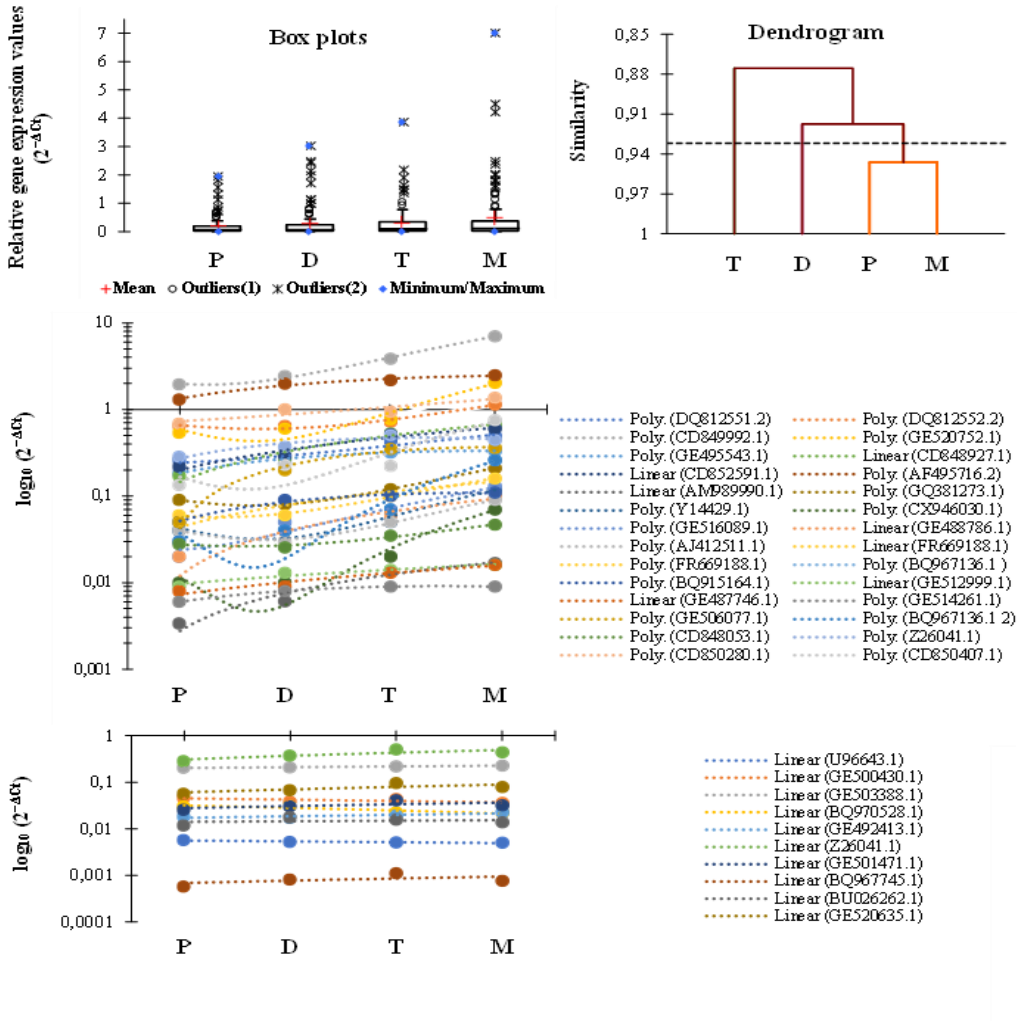


Figure 1. Variation in gene expression across microsporogenesis stages in sunflower anthers of the SW501 homozygous fertile line (boxplots, UPGMA-based hierarchical clustering and regression trend lines of relative expression values)

The relatively close medians observed in the boxplots suggest moderately variable gene expression between phases. At the same time, the extreme values (minimum and maximum) indicate a more pronounced dispersion of transcript levels, particularly in the tetrad phase, compared to the pachytene and division phases. The tetrad formation phase

is characterized by a higher degree of value dispersion, but with a symmetric distribution around the median, suggesting heterogeneous, but balanced gene expression. In contrast, the microspore phase shows the greatest variability, reflected by a high number of extreme values, indicating divergent expression levels both in comparison to other genes and to the expression of the same genes in earlier stages.

The hierarchical clustering analysis confirms the differentiation of gene expression data in the microspore phase, which is separated first, followed by the tetrad phase, which in turn shows a low degree of similarity with the pachytene and meiotic division phases. However, the overall high degree of similarity between phases (above 0.90) indicates a well-defined pattern of gene interaction and suggests the presence of fine regulatory mechanisms of gene expression during microsporogenesis in a physiologically normal (homeostatic) conditions.

In conclusion, the comparative analysis of gene expression values distributions across different phases of microsporogenesis in fertile anthers of the homozygous plants SW501 line, using heatmap, boxplot representations, and hierarchical clustering analysis, revealed:

- a moderate increase in gene expression variability during the tetrad and microspore stages compared to the earlier phases of microsporogenesis, such as pachytene and meiotic division,
- groups of co-expressed genes, which may be attributed to their involvement in the same metabolic pathways or physiological processes, supporting the validity of the functional category association results. Moreover, this gene expression correlation (co-expression) may reflect common regulatory factors or synchronized responses to internal signals associated with specific physiological states.

To gain a deeper understanding of the temporal dynamics of the studied gene expression during microsporogenesis and to identify genes with functional roles in the key developmental stages of microsporocytes and microspores, genes were classified based on natural patterns of expression variation under normal physiological conditions. Such an approach allows for the distinction between genes with relatively constant expression and those with variable expression (fluctuating, increasing, or decreasing), providing insights into the metabolic or regulatory processes in which these genes are involved.

Thus, in the next stage of the analysis, expression trends across the four studied phases (P, D, T and M) were examined, and the genes were clustered into three groups: those with progressively increasing expression levels, relatively constant expression, and oscillatory expression, potentially involved in the regulation of transient or cyclical processes and, implicitly, in physiological responses to various intra- or intercellular signals.

Group 1: Genes with a progressively increasing expression profile. Out of the 108 genes analyzed, 27 exhibited a progressively increasing expression pattern. These

genes belong to all five functional categories examined in this study, as follows:

- **category I – Redox homeostasis and cellular stress response:** *MSD1, MSD2, APX1, TRX2, BADH, GPX1, GSH, DHAR2, MATE.*
- **category II – Signal perception and transduction, hormonal interactions:** *NDPK1, NDK2, NDPK3, GA20-OX1, IAA27, PP2A.*
- **category III – Growth and development of reproductive structures:** *PEL18, TMT2, PME2.*
- **category IV – Mitochondrial activity and biogenesis:** *UCP1, ACP2, GAPB, VDAC, ATAD1B.*
- **category V – Genome integrity maintenance, DNA replication and repair, cell division:** *RAN3, NTH, MUTYH, MSH1*.

These genes exhibit a clear upward trend in transcript levels over time (P–M) (Figure 1). Additionally, based on their expression level values, the genes were conventionally divided into two subgroups:

- *Subgroup 1.1* includes genes that showed relatively high minimum and maximum expression values (relative expression range: 0.1 – 7.0). The genes in this subgroup are: *MSD1, MSD2, APX1, TRX2, NDPK1, NDPK3, TMT2, UCP1, ACP2, VDAC, RAN3, PP2A.*
- *Subgroup 1.2* includes genes with significantly lower relative expression levels—approximately 10- to 20-fold lower compared to the subgroup 1.1 (the relative expression range: 0.003–0.40). The genes in this subgroup are: *BADH, GPX1, GSH, DHAR2, MATE, GA20-OX1, IAA27, PEL18, PME2, GAPB, ATAD1B, NTH, MUTYH, MSH1, NDK2.*

Group 2: Genes with relatively constant expression. This group includes 9 genes that exhibit a relatively stable expression profile across the analyzed phases (fold change < 1.5), with relative expression values ranging from 0.0006 to 0.25. Their distribution across functional categories is as follows: five genes from category II (*RLPK, MRK3, GID1C, PERK1, CBL2*), two genes from category III (*MC1, GCL1*), one from category IV (*SDH1*) and one from category V (*MTSSB*).

Group 3: Genes with oscillatory expression. This group includes the largest number of genes (72), showing significant variation in expression across phases:

- **category I – Redox homeostasis and cellular stress response:** *SOD1CU, SOD2CU, PRXIIF, CATA2, CATA3, GST, CATA4, APX4, CATA, NTRB.*
- **category II – Signal perception and transduction, hormonal interactions:** *CAM, NTF4, RAC1, TIR1, GGPS. PI3K, SERK1, SERK2, MAPKKK7, MKP1, ACA9, GA2OX1, GAI1, RGL2, ABP1, GID2, TMK1, GDI1*
- **category III – Growth and development of reproductive structures:** *AG, BGAL5, EXPA8, EXPA1, GPAT1, DAD1, BAXI1, MC4, HXK1, HOG1, LSD1, KN1, KN2, HB-8, CDF3, MYB1, MYB-PHL7, MYB4, HEN1, GSL12, XTH6, BAG3, MYC2, XTH23,*

DRH.

- **category IV – Mitochondrial activity and biogenesis:** *NDB1, NDC1, DL1C, CYTC-1, NDA2.*
- **category V – Genome integrity maintenance, DNA replication and repair, cell division:** *HIS1, WHY2, Histone H3.2, DYAD, MMH-1, OGG1, MSH5, OSB2, RECA3, BFN1, SKP1-1B, CDC2A, H2B, PCNA.*

Based on expression level values, these genes were conventionally classified into three subgroups.

Subgroup 3.1 includes genes with expression levels ranging from 0.1 to 5,0: *SOD1CU, SOD2CU, CAM, HOG1, DRH, PRXIIF, SKP1-1B, CYTC-1, CDC2A, NTRB, BGAL5, BAXI1, GPAT1, HIS1, RAC1, DAD1, CATA, NDA2, NTF4, GID2, TMK1, H2B, MC4, WHY2, GGPS.*

Subgroup 3.2 comprises genes with moderate expression levels, ranging from 0.01 to 1. The expression values of these genes are approximately 5 to 10 times lower than those in subgroup 3.1: *EXPA8, EXPA1, HXK1, LSD1, PCNA, NDB1, PI3K, HEN1, SERK1, AG, TIR1, CATA3, GST, SERK2, MAPKKK7, MKP1, GA2OX1, ABP1, KN1, HB-8, CDF3, GAI1, MYB1, NDC1, DL1C, DYAD, MMH-1, OGG1, RGL2, OSB2, APX4, MYB-PHL7, XTH6, XTH23, RECA3, KN2, BFN1, GDI1, CATA4*.

Subgroup 3.3 includes genes with predominantly low expression values (< 0.01), specifically within the range of 0.0003 to 0.015: *CATA2, MYC2, MYB4, GSL12, BAG3, histone H3.2, ACA9, MSH5*. Compared to subgroup 3.2, the expression levels of genes in subgroup 3.3 are approximately 30 to 70 times lower.

The synthesis of the obtained data reveals that the majority of the analyzed genes (over 90%) exhibit oscillatory or dynamic expression patterns. The predominant group of oscillatory genes is mainly represented by those classified in Categories I, II and V, whose molecular functions are associated with signal transduction and hormonal interactions, growth and development of reproductive structures, as well as in cell division, genome integrity maintenance, DNA replication and repair. These findings suggest the presence of an active and adaptive temporal regulation in response to the physiological transitions occurring between successive stages of anther development.

A comparatively large number of genes with progressively increasing expression levels belong to Categories I and IV, indicating a gradual activation of processes involved in redox homeostasis, cellular stress responses, mitochondrial biogenesis and function. This may reflect the high metabolic activity within the microsporangium, particularly in tapetal cells, which play a key role in nourishing developing microspores.

Within this study, only a small number of genes exhibited either gradually decreasing expression levels across microsporogenesis stages (functional repression) or relatively stable expression throughout all four stages.

CONCLUSIONS

The comparative analysis of gene expression value distributions across different phases of microsporogenesis in fertile anthers of the homozygous plants SW501 line revealed a moderate increase in gene expression variability during the tetrad and microspore stages, compared to the earlier phases of microsporogenesis, such as pachytene and meiotic division.

The prevalence of dynamically expressed genes, whether progressively upregulated or fluctuating, within the transcriptomic profile of anthers during microsporogenesis highlights the involvement of complex regulatory mechanisms that maintain physiological homeostasis. Disruption of this biochemical and physiological equilibrium during male gametophyte development may result in partial or complete male sterility.

This study adds valuable scientific evidence concerning the experimental validation and functional characterization of candidate genes, thereby facilitating the functional the functional annotation process and the integration of these genes into molecular networks.

Acknowledgments: The research was carried out within the *Subprogram 011101: Genetic and biotechnological approaches to agroecosystem management under climate change* financed by the Ministry of Education and Research.

REFERENCES

1. CERONE L., NEUFELD Z. Differential Gene Expression Regulated by Oscillatory Transcription Factors. *PLOS ONE*, 2012, vol. 7(1): e30283. ISSN 1932-6203.
2. FERNANDEZ P., DI RIENZO J., FERNANDEZ L., HOPP H.E., PANIEGO N., HEINZ R.A. Transcriptomic identification of candidate genes involved in sunflower responses to chilling and salt stresses based on cDNA microarray analysis. *BMC Plant Biology*, 2008, vol. 8:11. ISSN 1471-2229.
3. SHI H., HOU J., LI D. et al. Comparative transcriptome and coexpression network analysis reveals key pathways and hub candidate genes associated with sunflower (Helianthus annuus L.) drought tolerance. *BMC Plant Biology*, 2024, vol. 24:224. ISSN 1471-2229.
4. SISOU D., TADMOR Y., PLAKHINE D., ZIADNA H., HÜBNER S., EIZENBERG H. Biological and Transcriptomic Characterization of Pre-Haustorial Resistance to Sunflower Broomrape (Orobanche cumana W.) in Sunflowers (Helianthus annuus). *Plants (Basel)*, 2021, vol. 10(9):1810. ISSN 2223-7747.
5. LI J., LIU A., NAJEEB U., ZHOU W., LIU H., YAN G., GILL R.A., YUN X., BAI Q., XU L. Genome-wide investigation and expression analysis of membrane-bound fatty acid desaturase genes under different biotic and abiotic stresses in sunflower (Helianthus annuus L.). *International Journal of Biological Macromolecules*, 2021, vol. 175, pp. 188–198. ISSN 0141-8130.
6. LIU H., SUN Z., HU L., LI C., WANG X., YUE Z., HAN Y., YANG G., MA K., YIN G. Comparative Transcriptome Analysis of Male Sterile Anthers Induced by High Temperature in Wheat (*Triticum aestivum* L.). *Frontiers in Plant Science*, 2021, vol. 12:727966, ISSN

1664-462X.

7. TOMKOWIAK A., JAMRUSZKA T., BOCIANOWSKI J., SOBIECH A., JARZYNIAK K., LENORT M., MIKOŁAJCZYK S., ŻUREK M. Transcriptomic Characterization of Genes Harboring Markers Linked to Maize Yield. *Genes (Basel)*, 2024, vol. 15(12):1558, ISSN 2073-4425.
8. PORT A. Analiza fenotipului citohistologic al anterelor la floarea-soarelui cu ASI-AG3 în contextul mitotipului PET1 și genotipului alelic pentru gena Rf_1. In: *Revista de Ştiinţă, Inovare, Cultură şi Artă „Akademos”*, 2024, nr. 1(72), pp. 25-35. ISSN 1857-0461.