# Transcriptomic analysis of radish (*Raphanus sativus* L.) spontaneous tumors

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# Purpose of this study


Figure 1. Taproots of plants of line 19 with spontaneous tumours and of related non-tumorous line 18.

Spontaneous tumors can develop in different organs of various plant species without any pathogen infection and, as a rule, appear in plants with a genotype of mutants or interspecific hybrids. In particular, among the inbred lines of radish (*Raphanus sativus* L.), lines that form spontaneous tumors on the taproot during the flowering period were obtained many years ago. **In this work, we analyzed the differential gene expression in the spontaneous tumors of radish versus the lateral roots using the RNA-seq method.** Data were obtained indicating the increased expression of genes associated with cell division and growth (especially genes that regulate G2-M transition and cytokinesis) in the spontaneous tumor. Among genes downregulated in the tumor tissue, genes participating in the response to stress and wounding, mainly involved in the biosynthesis of jasmonic acid and glucosinolates, were enriched. Our data will help elucidate the mechanisms of spontaneous tumor development in higher plants.

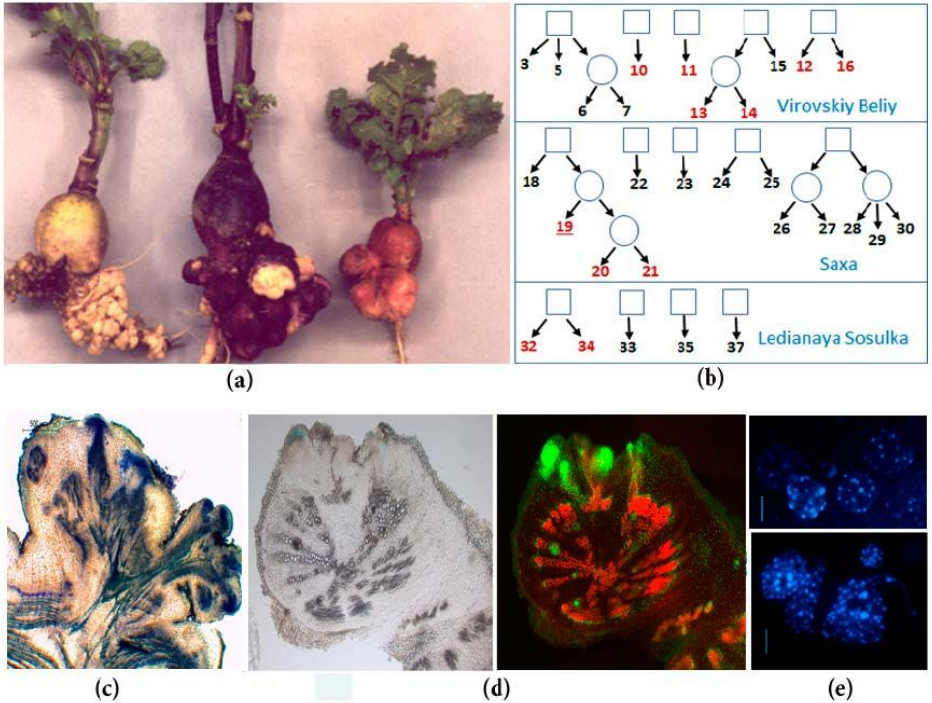


Figure 2. Spontaneous tumors of radish inbred lines: (a) Spontaneous tumors on the taproots of different inbred lines of SPbU genetic collection of Raphanus sativus (from left to right: lines 34, 16, and 19); (b) the origin of inbred radish lines of SPbU genetic collection. Lines with spontaneous tumor formation are marked in red; (c) anatomy of mature spontaneous tumors in radish: Mass of undifferentiated cells in the periphery of tumor, vascularization of the proximal part of tumor, and the connection of tumor and plant vascular systems; (d) analysis of cell proliferation intensity in the radish taproot with young tumors: Cells with active DNA synthesis were incorporated EdU (5-ethynyl-2'-deoxyuridine) and fluorescently labeled with Alexa Fluor-488 (right); (e) cytological analysis of roots and tumors in radish inbred line 19 (DAPI staining): Tumor cells (below) have an increased number of chromocenters, which indicate an increased level of ploidy in comparison with taproot cells (above).

# MATERIALS AND METHODS

The object of our research is spontaneous tumorigenesis in inbred lines of radish (*Raphanus sativus* var. radicula Pers.). The genetic collection of radish was created in St. Petersburg State University by selfing individual plants of four cultivars, and now it contains 32 highly inbred lines.

To compare transcriptomes of spontaneous tumors and lateral roots of radish, we isolated the RNA from tumors and lateral roots of radish plants of tumor-producing line 19 and subjected it to sequencing. Total RNA from three replicates of root and tumor samples was sequenced with an Illumina HiSeq 2500 sequencer resulting in 190.3 million paired-end reads. After all adapter trimming and contamination removal steps, 73.8 million paired-end reads were used for differential expression estimation and all downstream analyses. We have found that 425 genes were significantly upregulated in young tumors compared to lateral roots, while 1203 genes were significantly downregulated (adjusted p-value <0.05). Data on differential expression of genes were further analyzed using the gene set enrichment analysis **(GSEA)** method with the **fgsea package**. Three hundred and forty-four groups of genes (out of 9831 groups taken for the analysis) were significantly enriched among the genes with increased expression in radish tumor samples, and 132 groups of genes were significantly enriched among the genes with reduced expression in the tumor compared to the lateral root (adjusted p-value < 0.05). The **gene ontology enrichment analysis** performed with the **clusterProfiler package** on significantly differentially expressed genes has shown that 76 biological process terms were enriched among upregulated genes and 99 were enriched among downregulated genes with adjusted p-value < 0.05, whereas the **KEGG enrichment analysis** has shown that 8 and 15 pathways were enriched among upregulated and downregulated genes, respectively (adjusted p-value < 0.05).

# RESULTS

The gene ontology enrichment analysis performed with the clusterProfiler package on significantly differentially expressed genes has shown that 76 biological process terms were enriched among upregulated genes and 99 were enriched among downregulated genes with adjusted p-value < 0.05 (Figure 3), whereas the KEGG enrichment analysis has shown that 8 and 15 pathways were enriched among upregulated and downregulated genes, respectively (adjusted p-value < 0.05). We analyzed GO, GSEA, and KEGG pathways in lateral roots and spontaneous tumors of radish line 19.

## GO pathways analysis

**Upregulated genes (Figure 3a):**
pathways associated with the hyperplasia regulation:

- cell cycle,
- DNA replication,
- cytokinesis,
- spindle assembly,
- cytoskeleton rearrangement,
- cell expansion,
- cell wall modifications,
- chromatin modification;
- gene silencing;
- response to hormones (CK and GA),
- amino acid and protein modifications,
- DNA metabolism,
- organelle development,
- and morphogenetic processes.

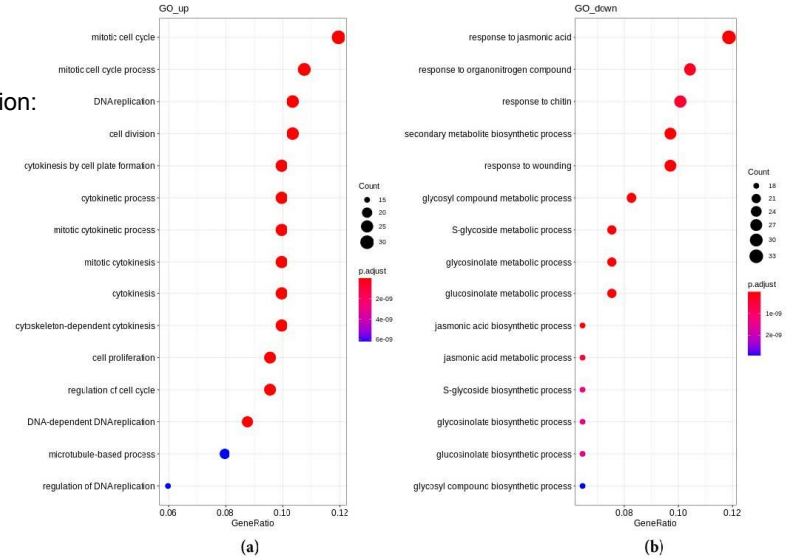


Figure 3. Overrepresented “biological process” GO pathways in genes upregulated (a) and downregulated (b) in the spontaneous tumors of radish in comparison with lateral roots. The count is the number of DEGs included in the respective pathway.

**Downregulated genes (Figure 3b):**
numerous stress-related pathways:

- response to wounding;
- response to abiotic stress;
- immune response (hypersensitive response (HR) and systemic acquired resistance (SAR) reactions);
- cell death;
- pathways of biosynthesis, metabolism, and signaling of such phytohormones as jasmonic acid (JA), salycilic acid (SA), ethylene, IAA, and even karrikins.
- pathways of biosynthesis and metabolism of ROS compounds, phenylpropanoids, flavonoids, glucosinolates, and polyamines;
- amino acid metabolism and transport;
- transport of organic acids and anions.

# RESULTS

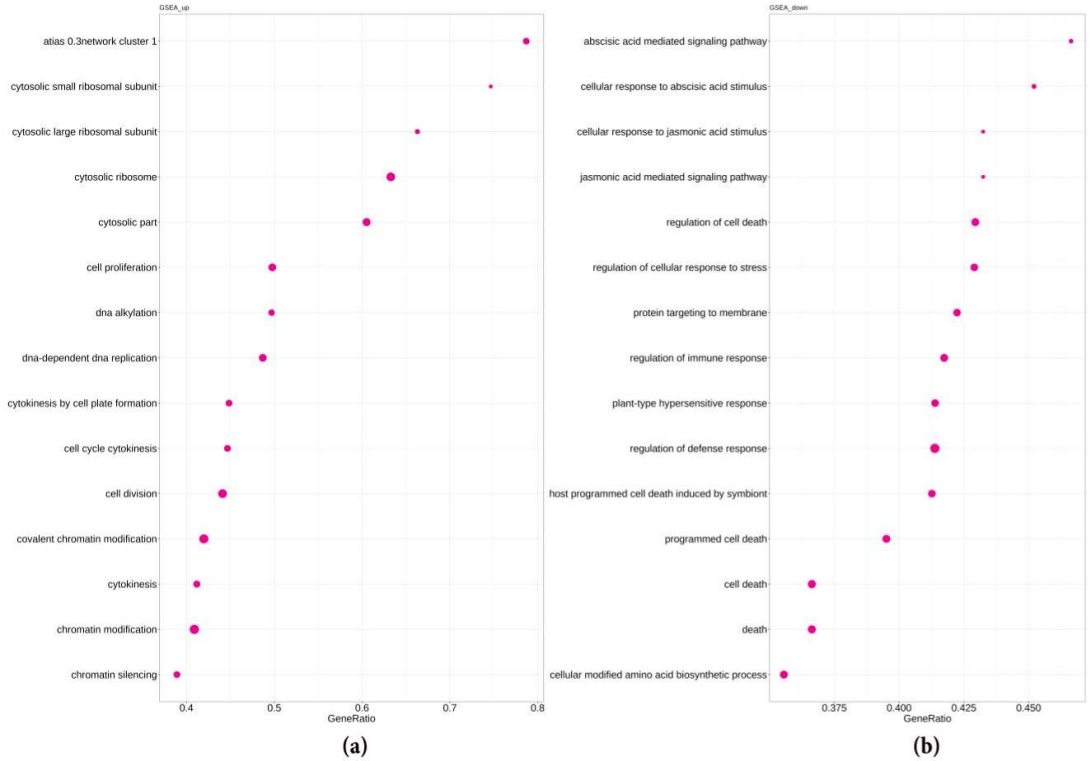


Figure 4. Overrepresented “biological process” GSEA pathways in genes upregulated (a) and downregulated (b) in the spontaneous tumors of radish in comparison with lateral roots. The count is the number of DEGs included in the respective pathway, the adjusted p-value is $3.4 \times 10^{-9}$ .

The data obtained in the analysis of the GSEA and KEGG pathways, in general confirm the data obtained for the GO pathways.

**Upregulated GSEA pathways** in spontaneous tumors (Figure 4):

- **cell division and chromatin modification**
- ribosome biogenesis
- development of meristems and floral organs.

**Downregulated GSEA pathways:**

- response to JA, ABA;
- stress and immune responses.

**KEGG pathways:**

⇧ cell division (e.g., DNA replication and mismatch repair),

⇩ biosynthesis of glucosinolates and phenylpropanoides.

Thus, according to the data on enriched pathways determined by different classifications (GO, GSEA, and KEGG pathways analyses), most of the upregulated pathways in spontaneous tumors were related with cell division and chromatin modification, and most of the downregulated pathways were associated with stress response.

# RESULTS

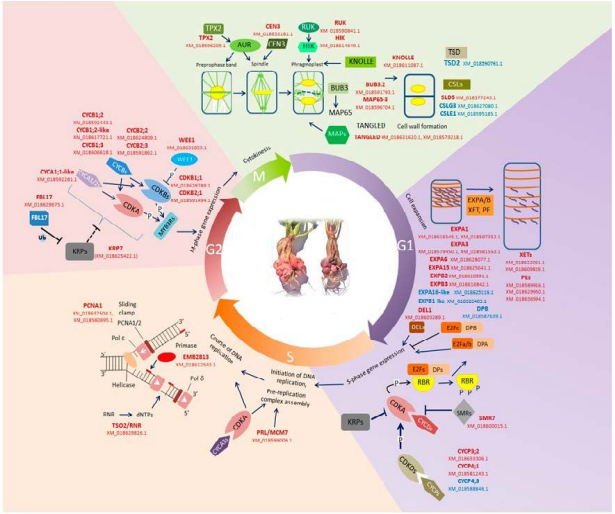

Figure 5. Genetic control of the plant cell cycle; genes whose homologues were differentially expressed in spontaneous tumors of radish are marked in color (red—upregulated genes, blue—downregulated genes).

In our experiment, between the spontaneous tumor and the lateral root 425 genes were significantly upregulated and 1203 genes were downregulated in the spontaneous tumors of radish. The exact functions of these genes in radish are unknown, however, the functions of their closest homologues in *Arabidopsis* were previously studied, and we can assume that the radish DEGs identified by us probably perform functions similar to those of their homologues in *Arabidopsis*.

**Cell Division and Cell Expansion Genes: Mostly Upregulated:**

Genes involved in the control of different stages of the cell cycle and cytokinesis: genes encoding cyclins of different classes, cyclin-dependent kinase B (CDKB), E2F and DP transcription factors, WEE1 kinase, cell cycle inhibitors of KRP and SMR families, proteins involved in the DNA replication and in the cytoskeleton dynamics during mitosis, and also genes, encoding cell wall loosening enzymes, which are essential for cell expansion (Figure 5).

**Stress Response Genes: Mostly Downregulated**

Genes involved in the biotic stress and wounding response are widely represented (Figure 6):

- **Genes of biosynthesis and signalling of jasmonic acid (JA):** phospholipase A1 (PLA1)/ DEFECTIVE IN ANTHER DEHISCENCE 1 (DAD1), lipoxygenases (LOX), allene oxide cyclases (AOCs), DAD1-like Lipase 3 (DALL3), LOX3, LOX4, LOX6, and AOC3, homologs of *OPCL1* and *CYP94B3* genes;
- **Upstream regulators of JA biosynthesis:** gene encoding octadecanoid-responsive AP2/ERF-domain TF ORA47, genes encoding TFs involved in the response to JA - MYC2 and AIF1, JA-responsive genes of transcriptional repressors JAZ6, JAZ8, and JAZ9;
- **Stress-responsive genes acting independently of JA**: metacaspase AMC6 and GLUTATHIONE-S-TRANSFERASE GSTF11, glucosinolates, enzymes of the phenylpropanoid biosynthetic pathway (phenylalanine ammonialyases ATPAL1 and ATPAL2, cinnamoyl CoA reductase, cinnamyl alcohol dehydrogenase and dirigent-like family protein involved in lignans synthesis);
- **Genes for glucosinolate biosynthesis enzymes**: cytochrome P450s CYP83A1 and SPS1/BUS1, methylthioalkylmalate synthases IMS3/MAM1 and IMS2/MAM3, isopropylmalate isomerase LEUD1, methionine-oxo-acid transaminase BCAT4, C-S lyase SUR1/ALF1, and 3-isopropylmalate dehydrogenase ATIMD1 and also MYB28.

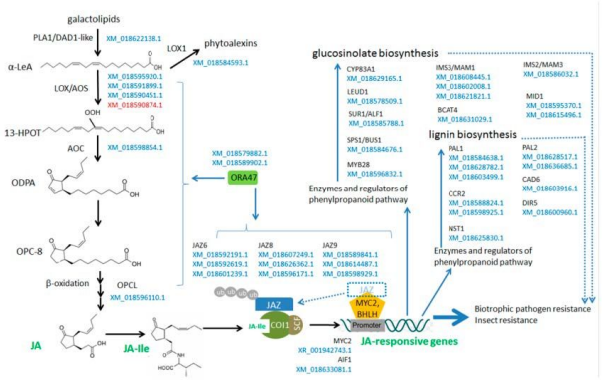


Figure 6. Several pathways of the plant stress response; genes whose homologues were differentially expressed in spontaneous tumors of radish are marked in color (red—upregulated genes, blue—downregulated genes).

# Verification of Differential Expression Analysis Data with QPCR

Next, we performed the qPCR expression analysis for genes showed an increase or decrease in their expression levels in the spontaneous tumors of radish according to the transcriptome analysis. In total, we analyzed 12 upregulated and 12 downregulated genes by qPCR. To order to conduct qPCR, we selected genes involved in different pathways and associated with varied functions. Among **upregulated genes**, we analyzed the expression of genes involved in the control of **cell cycle (CYCA1;1, CYCB1;2, DEL1)** and **cell growth (EXPA3)**, biosynthesis of **GA (GA20OX3)**, negative regulation of cell response to **cytokinin (ARR4 and ARR5)**, genes encoding **peptide phytohormone CLE46**, TFs of different families **(LBD25, LBD38, WRKY9)**, and also the homolog of unique bifunctional gene **ENO2/MBP1** prevailed over those upregulated in spontaneous tumors. Among genes **downregulated** in the tumor tissue, we measured the expression levels of genes involved in the cell cycle regulation **(DPB)**, genes encoding enzymes of **JA (AOC3), IAA (YUC8), CK (IPT5 and IPT7), and glucosinolates (IMS3) biosynthesis** , **TF-encoding genes** (TCP2, RL3, NAC090, SOC1, BEL-like 4), and also the **gene encoding FT-like protein BFT**. In general, the obtained results of qPCR confirmed the transcriptome data: genes that have been identified as upregulated and downregulated in the RNA-seq experiment, demonstrated a significant increase or decrease of expression levels in the qPCR analysis, respectively.

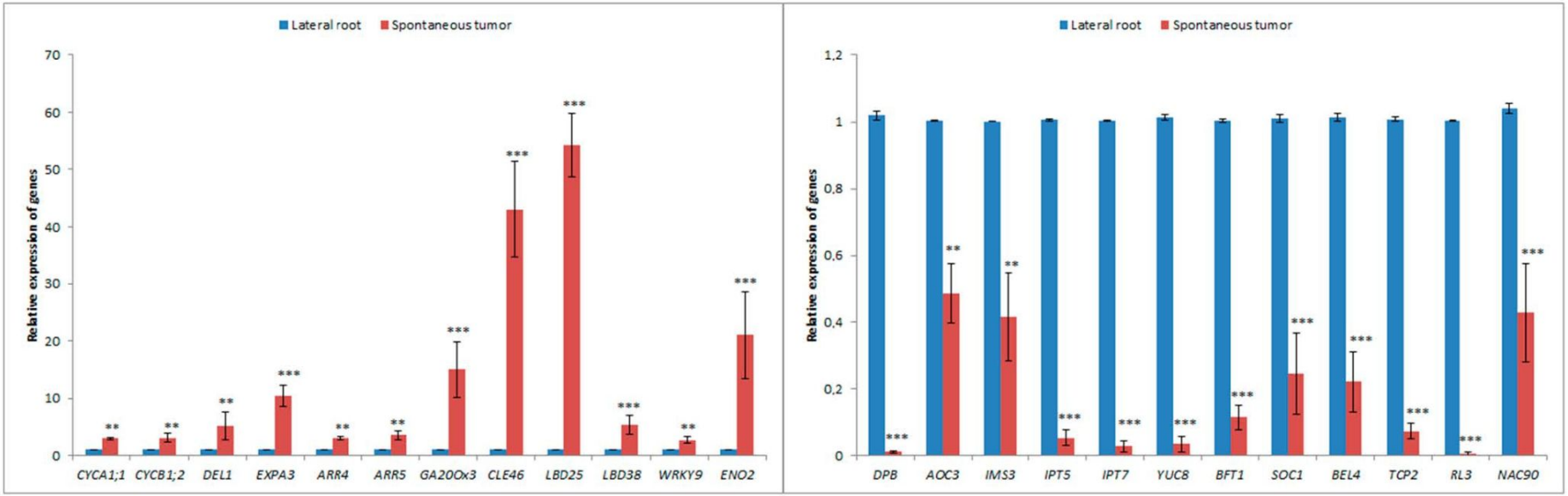


Figure 7. Gene expression analysis by qPCR: (a) Genes which were upregulated in the RNA-seq experiment; (b) genes which were downregulated in the RNA-seq experiment. Error bars indicate the standard deviation of three technical repeats (p-value < 0.01—**, p-value < 0.001—***).

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# Thank you for your attention!