



Transcriptomic response of apple (*Malus × domestica* Borkh. cv. Húsvéti rozmaring) shoot explants to in vitro cultivation on media containing thidiazuron or 6-benzylaminopurine riboside

Dóra Farkas¹ · Viktor Ambrus² · Anita Király¹ · Neama Abdalla³ · Judit Dobránszki¹

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Abstract

One of the most critical factors in maintaining healthy in vitro axillary apple shoot cultures is the cytokinin content of the medium. The effects of two cytokinins applied in the medium, thidiazuron and 6-benzylaminopurine riboside, each applied in a quantity of 4.5 μM , were studied on mRNA transcription of in vitro apple shoots. Transcriptomic response of in vitro apple shoots was investigated at two time points; at 48 h and 4 weeks (at the end of the subculture) after the cytokinin application by mRNA-seq, bioinformatics analysis, GO annotation and KEGG mapping. This study revealed that different expression intensities of DEGs were related to TF families (mainly WRKY, MYB, AP2/ERF, bHLH) associated with plant growth and development, in addition to enzymes involved in genetic and environmental information processing, cell motility, the zeatin biosynthesis, the tryptophan metabolism, the phenylpropanoid biosynthesis, and cutin, suberin, and wax biosynthesis. A putative roadmap based on changes in gene expression intensity that may be in association with various in vitro apple shoot development and growth disorders caused by different cytokinin supplies was conceived.

Key message

Transcription related to environmental information processing changed by both cytokinins, but differently. Only TDZ affected significantly the cell motility and DNA replication compared to the control group. Transcriptomic changes were in connection with the shoot characteristics.

Keywords Plant hormone signal transduction · Zeatin biosynthesis · DNA replication · Transcription factors · *Malus × domestica* Borkh.

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✉ Judit Dobránszki
dobranszki@freemail.hu

Dóra Farkas
farkas.dora@agr.unideb.hu

Viktor Ambrus
ambrus.viktor@science.unideb.hu

Anita Király
gonda-kiraly.anita@agr.unideb.hu

Neama Abdalla
neama_ncr@yahoo.com

¹ Centre for Agricultural Genomics and Biotechnology, University of Debrecen, Debrecen, Hungary

² Department of Biotechnology and Microbiology, Faculty of Science and Technology, University of Debrecen, Debrecen, Hungary

³ Plant Biotechnology Department, Biotechnology Research Institute, National Research Centre, 33 El Buhouth St., Dokki, Giza 12622, Egypt

Introduction

Apple (*Malus × domestica* Borkh., Rosaceae family), is one of the most economically important fruit crops, which is widely grown in temperate climate zones of the world (Baldi et al. 2024). Apples are a rich source of many essential phytonutrients, such as minerals, vitamins, antioxidants, dietary fibres, organic acids and sugars (Oyenihi et al. 2022), as a result of having been developed by many technologies of breeding and preservation (Itana and Habib 2022). Apples offer numerous health benefits, and their consumption prevents cardiovascular diseases as atherosclerosis and coronary artery disease (Ferretti et al. 2014), reduces the risk of diabetes, and also provides anti-asthmatic and anti-allergic effects (Mierczak and Garus-Pakowska 2024).

Micropropagation represents a widely known method for plant production, conservation and improvement, and has the highest currently available capacity to multiply the plant material all over the year irrespective of cultivation season (Shi et al. 2021). In vitro micropropagation of apples has played a remarkable function in the production of genetically uniform, healthy, pathogen-free scions and rootstocks, and in quick, mass propagation of fruit trees with suitable traits (Dobránszki and Teixeira da Silva 2010; Kumar et al. 2023; Abdalla and Dobránszki 2024). Tissue culture methods have been successfully applied for the propagation of various *Malus* species (Butiuc-Keul et al. 2010; Teixeira da Silva et al. 2019) and have been broadly used for providing multiple clones of apple scions and rootstocks (Bhatti and Jha 2010).

Shoot multiplication is one of the key processes, and the most important aspect of an efficient micropropagation protocol (Kaur et al. 2018). The success and usefulness of an apple micropropagation protocol for commercial production in large scale largely depends on the mode and rate of shoot multiplication because this is a key phase in whether in vitro shoots of a suitable quantity and quality (suitable for rooting) can be produced (Dobránszki and Teixeira da Silva 2010). The efficacy of shoot multiplication is influenced by several factors, such as genotype, media composition as well as in vitro environmental conditions. However, the main controllers of the process are cytokinins (CKs); their type plays a decisive role in the in vitro shoot development (Magyar-Tábori et al. 2010; Dobránszki 2014; Teixeira da Silva et al. 2019).

Cytokinins are phytohormones and there are some plant growth regulators (PGRs) with cytokinin-like activity that regulate a great variety of biological processes and affect plant growth and development, including cell division, proliferation and differentiation, shoot branching, growth, and development of both shoots and roots (Dobránszki 2014; Hu and Shani 2023). Thidiazuron (TDZ), as one of the most

active cytokinin-like substances, could stimulate in vitro shoot multiplication of a wide range of apple scions and rootstocks (reviewed in: Dobránszki and Teixeira da Silva 2010). Although TDZ is one of the most active cytokinin-like substances, in woody plants it may cause a series of undesirable side-effects when applied exogenously (Huetteman and Preece 1993). In many cases, the formation of large shoot clumps, fasciated, dwarf shoots were observed and some of these shoots seemed to be adventitious, which is unfavourable in true-to-type micropropagation protocols (Hazarika 2006; Polivanova and Bedarev 2022). Moreover, it had a strong after-effect on rooting by inhibiting the root formation of in vitro shoots propagated on TDZ-containing medium (Huetteman and Preece 1993; Dobránszki and Teixeira da Silva 2010). TDZ was the most efficient CK for enhancing the number of shoots/explants in apple scion cv. Húsvéti Rozmaring, but resulted in shoots of low quality as they were stunted and thickened with small leaves (Abdalla and Dobránszki 2024).

Beyond TDZ, some sugar conjugates of aromatic CKs, such as 6-benzylaminopurine riboside (BAR), were proven to be very active in the in vitro shoot growth and development of apple (Dobránszki and Teixeira da Silva 2010; Vylíčilová et al. 2020). BAR is an N⁹-riboside derivate of 6-benzylaminopurine that is biologically active (Vylíčilová et al. 2020). It can be used with better efficiency than 6-benzylaminopurine (BA), i.e. a better multiplication rate could be achieved, and in vitro shoots developed on BAR-containing medium did not show the disorders occurring in case of TDZ-induced in vitro shoots. Moreover, rooting inhibition, which is a very frequent side-effect after application of TDZ and BA, cannot be detected (Dobránszki and Teixeira da Silva 2010).

The effects of TDZ and BAR on in vitro shoot development of apple are well documented, but the transcriptomic changes underlying them are still unexplored. The aim of this study was to investigate the transcriptomic response of in vitro apple shoots induced on medium containing TDZ or BAR.

Materials and methods

Plant material, in vitro growing conditions and sample collection

20-mm-long shoot explants from in vitro maintained shoot cultures of apple (*Malus x domestica* Borkh. cv. Húsvéti rozmaring) were used as the source for the experiments. In vitro shoots served as explant source were maintained on medium containing MS macro- and micronutrients and vitamins (Murashige and Skoog 1962) supplemented

with 2.22 μM 6-benzylaminopurine (BA), 0.49 μM indole butyric acid (IBA), 0.58 μM gibberellic acid (GA_3), 3% (w/v) sucrose, and 0.7% (w/v) agar-agar. Before autoclaving for 20 min, at 121 $^\circ\text{C}$ and 10^5 Pa, the pH of the medium was adjusted to 5.8. Each culture vessel contained 70 ml of medium and five shoot explants. Shoot cultures were grown at 23 ± 2 $^\circ\text{C}$; under 16/8 photoperiod, at light intensity of $106 \mu\text{mol s}^{-1}\text{m}^{-2}$. However, in the last subculture before the experiments, shoots were grown in vitro on cytokinin-free medium to avoid the after-effects of cytokinin in the experiments.

4-week-old in vitro shoots grown on cytokinin-free medium were separated from in vitro mother shoots, cut into 20-mm-long segments and placed on experimental media. Except for the cytokinin content, the experimental media were the same as the medium used for maintenance described above. Three different cytokinin-containing media were used during the experiments; medium without cytokinin (control), media containing 4.5 μM thidiazuron (TDZ) and 4.5 μM 6-benzylaminopurine riboside (BAR), respectively. Culture conditions were the same as were applied for maintenance of shoot cultures. Shoots were collected from experimental cultures two times; 48 h after placing in vitro shoot explants on the experimental media (48 h) and at the end of the subculture, at 4th week (4 wks). Plant materials were collected from three different culture vessels from each cytokinin treatment. Collected samples were stored at -80 $^\circ\text{C}$ until RNA isolation.

Isolation of mRNA and sequencing

Total RNA was purified from the samples as three biological replicates each using Quick-RNA™ Plant Miniprep kit (Zymo Research, Irvine, CA, USA) based on the manufacturer's protocol. The purity and quantity of the isolated RNA samples were assessed via microcapillary electrophoresis using an Implen n50 nanophotometer (Implen, Munich, Germany) and by fragment analysis with an Agilent Bioanalyzer 2100 system using RNA 6000 Nano kit (Agilent, Santa Clara, CA, USA).

Following mRNA library preparation protocol (poly A enrichment and cDNA library construction), paired-end 150 bp strategy was applied to sequence the samples using Illumina Sequencing Platform of NovaSeq X Plus Series (PE150). All raw sequences have been deposited into the NCBI Gene Expression Omnibus (GEO) repository under GSE287596 accession number.

Bioinformatic analysis and functional annotation of dataset

Quality control of raw sequence reads were carried out with fastp software. After adapter and low quality base removal, the clean reads were mapped to the GCA_004115385.1 *Malus domestica* reference genome using Hisat2 (v2.0.5). Gene level read quantification was performed with featureCounts (v1.5.0-p3).

Differential expression analysis was conducted with the DESeq2 R package (v.1.20.0), and differentially expressed genes (DEGs) were defined by the adjusted p-value (FDR) < 0.05 and $\log_2(\text{Fold Change}) \geq 3$.

Gene enrichment analysis was performed with the clusterProfiler package (v.3.8.1) using the gene set for all genes that were analyzed for significant differences and annotated to the GO or KEGG database as background genes. GO terms and pathways with adjusted p-value < 0.05 were considered significantly enriched. Visualization of the enriched GO terms were carried out with OmicsBox.

Results

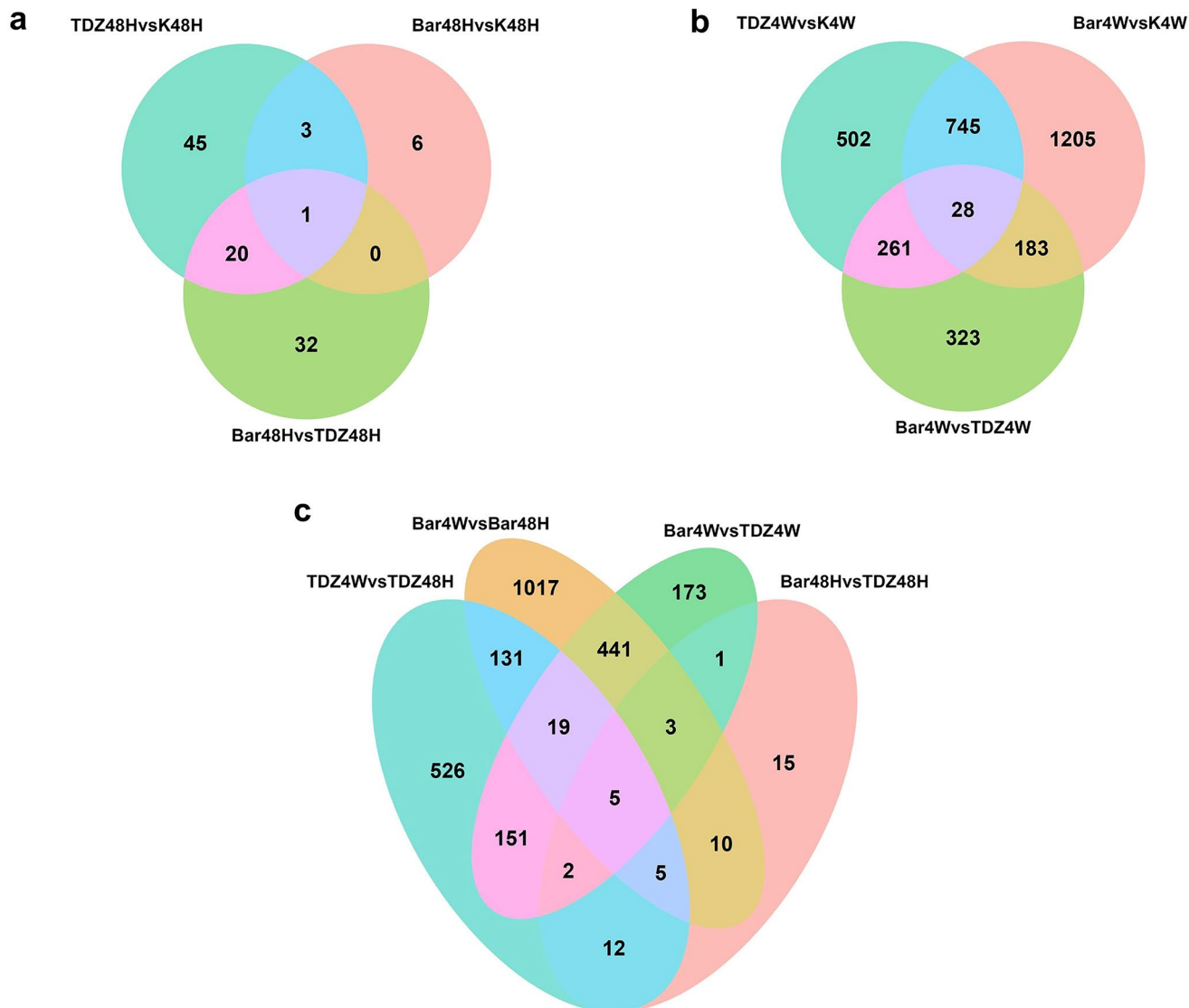
Assessment of global changes in RNA expression profile

The expression intensity of 32,834 genes including their promoters and coding regions were assessed in three comparisons based on exogenous cytokinin concentration (TDZ, BAR or without cytokinin) of the shoot multiplication medium, i.e. TDZ vs. control (no cytokinin added), BAR vs. control and BAR vs. TDZ. A heat map based on the expression intensity of genes was generated (Table S1; Fig. S1). A total of 69, 10 and 53 sequences were significantly up- or down-regulated 48 h after in vitro cultivation in comparisons of TDZ vs. control, BAR vs. control and BAR vs. TDZ, respectively. By the end of the subculture (4th week), the number of significantly up- or down-regulated sequences increased to 1,536, 2,161, and 795 in the same comparisons, respectively (Table 1). Volcano plots generated based on cytokinin supply (control, TDZ, BAR) and sampling time (48 h, 4 wks) show up- and down-regulated genes for each comparison (TDZ vs. control, BAR vs. control and BAR vs. TDZ) (Fig. S2).

Considering all, up- or down-regulated DEGs; at 48 h, three DEGs were identical in comparisons of TDZ vs. control and BAR vs. control, while 20 DEGs in comparisons of TDZ vs. control and BAR vs. TDZ. There was no identical DEG in comparisons of BAR vs. control and BAR vs. TDZ. However, the number of identical DEGs increased by the 4th week. The values were 745, 261 and 183 in comparisons

Table 1 Number of significantly up or down-regulated DEGs, in TDZ vs. Control, BAR vs. Control and BAR vs. TDZ comparisons 48 h and 4 weeks after culturing in vitro shoots on shoot multiplication media containing different types of cytokinin (TDZ or BAR) or no (control) cytokinin

Treatment comparisons	TDZ-Control comparison		BAR-Control comparison		BAR-TDZ comparison	
	↑ Up-regulated genes	↓ Down-regulated genes	↑ Up-regulated genes	↓ Down-regulated genes	↑ Up-regulated genes	↓ Down-regulated genes
48 h	19	50	7	3	45	8
4 weeks	1439	97	1401	760	60	735

**Fig. 1** Distribution of significantly differentially expressed genes based on comparisons of TDZ vs. control and BAR vs. control, and BAR vs. TDZ, 48 h (a) and 4 weeks (b) after various cytokinin supplies applied in the shoot multiplication medium of in vitro apple (cv. Hús-

véti rozmaring) shoots, as well as in multiple comparison (c) including all sampling times and cytokinin supplies (TDZ: thidiazuron; BAR: 6-benzylaminopurine riboside)

of TDZ vs. control and BAR vs. control, TDZ vs. control and BAR vs. TDZ, and BAR vs. control and BAR vs. TDZ, respectively (Fig. 1a, b). The number of identical DEGs was presented in multiple comparisons in Fig. 1c; a total of five DEGs were identical considering all comparisons.

Changes in biological processes, cellular components, and molecular function

Assessment of the most important processes related to molecular function, cellular components and biological

processes was carried out based on comparing significant up-, and downregulation in various comparisons (Fig. S3). Significantly enriched Gene Ontology terms among the DEGs were identified based on an adjusted p-value threshold of <0.05.

48 h after placing shoot explants on various cytokinin- (TDZ- or BAR-) containing medium, only two biological processes were affected compared to control (i.e. shoots from cytokinin-free medium). DEGs were limited to two biological processes, a cytokinin metabolic process and a hormone metabolic process in 50–50%. Three cellular components were affected, the cell wall, the apoplast and the peroxisome in 40, 40 and 20%, respectively. TDZ application affected mainly (> 10%) four out of ten molecular functions, i.e. cytokinin dehydrogenase activity (10.3%), heme binding (13.8%), iron ion binding (13.8%) and flavin adenine dinucleotide binding (13.8%). In case of BAR supply, four molecular functions were affected, i.e. cytokinin dehydrogenase activity (33.3%), flavin adenine dinucleotide binding (33.3%), terpene synthase activity (16.7%) and magnesium ion binding (16.7%) (Fig. S3).

At the end of the subculture (4 wks) three biological processes were affected after both cytokinin treatments. TDZ affected DEGs related to the phosphorelay transduction system, the regulation of cyclin-dependent serine/threonine kinase activity and microtubule-based movement in 31.7, 9.8 and 58.5%, respectively, while BAR affected the biological processes of the response to oxidative stress, cell wall organization and the response to auxin in 40, 21.8 and 38.2%, respectively. As for cellular components, in case of BAR supply, only the photosystem I (100%) was affected, while in case of TDZ supply, the nucleosome (32.3%), the photosynthetic membrane (25.8%), the apoplast (16.1%), the cell wall (16.1%) and the proton-transporting ATP synthase complex, coupling factor F(o) (9.7%). DEGs were related to five molecular functions (hydrolase activity, hydrolyzing O-glycosyl compounds in 25.4%, heme binding in 25.4%,

microtubule motor activity in 17.4%, microtubule binding in 19.6% and in electron transfer activity in 12.3%) 4 weeks after TDZ treatment, while to six ones (heme binding in 20.3%, hydrolase activity, hydrolyzing O-glycosyl compounds in 18.4%, DNA-binding transcription factor activity in 19.6%, oxidoreductase activity, iron ion binding in 14.6% and hexosyltransferase activity in 12.3%) 4 weeks after BAR treatment (Fig. S3).

When comparing BAR vs. TDZ, DEGs were found in three biological processes (the glucan metabolic process in 42.9%, the amine metabolic process and cell wall modification in 28.6–28.6%), connected to two cellular components (the cell wall and the apoplast in 50–50%) and only one molecular function (xyloglucan: xyloglucosyl transferase activity). In 4-week-old shoots, however, six biological processes were affected by DEGs; mainly (> 20%) microtubule-based movement, the phosphorelay transduction system and chromosome organization, while in less extent (< 15%) DNA replication initiation, the cytokinin metabolic process and the hormone metabolic process. All DEGs related to nucleosomes (Fig. S3).

Transcription factor-related up- and down-regulated DEGs

Identification of DEGs related to transcription factors (TFs) was assessed using NCBI and GO descriptions. Based on Hong (2016), these TFs were grouped into TF families for easier transparency (Fig. 2; Table S1).

When comparing treated (either with BAR or TDZ) plants to the control group at the 48 h mark, no significant TF DEGs were detected. However, after 48 h of cultivation, analyzing BAR vs. TDZ groups, only one TF, the ARATH Transcription factor ORG2, a member of the bHLH (basic helix-loop-helix) TF family, was significantly up-regulated. Compared to the control group, when the medium contained TDZ after 4 weeks, 21 TF DEGs in the bHLH family were

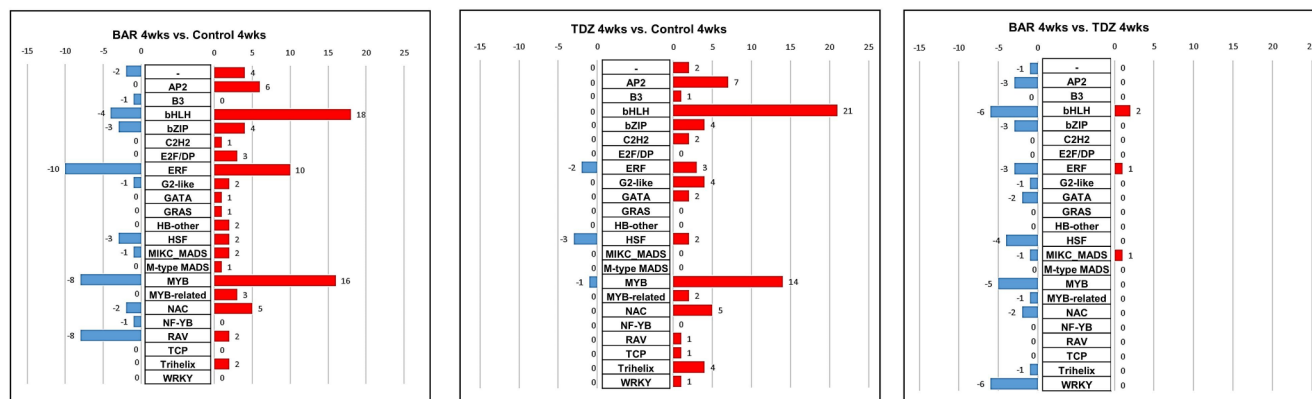


Fig. 2 Transcription factors (TFs) affected by cytokinin treatments grouped based on TF families at 4 weeks in comparisons of treatments (BAR; TDZ) vs. Control and both cytokinin treatments to each other (TDZ: thidiazuron; BAR: 6-benzylaminopurine riboside)

up-regulated, and in the BAR vs. control comparison only 18 TFs were up-regulated, meanwhile four TFs were down-regulated. Drawing a parallel between the BAR and TDZ groups at the 4-week mark, six bHLH TFs were down-regulated and two DEGs were up-regulated (Fig. 2).

4 weeks after cultivating *in vitro* shoots on BAR-containing media compared to the control group, 10 ERF (ethylene-responsive factor) TFs were up-, and 10 were down-regulated. However, at the same time, in the TDZ vs. control comparison only three TFs were up-, and two were down-regulated. If we compare the TDZ and BAR groups, only one ERF TF was up- and three were down-regulated. In both BAR and TDZ groups compared to control, the AP2 (APETALA2) TF-s were up-regulated by the 4th week (six and seven DEGs, respectively). On the contrary, regarding the BAR vs. TDZ comparison, three TFs were down-regulated at the same time. There was no difference in the expression of RAV (related to ABI3 and VPI) TFs comparing BAR and TDZ at 4 weeks, but regarding the BAR vs. control comparison, two TFs were up-, and eight were down-regulated, and as for the TDZ vs. control comparison, only one TF was up-regulated (Fig. 2).

After 4 weeks of *in vitro* cultivation of shoots on media supplemented with BAR vs. control, 16 MYB (myeloblastosis) TFs were up- and eight were down-regulated, while regarding the TDZ vs. control comparison, 14 TFs were up-, and one was down-regulated. Five MYB TFs were down-regulated by the 4th week comparing BAR vs. TDZ (Fig. 2).

No TFs from the WRKY family were affected by BAR after 4 weeks compared to the control group, and only one TF was up-regulated in the TDZ vs. control comparison. Regarding the BAR vs. TDZ comparison, six WRKY TFs were down-regulated, respectively (Fig. 2).

KEGG mapping of up- and down-regulated DEGs

48 h after cultivation of *in vitro* shoots on different media with various cytokinin content, up-, and down-regulation of metabolic processes related to DEGs were detected. In response to TDZ supply in the medium, three metabolic processes were affected. 25%, 25% and 50% of DEGs related to the biosynthesis of unsaturated fatty acids, sesquiterpenoid and triterpenoid biosynthesis, and zeatin biosynthesis, were up- or down-regulated, respectively. However, in response to BAR supply only DEGs related to zeatin biosynthesis was altered.

After 4-week-long *in vitro* shoot cultivation, not only did the application of various cytokinins in the plant tissue culture medium affect the metabolic processes, but they even act on cell motility, genetic information processing and environmental information processing as well (Table 2; Table S2).

4 weeks after culturing shoots on TDZ-containing medium, all 118 DEGs mapped to different pathways were up-regulated. More than 70% of all up-regulated DEGs was related to the plant hormone signal transduction (33%), motor proteins (20%) and phenylpropanoid biosynthesis (18%). In addition, pentose and glucuronate interconversions (16 DEGs, 18%), fatty acid degradation (9 DEGs, 8%) and DNA replication (9 DEGs, 8%) were up-regulated in response to the TDZ in the medium (Table 2).

When the medium contained BAR, 4 weeks after culturing shoots on it, 10 pathways were affected both by up- or down-regulation. A total of 122 up-regulated and 80 down-regulated DEGs were mapped by using KEGG (Table 2). Plant hormone signal transduction was the most affected pathway (26% of DEGs related to this pathway), with 41 up-, and 14 down-regulated DEGs encoding nine and seven enzymes, respectively. (Table S2; Fig. S4). 14–15% of DEGs (both up-, and down-regulated) were related to phenylpropanoid biosynthesis, and the starch and sucrose metabolism, respectively. In addition, other pathways, such as ABC transporters, the biosynthesis of various plant secondary metabolites, the cutin, suberine and wax biosynthesis, the cyanoamino acid metabolism, the galactose metabolism, pentose and glucuronate interconversions and the zeatin biosynthesis, were significantly affected (in 5–8% of all DEGs, respectively).

Up- and down-regulated DEGs related to metabolic processes

Eight DEGs could be detected 48 h after placing the shoot explants on TDZ-containing medium. They were related to the biosynthesis of unsaturated fatty acids, sesquiterpenoid and triterpenoid biosynthesis and zeatin biosynthesis. The biosynthesis of unsaturated fatty acids was attenuated by down-regulation of two DEGs (stearoyl-[acyl-carrier-protein] 9-desaturase 6, chloroplastic-like, EC:1.14.19.2 1.14.19.11 1.14.19.26, and peroxisomal acyl-coenzyme A oxidase 1, EC:1.3.3.6, respectively), while sesquiterpenoid and triterpenoid biosynthesis was elevated by up-regulation of two DEGs related to the biosynthesis of germacrene D (EC:4.2.3.75: germacrene D synthase-like). Zeatin biosynthesis was affected multiple. A DEG related to two enzymes (EC:2.5.1.27 and EC:2.5.1.112, adenylate isopentenyltransferase-like) that catalyze the synthesis of isopentenyl-AMP, -ADP and -ATP were down-regulated, while three DEGs related to the cytokinin dehydrogenase 4-like enzyme (EC:1.5.99.12) were up-regulated. The latter, up-regulated enzyme acts as a cytokinin-oxidase (Table 2; Table S2; Fig. S4).

Only two DEGs could be detected 48 h after shoot explants were put onto BAR-containing medium. They encode the

Table 2 Number of significantly up (↑)-, and down (↓)-regulated DEGs, as well as the number of enzymes they encode (in brackets) based on the KEGG mapping, related to the metabolic and information processing pathways, as well as cell motility

Pathways	48 h			4 weeks		
	TDZ vs. Control	BAR vs. Control	BAR vs. TDZ	TDZ vs. Control	BAR vs. Control	BAR vs. TDZ
<i>Metabolic processes</i>						
ABC transporters					↑ 10 (3)	↓ 6 (1)
Biosynthesis of unsaturated fatty acids		↓ 2 (4)				
Biosynthesis of various plant secondary metabolites					↑ 6 (4)	↓ 6 (3)
Cutin, suberine and wax biosynthesis					↑ 7 (8)	↓ 3 (4)
Cyanoamino acid metabolism					↑ 7 (2)	↓ 8 (3)
						↑ 3 (1)
Fatty acid degradation				↑ 9 (3)		
Galactose metabolism					↑ 4 (2)	↓ 9 (3)
Pentose and glucuronate interconversions				↑ 16 (3)	↑ 12 (2)	↓ 4 (1)
Phenylpropanoid biosynthesis				↑ 21 (6)	↑ 19 (3)	↓ 12 (5)
						↓ 21 (10)
Sesquiterpenoid and triterpenoid biosynthesis	↑ 2 (1)					
Starch and sucrose metabolism					↑ 15 (6)	↓ 14 (6)
Tryptophan metabolism						↓ 9 (2)
Zeatin biosynthesis	↑ 3 (1)	↓ 1 (2)	↑ 2 (1)		↑ 1 (1)	↓ 4 (3)
						↑ 1 (2)
<i>Cell motility</i>						
Motor proteins				↑ 24 (6)		↓ 16 (6)
<i>Genetic information processing</i>						
DNA replication				↑ 9 (7/4)		↓ 13 (11/5)
<i>Environmental information processing</i>						
Plant hormone signal transduction				↑ 39 (14)	↑ 41 (9)	↓ 14 (7)
						↑ 3 (3)
						↓ 25 (10)

cytokinin dehydrogenase 4-like enzyme (EC:1.5.99.12) that catalyzes the oxidation of cytokinins, and similarly to 48 h after the application of TDZ, they were up-regulated (Table 2; Table S2; Fig. S4).

The TDZ content of the medium led to the modification of gene expression intensity in DEGs related to three metabolic processes when compared to the control shoots grown on cytokinin-free medium. In fatty acid degradation, three enzymes (alcohol dehydrogenase 1-like (EC:1.1.1.1), aldehyde dehydrogenase (EC:1.2.1.3), long chain acyl-CoA synthetase 4 (EC:6.2.1.3)) encoded by nine up-regulated DEGs were affected. The pentose and glucuronate interconversions pathway was affected by up-regulation of 16 DEGs encoding three enzymes (pectate lyase 8 (EC:4.2.2.2), pectinesterase 53 (EC:3.1.1.11); sorbitol dehydrogenase-like (EC:1.1.1.14)). The highest number of DEGs were related to phenylpropanoid biosynthesis among metabolic processes; each of them were up-regulated. They encode six enzymes (caffeic acid 3-O-methyltransferase-like, (EC:2.1.1.68 2.1.1.4), caffeoyl shikimate esterase-like (EC:3.1.1.-), peroxidase 64-like (EC:1.11.1.7), peroxidase

64-like (EC:1.11.1.7), caffeoyl-CoA O-methyltransferase At4 g26220 (EC:2.1.1.104), mannitol dehydrogenase isoform X1 (EC:1.1.1.195)). Their up-regulation may shift the biosynthesis of phenylpropanoids towards the end products of syringil lignin, guaiacyl lignin and p-hydroxyl-phenyl lignin (Table S2; Fig. S4).

Seven metabolic processes were affected after 4-week-long subculture in in vitro shoots grown on BAR-containing medium compared to the ones grown on cytokinin-free medium (control). Both up- and down-regulated DEGs were detected in all affected metabolic processes (Table 2).

DEGs encoding different ABC transporters were up- and down-regulated. ABC B (EC:7.6.2.2), ABC C and ABC G family members were up-regulated, while PDR was down-regulated. In the various plant secondary metabolites pathway, the nicotianamine synthase-like enzyme (EC:2.5.1.43) and beta-glucosidase (EC:3.2.1.21) were down-regulated, while bifunctional 3-dehydroquinate dehydratase (EC:4.2.1.10), shikimate dehydrogenase, (1.1.1.25), and S-adenosylmethionine synthase 1 (EC:2.5.1.6) were up-regulated (Table S2; Fig. S4).

Ten DEGs were affected in cutin, suberine and wax biosynthesis. DEGs encoding cytochrome P450 77 A3-like, cytochrome P450 86 A8, fatty acyl-CoA reductase 3-like (EC:1.2.1.84), omega-hydroxypalmitate O-feruloyl transferase (EC:2.3.1.188) and protein HOTHEAD were up-regulated, while DEGs encoding 86B1, 86 A1 and 94 A1-like of cytochrome P450 were down-regulated (Table 2; Table S2; Fig. S4).

In the cyanoamino acid metabolism, eight DEGs related beta-glucosidase 40 (EC:3.2.1.21) were either up- or down-regulated. DEGs encoding L- 3-cyanoalanine synthase 1 (EC: 4.4.1.9), (R)-mandelonitrile lyase 2-like (EC:4.1.2.10) enzymes were down-regulated, while DEGs encoding beta-glucosidase 12-like (EC:3.2.1.118) enzyme were up-regulated.

In the galactose metabolism, four up- and three down-regulated DEGs were identified. Reactions catalyzed by alpha-galactosidase 1-like (EC:3.2.1.22) and ATP-dependent 6-phosphofructokinase 6-like (EC:2.7.1.11) enzymes were up-regulated, while reactions catalyzed by probable galactinol-sucrose galactosyltransferase 6 (EC:2.4.1.82), galactinol synthase 1-like (EC:2.4.1.123) and acid β -fructofuranosidase 2 (EC:3.2.1.26) were down-regulated (Table 2; Table S2; Fig. S4).

In the pentose and glucuronate interconversions pathway, 12 up-regulated and 4 down-regulated DEGs were detected. Accordingly, in the pathway, reactions catalyzed by pectate lyase (EC:4.2.2.2) and pectinesterase (EC:3.1.1.11) were up-regulated, while polygalacturonase (EC:3.2.1.15) catalyzed reaction was down-regulated (Table 2; Table S2; Fig. S4).

Concerning the metabolic processes, the most affected DEGs were related to the phenylpropanoid biosynthesis (31 DEGs up- or down-regulated), and starch and sucrose metabolism (29 DEGs up- or down-regulated). In phenolpropanoid biosynthesis reactions catalyzed by caffeoyl shikimate esterase-like (EC:3.1.1.-), peroxidase 64-like (EC:1.11.1.7), and probable mannitol dehydrogenase isoform X1 (EC:1.1.1.195) were up-regulated, while reactions catalyzed by 4-coumarate-CoA ligase-like 9 (EC:6.2.1.12), anthocyanidin 3-O-glucosyltransferase 5-like (EC:2.4.1.111), cytochrome P450 98 A2 (EC:1.14.14.96), trans-cinnamate 4-monooxygenase (EC:1.14.14.91) and shikimate O-hydroxycinnamoyltransferase-like (vinorin synthase) (EC:2.3.1.133) were down-regulated (Table 2; Table S2; Fig. S4).

In the starch and sucrose metabolism, 29 DEGs were up- or down-regulated. Reactions of the pathway catalyzed by acid beta-fructofuranosidase 2 (EC:3.2.1.26), alpha-amylase 3 (EC:3.2.1.1), glucose- 1-phosphate adenyltransferase small subunit 2 (EC:2.7.7.27), isoamylase 3 (EC:3.2.1.68), ectonucleotide pyrophosphatase/phosphodiesterase family member 3-like (EC: 3.6.1.9), and alpha-trehalose-phosphate synthase [UDP-forming] 9 (EC:2.4.1.15) were down-regulated.

However, reactions catalyzed by 4-alpha-glucanotransferase (EC:2.4.1.25), beta-glucosidase 40 (EC:3.2.1.21), endoglucanase 25-like (EC:3.2.1.4), glucan endo- 1,3-beta-glucosidase 5 (EC:3.2.1.39), granule-bound starch synthase 1 (EC:2.4.1.242) and sucrose synthase (EC:2.4.1.13) were up-regulated (Table 2; Table S2; Fig. S4).

The zeatin biosynthesis was affected by the down-regulation of DEGs encoding cytokinin dehydrogenase 4-(EC:1.5.99.12), cytokinin hydroxylase-like and zeatin O-glucosyltransferase-like (EC:2.4.1.215) enzymes, and by the up-regulation of DEGs encoding adenylate isopentenyltransferase (EC:2.5.1.27, 2.5.1.112) (Table 2; Table S2; Fig. S4).

When comparing the effects of BAR vs. TDZ on the expression intensity of DEGs related to various metabolic processes, down-regulated DEGs were detected in the galactose metabolism, pentose and glucuronate interconversions, the phenylpropanoid biosynthesis and the tryptophan metabolism. However, in the cyanoamino acid metabolism and the zeatin biosynthesis, both up- and down-regulated DEGs were detected (Table 2; Table S2; Fig. S4).

Up- and down-regulated DEGs related to information processing pathways and cell motility

When TDZ was applied in the medium, even cell motility (motor protein) and genetic information processing (DNA replication) were up-regulated in 4-week-old shoots. Cell motility was affected by the up-regulation of DEGs encoding kinesins (kinesin-like protein KIN- 12B, KIN- 5B, KIN- 7B, KIN- 8B isoform X1) and microtubule (tubulin alpha chain (TUBA), tubulin beta chain (TUBB)). As for DNA replication, DEGs encoding alpha catalytic subunit and epsilon subunit B of DNA polymerase (EC:2.7.7.7), small subunit of DNA primase (EC:2.7.7.102), DNA replication ATP-dependent helicase/nuclease DNA2 (EC:5.6.2.3 3.1.-.-) and DNA replication licensing factor MCM2-like (EC:5.6.2.3) (in MCM complex) were up-regulated (Table 2; Table S2; Fig. S4). Neither of these pathways were affected after BAR application (Table 2). Accordingly, when comparing BAR vs. TDZ applications, DEGs of kinesins and microtubule proteins related to the cell motility, moreover, DEGs encoding alpha catalytic subunit and epsilon subunit of DNA polymerase, helicase and MCM complex related to DNA replication were down-regulated (Table 2).

Application of any investigated cytokinin affected the environmental information processing, i.e. DEGs related to plant hormone signal transduction in 4-week-old in vitro shoots, but in a different way depending on the cytokinin applied (Table 2). In response to TDZ in the medium, all 39 detected DEGs were up-regulated. DEGs encode to 14 proteins, such as auxin transporter-like proteins (3 and 15 A-like), IAA20-like (AUX/IAA) and SAUR50-like auxin-responsive proteins (SAUR), BTB/POZ domain and ankyrin repeat-containing

protein NPR2-like, cyclin-D3 -1-like (CYCD3), ETHYLENE INSENSITIVE 3-like 1 protein (EIN3), histidine kinase 3-like, auxin response factor 9-like (ARF), probable indole-3-acetic acid-amido synthetase GH3.1, protein TIFY 10 A-like, pseudo histidine-containing phosphotransfer protein 5-like, serine/threonine-protein kinase BSK2-like and two-component response regulator ARR5 (Table 2; Table S2; Fig. S4).

When BAR was in the medium, 55 DEGs related to plant hormone signal transduction were detected (41 up- and 14 down-regulated) in 4-week-old in vitro shoots. These DEGs encode the following proteins: abscisic acid receptor PYR1-like, auxin response factor 9-like, auxin-induced protein 15 A-like, auxin-responsive protein IAA20-like (AUX/IAA), SAUR50, and SAUR71-like (SAUR), DELLA protein GAI-like, ETHYLENE INSENSITIVE 3-like 1 protein (EIN3), ethylene-responsive transcription factor 1B-like, histidine kinase 3-like, indole-3-acetic acid-induced protein ARG7, pathogenesis-related protein 1-like, probable indole-3-acetic acid-amido synthetase GH3.1, protein phosphatase 2 C 56-like, protein TIFY 10 A-like, pseudo histidine-containing phosphotransfer protein 5-like, two-component response regulator ARR1-like isoform X1, and two-component response regulator ARR5 (Table 2; Table S2; Fig. S4).

In BAR vs. TDZ comparison, 25 DEGs in the plant hormone signal transduction pathway were down-regulated. They encode 10 proteins/enzymes (ETHYLENE INSENSITIVE 3-like 1 protein (EIN3), histidine kinase 3-like, pathogenesis-related protein 1-like, probable indole-3-acetic acid-amido synthetase GH3.1, protein FD, protein phosphatase 2 C 56-like, pseudo histidine-containing phosphotransfer protein 5-like, transcription factor TGA7, two-component response regulator ARR5, and SAUR50-like auxin-responsive proteins (SAUR)). In addition, three DEGs encoding the ethylene-responsive transcription factor 1B-like (ERF1), the SAUR71-like auxin-responsive protein (SAUR) and the auxin-induced protein 15 A-like protein were up-regulated (Table 2; Table S2; Fig. S4).

Discussion

TDZ and BAR supplementation in the culture medium affected apple in vitro shoot development differently, as described in several previous studies (Huetteman and Preece 1993; Dobránszki and Teixeira da Silva 2010; Magyar-Tábori et al. 2010; Teixeira da Silva et al. 2019; Abdalla and Dobránszki 2024). Consistent with these earlier findings on in vitro shoot development, the morphology of the developed new shoots was different, on cytokinin-free, TDZ-containing and BAR-containing media (Fig. 3). The present study revealed some transcriptomic changes behind the various shoot development and developmental disorders caused by different CK-supply of the multiplication media. The developmental characteristics observed

on different CK-containing media were related to the different expression intensities of genes encoding TFs and enzymes involved mainly in genetic and environmental information processing, cell motility, the zeatin biosynthesis, the tryptophan metabolism, the phenylpropanoid biosynthesis, and cutin, suberin, and wax biosynthesis (Fig. 3).

In 4-week-old in vitro apple shoots, the type of CK applied in the multiplication medium affected the expression intensities of some genes related to the plant hormone signal transduction (Table 2; Fig. 3; Table S2; Fig. S4). DEGs encoding auxin transporter-like (auxin transporter-like 3 and 15 A-like) proteins were upregulated in shoots grown on TDZ-containing medium compared to control shoots grown on CK-free medium while that was not detectable in response to BAR. Cellular/tissue accumulation of auxin regulates all aspects of plant growth and development in the course of which it is regulated by different transport processes (Hammes et al. 2022). Furthermore, in TDZ vs. control comparison signal transduction pathways leading to cell enlargement and plant growth (early response auxin genes, like genes of AUX/IAA, SAUR, ARF, indole-3-acetic acid-amido synthetase GH3.1) (Staswick et al. 2005; Dreher et al. 2006; Li et al. 2016; Zhang et al. 2021), cell division and shoot initiation, ubiquitin mediated proteolysis (BTB/POZ domain and ankyrin repeat-containing protein NPR2-like) were up-regulated. In addition, up-regulation of DEG related to CYCD3 can activate the G1/S phase transition in response to the hormone signal cytokinin (Riou-Khamlichi et al. 1999; Menges et al. 2006) supporting cell division. In shoots grown on BAR-containing medium, however, there was no change detected in the expression intensity of the DEG encoding CYCD3. This is consistent with finding that DEGs encoding enzymes related to DNA replication were up-regulated in TDZ-applied medium compared to the control, but down-regulated when BAR was applied compared to TDZ application in 4-week-old shoots. Exogenous cytokinin application (to some organs that normally lack this hormone) has been demonstrated to induce cell division and cytokinins have been linked to all stages of the cell cycle (reviewed in D'Agostino and Kieber 1999; Frank and Schmülling 1999; den Boer and Murray 2000). In *Arabidopsis*, cytokinin rapidly up-regulates the expression of CYCD3, a cyclin that plays a key role in the regulation of plant cell division both in vitro and in vivo (Soni et al. 1995; Riou-Khamlichi et al. 1999). Cytokinins may also play a role in the G2/M transition (Hare and Staden 1997; Francis 2011; Lipavská et al. 2011). The up-regulation of other proteins affected the jasmonate signaling (protein TIFY 10 A-like, Chini et al. 2007) and brassinosteroid mediated signaling (serine/threonine-protein kinase BSK2-like, Afzal et al. 2008) pathways and cytokinin perception and phosphorelay (histidine kinase 3-like, histidine-containing phosphotransfer protein 5-like; ARR5) (To et al. 2007; Abriata et al. 2017). In the BAR vs. control comparison, in the pathways leading to cell enlargement and plant growth,

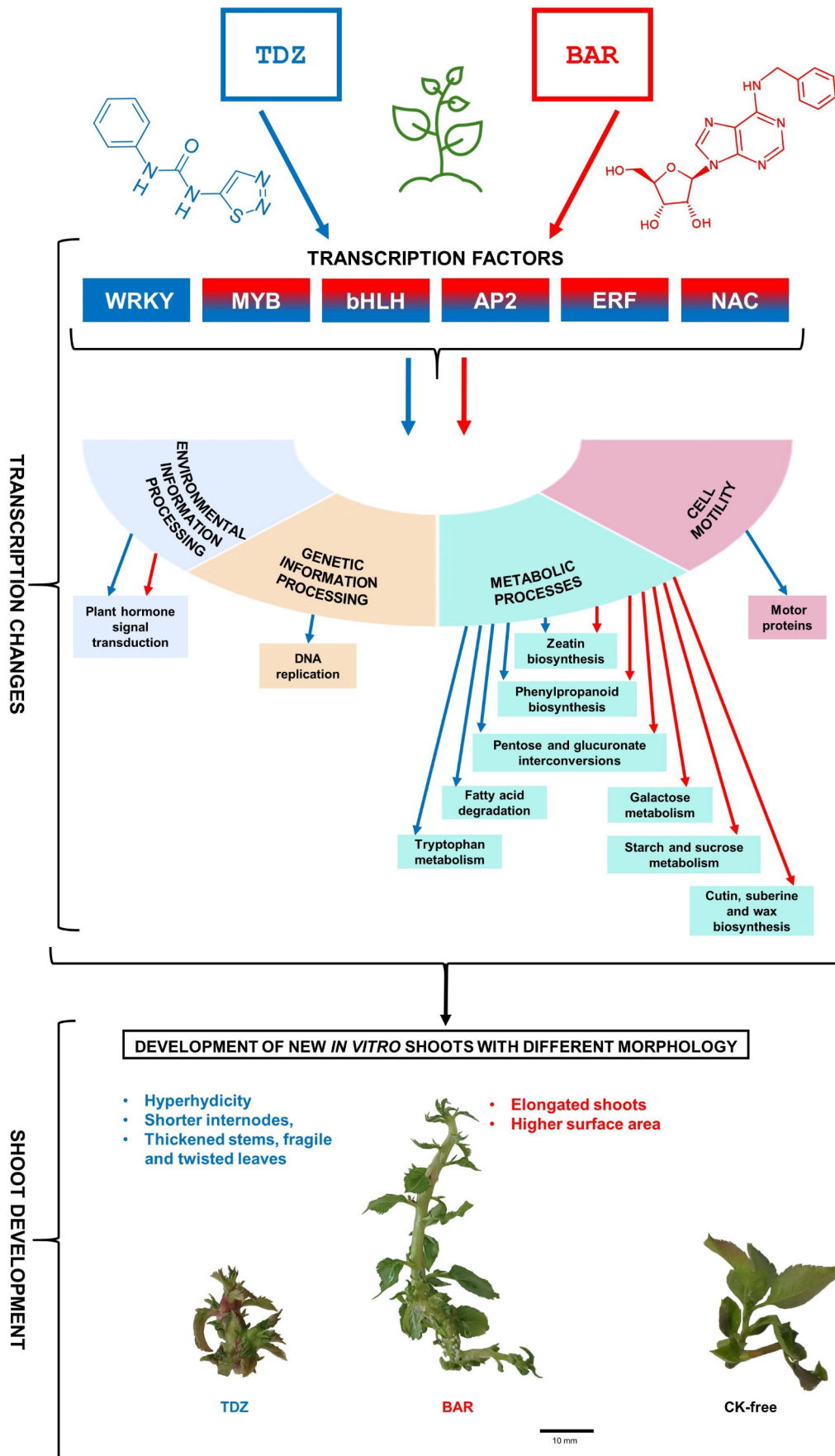


Fig. 3 Putative roadmap of main transcriptomic responses of in vitro apple (*Malus × domestica* Borkh.) cv. Húsvéti rozmaring shoots cultured on medium supplemented with either thidiazuron (TDZ) or 6-benzylaminopurine riboside (BAR). Effects of TDZ are indicated with blue colors while effects of BAR are indicated with red colors (arrows, box color of transcription factors, and description of shoot morphology). Detailed description is in the text

out of early response auxin genes AUX/IAA, SAUR, ARF and others involved in the response to auxin (like indole-3-acetic acid-induced protein ARG7) were upregulated similarly as in TDZ treatment. However, on the contrary, GH3 was downregulated. The GH3 enzyme catalyzes the synthesis IAA-amino acid conjugates and thus participates in plant auxin homeostasis by removing excess free auxin (Staswick et al. 2005). Its down-regulation may have led to a change in the ratio of CK and auxin in BAR-treated shoots compared to both TDZ-treated and control shoots. In contrast to the effect of TDZ, BAR caused the down-regulation of the DEG of EIN3, which acts as a TF, regulating ethylene response pathway (Chao et al. 1997). Its down-regulation down-regulated the pathway leading to ubiquitin mediated proteolysis in BAR-treated shoots compared both to control and TDZ-treated shoots. The latter process plays an important role—among others—in the regulation of TFs and the abundance and desensitization of receptors, thus in the signal transduction of cytokinins, auxins and other plant hormones (Frugis and Chua 2002). The ERF subfamily of APETALA2/Ethylene Responsive Factor (AP2/ERF) family TFs is involved in floral development and also enhances abiotic stress tolerance via exhibiting different response patterns under abiotic stresses. The AP2 subfamily plays a vital role in organ architecture and development in plants (including leaf and embryonic development) (Kerstens et al. 2020). Transmissions between developmental stages, hormone signaling and stress responses are regulated by the ethylene signaling pathway, which is under control or supervision—not exclusively—by the RAV subfamily (Alonso and Stepanova 2004). Cytokinin perception (histidine kinase 3-like) was up-regulated in BAR treatment when compared to the control, similarly as in TDZ treatment. However, both cytokinin perception and phosphorelay (histidine kinase 3-like, histidine-containing phosphotransfer protein 5-like; ARR5) were down-regulated in BAR treatment compared to TDZ-treatment. In response to exogenous BAR supply, the up-regulation of the DEG encoding the DELLA protein GAI-like suggested that exogenous BAR affected gibberellin (GA) signaling and homeostasis (Fukazawa et al. 2014), microtubules (MTs) dissociation (Locascio et al. 2013), which could not be detected in TDZ treatment. Different GA signaling upon exogenous TDZ and BAR treatment may also have contributed to the different morphology and development of newly developed in vitro apple shoots after 4 weeks of subculture. This process is related to other findings of this study that in the TDZ-supplied medium, up-regulated DEGs relating to motor proteins were detected in

4-week-old shoots compared to the control, while in the BAR-supplied medium, the down-regulation of these was detected (Table 2; Table S2; Fig. S4). Cortical microtubules (CMTs), located in the cell cortex in non-dividing cells (Lucas and Shaw 2008), play an important role in delivering and depositing new cell wall components and maintaining the cell shape (Hashimoto 2015; Elliott and Shaw 2018). The regulation of the CMT network involves type-B response regulators (ARRs) that mediate cytokinin signaling in *Arabidopsis*. Increasing cytokinin activity (either endogenously or exogenously) has an effect on the dynamics of the microtubular cytoskeleton; CMTs are reorganized earlier before differentiation (Montesinos et al. 2020). The up-regulation of certain genes related to DNS replication and motor proteins when supplying TDZ may have been a result of the connection existing between cytokinin signaling and the regulation of the cell cycle and microtubules.

Plant bHLH TFs were highly affected by the supply of exogenous CK, either BAR or TDZ (Fig. 2). They are involved in a wide range of biological processes including growth and development, hormone signaling, cellular differentiation, environmental interactions/adaptation, and play a regulatory role in the response to various stresses (Zhai et al. 2016; Hong 2016; Li et al. 2021). Furthermore, bHLH TFs also regulate complex physiological processes (incorporating biomass, lignin, anthocyanin and flavonoid biosynthesis) and have a vital role in hormone signal transduction involving jasminic acid, salicylic acid, ethylene, gibberellin, auxin and abscisic acid (Murre et al. 1989; Feller et al. 2011; Kazan and Manners 2013).

The WRKY TF family participates in several physiological and developmental processes including plant hormone signaling, is involved in various stress responses, and also takes part in numerous cytoplasmic and nuclear processes (e.g. signaling events from organelles or the cytoplasm to the nucleus) (Wani et al. 2021). WRKY TFs were detected to be upregulated in response to exogenous TDZ-supply (Fig. 2).

The expression intensity of genes involved in the zeatin biosynthesis in in vitro apple shoots was significantly influenced by the CK content of the culture medium, to a CK-dependent extent (Table 2; Table S2; Fig. S4). Addition of exogenous CKs, either TDZ or BAR, to the culture medium led to the increase of the expression intensity of DEGs encoding the cytokinin dehydrogenase 4-like enzyme (EC:1.5.99.12) 48 h after shoot explants being placed on CK-containing medium (Table 2; Table S2; Fig. S4). This enzyme catalyzes the oxidation of CKs. This finding is consistent with previous studies where transient increases in cytokinin oxidase/dehydrogenase activity were observed in plant tissue cultures of other plants after application of exogenous CKs (Chatfield and Armstrong 1986; Motyka et al. 2003; Schmülling et al. 2003). Furthermore, DEGs related to the synthesis of isopentenyl-AMP, isopentenyl-ADP and isopentenyl-ATP were down-regulated

upon TDZ supply, ultimately leading to a down-regulation in the iPMP-dependent biosynthetic pathway of endogenous trans-zeatin (Astot et al. 2000) (Table 2; Table S2; Fig. S4). However, in 4-week-old shoots grown on TDZ containing medium, no changes in the expression intensities of genes related to the zeatin biosynthesis were detected. On the contrary, BAR supply affected the zeatin biosynthetic pathway in 4-week-old shoots compared to shoots grown on both the CK-free and the TDZ-containing media. BAR-containing medium inhibited the oxidation (i.e. degradation) and the O-glycosylation (i.e. inactivation) (Schmülling 2004) of endogenous CK (zeatin) by the down-regulation of DEGs encoding cytokinin dehydrogenase 4-(EC:1.5.99.12), cytokinin hydroxylase-like and zeatin O-glucosyltransferase-like enzymes EC:2.4.1.215), in 4-week-old shoots (Table 2; Table S2; Fig. S4). Thereby it may have contributed to a higher level of endogenous cis/trans-zeatin compared to shoots grown on CK-free or even on TDZ-containing media. In shoots grown on TDZ-containing medium the CK degradation was down-regulated while DEGs related to the synthesis of isopentenyl-AMP/ADP/ATP were up-regulated compared to shoots grown on BAR-containing medium (Fig. 3).

Changes in the tryptophan metabolism may be important for the endogenous hormone balance and homeostasis in plants since tryptophan being a common precursor of both melatonin and indoleacetate biosynthesis (Posmyk and Janas 2009; Erland and Saxena 2018). The type of CK applied in the culture medium modified the pathway; the formation of both melatonin and indolacetate were down-regulated in 4-week-old shoots grown on BAR-containing medium compared to ones grown on TDZ-containing medium, via the down-regulation of DEGs encoding indole-3-pyruvate monooxygenase YUCCA2-like (C:1.14.13.168) and caffeic acid 3-O-methyltransferase-like (EC:2.1.1.68 2.1.1.4) (Table 2; Table S2; Fig. S4). As melatonin is a master regulator and indoleacetate is an auxin in plants, this metabolic process is closely linked to the regulation of plant growth and development (Fig. 3) (Erland et al. 2015; Arnao and Hernández-Ruiz 2019).

Changes in the ultrastructure of the cuticle may lead to abnormal uptake, transport and evaporation of water thus causing hyperhydricity or other disorders affecting mainly the leaves and stems of the explants (Hazarika 2006; Polivanova and Bedarev 2022). High concentrations of cytokinins can also lead to hyperhydricity resulting in shorter internodes, thickened stems, fragile and twisted leaves as shown in Fig. 3 regarding TDZ (Beisson et al. 2012). The BAR content of the medium shifted the cutin, suberine and wax biosynthesis towards long-chain wax ester (wax biosynthesis), common cutin and suberin monomers (polyhydroxy-fatty acid, α,ω -dicarboxylic acid), and compounds of long-chain fatty acids, such as 10,16-dihydroxypalmitate and 16-feruloyoxypalmitic acid (Table 2; Table S2; Fig. S4). The shoots derived from

BAR-containing medium were elongated compared to the ones on TDZ-containing medium (Fig. 3), resulting in a higher surface area expansion. The cuticular composition and wax load in general remain constant during elongation, which entails our observation in the case of BAR-supplied medium that the biosynthetic flux shifting into cutin, suberine and waxes closely matched this surface area expansion (Suh et al. 2005). Furthermore, over- (or under-) expression of TFs (mainly AP2/ERF and MYB TFs) involved in cuticle biosynthesis, which was observed in both CK treatments, but in a different way, may have induced cuticle composition and structure changes and also negatively influenced plant development (Figs. 2, 3) (Nikolai et al. 2014; Licausi et al. 2010). The MYB TF family is involved in the regulation of plant growth and development, biotic/abiotic stress resistance, organogenesis, signaling pathways, metabolite (e.g. anthocyanin) biosynthesis and acting as a link between different phytohormones while also regulating their levels (Wu et al. 2024).

Between organs, cell types, cell layers, developmental stages and different growth conditions, the amount, and to a lower extent the composition of lignin units (radicals) differs widely (Boerjan et al. 2003; Rinaldi et al. 2016; Meents et al. 2018; Cesarino 2019). Perturbations of lignin biosynthesis is often caused by changes in the environmental conditions (f.e. hypolignification as a cause of hyperhydricity in *in vitro* cultures) (Polivanova and Bedarev 2022). Production of guaiacyl lignin, 5-hydroxyguaiacyl lignin, syringin and p-hydroxyphenyl lignin, that are capable of spontaneous polymerization to lignin, were down-regulated regarding the shoots derived from BAR-containing medium compared to the effect of TDZ (Table 2; Table S2; Fig. S4). Regarding the modulation of cell wall features and chemical composition, phytohormones, such as CKs, are involved in cell wall development regulation for wood formation (Jang and Choi 2018). These observations further prove that different CKs have dissimilar effects on lignin biosynthesis and wood formation in vascular plants. Furthermore, upon hormonal signaling by CKs, MYB and NAC TFs, that are also regulators of the lignin pathway (Xiao et al. 2021; Li et al. 2024), were differentially expressed in response to both CK-supplies, either BAR or TDZ (Fig. 2). The detected changes in the expression intensity of genes linked to the phenylpropanoid biosynthesis may be one of the reasons of growth and developmental disorders obtained upon various CK-supplies, mainly in the case of TDZ in our study (Fig. 3).

It is still worth mentioning that BAR supply affected the starch and sucrose metabolism and galactose metabolism in 4-week-old shoots, while TDZ-supply did not affect them. On the contrary, TDZ supply modified the fatty acid degradation via its up-regulation but BAR-supply did not affect it. In addition, the pentose glucuronate pathway was affected by both CKs differently (Table 2; Table S2; Fig. S4).

Experiments regarding the CK content of the shoot multiplication medium have been focusing on the morphological, physiological and biochemical changes (Huetteman and Preece 1993; Hazarika 2006; reviewed in Dobránszki and Teixeira da Silva 2010; Dobránszki 2014; Polivanova and Bedarev 2022; Hu and Shani 2023; Abdalla and Dobránszki 2024), however, their transcriptomic background was not covered yet. The present investigation provided evidence for the transcriptomic changes within in vitro apple shoots in response to exogenous CK supply. The present study allowed us to create a putative roadmap of transcriptomic changes (Fig. 3) in in vitro apple shoots in response to different CK supplies which may contribute to better understanding the molecular background of in vitro shoot developmental characteristics, and growth and developmental disorders.

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Author contributions JD conceptualization; DF, AK, NA, VA, JD performed the experiments; VA, JD, AK and DF analyzed and interpreted the data; all authors were involved in the preparation of the manuscript, all authors have approved the manuscript.

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Data availability The transcriptomics data has been deposited at the NCBI GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), where it is available under the identifier GSE287596. Apart from that, all relevant data can be found within the manuscript and its supporting materials.

Declarations

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could appear to influence the work reported in this paper.

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References

- Abdalla N, Dobránszki J (2024) Meta-Topolin as an effective benzyladenine derivative to improve the multiplication rate and quality of in vitro axillary shoots of *húsvéti Rozmaring Apple* scion. *Plants (Basel)* 13(11):1568. <https://doi.org/10.3390/plants13111568>
- Abriata LA, Albanesi D, Dal Peraro M, De Mendoza D (2017) Signal sensing and transduction by histidine kinases as unveiled through studies on a temperature sensor. *Acc Chem Res* 50(6):1359–1366. <https://doi.org/10.1021/acs.accounts.6b00593>
- Afzal AJ, Wood AJ, Lightfoot DA (2008) Plant receptor-like Serine threonine kinases: roles in signaling and plant defense. *Mol Plant Microbe Interact* 21(5):507–517. <https://doi.org/10.1094/MPMI-21-5-0507>
- Alonso JM, Stepanova AN (2004) The ethylene signaling pathway. *Science* 306(5701):1513–1515
- Arnao MB, Hernández-Ruiz J (2019) Melatonin: A New Plant Hormone and/or a Plant Master Regulator? *Trends Plant Sci* 24(1):38–48. <https://doi.org/10.1016/j.tplants.2018.10.010>
- Astot C, Dolezal K, Nordström A, Wang Q, Kunkel T, Moritz T, Chua NH, Sandberg G (2000) An alternative cytokinin biosynthesis pathway. *Proc Natl Acad Sci USA* 97(26):14778–14783. <https://doi.org/10.1073/pnas.260504097>
- Baldi P, Buti M, Gualandri V, Khomenko I, Farneti B, Biasioli F, Paffetti D, Malnoy M (2024) Transcriptomic and volatilmic profiles reveal *Neofabraea vagabunda* infection-induced changes in susceptible and resistant apples during storage. *Postharvest Biol Technol* 212(112889):0925–5214. <https://doi.org/10.1016/j.postharvbio>
- Beisson F, Li-Beisson Y, Pollard M (2012) Solving the puzzles of cutin and suberin polymer biosynthesis. *Curr Opin Plant Biol* 15(3):329–337. <https://doi.org/10.1016/j.pbi.2012.03.003>
- Bhatti S, Jha G (2010) Current trends and future prospects of biotechnological interventions through tissue culture in Apple. *Plant Cell Rep* 29:1215–1225. <https://doi.org/10.1007/s00299-010-0907-8>
- Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. *Annu Rev Plant Biol* 54(1):519–546. <https://doi.org/10.1146/annurev.arplant.54.031902.134938>
- Butiuc-Keul AL, Halmagyi A, Isac V, Crăciunaş C, Carpa R (2010) Apple shoot multiplication and plantlets reaction to in vitro culture. *Analele Universitatii Din Oradea: Fascicula Biol*, 17 (1). pp.70–75. Print-ISSN: 1224–5119, e-ISSN: 1844–7589, CD-ISSN: 1842–6433
- Cesarino I (2019) Structural features and regulation of lignin deposited upon biotic and abiotic stresses. *Curr Opin Biotech* 56:209–214. <https://doi.org/10.1016/j.copbio.2018.12.012>
- Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR (1997) Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. *Cell* 89(7):1133–1144. [https://doi.org/10.1016/s0092-8674\(00\)80300-1](https://doi.org/10.1016/s0092-8674(00)80300-1)
- Chatfield JM, Armstrong DJ (1986) Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. Cv. Great Northern. *Plant Physiol* 80:493–499. <https://doi.org/10.1104/pp.80.2.493>
- Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing

- link in jasmonate signalling. *Nature* 448(7154):666–671. <https://doi.org/10.1038/nature06006>
- D'Agostino IB, Kieber JJ (1999) Molecular mechanisms of cytokinin action. *Curr Opin Plant Biol* 2:359–364. [https://doi.org/10.1016/S1369-5266\(99\)00005-9](https://doi.org/10.1016/S1369-5266(99)00005-9)
- den Boer BG, Murray JA (2000) Triggering the cell cycle in plants. *Trends Cell Biol* 10:245–250. [https://doi.org/10.1016/S0962-8924\(00\)01765-7](https://doi.org/10.1016/S0962-8924(00)01765-7)
- Dobránszki J (2014) Cytokinins—importance, structure, effects in vitro. In: Dobránszki J ed. *Aromatic cytokinins applied exogenously in plant tissue culture*, University of Debrecen Centre for Agricultural Sciences Research Institute of Nyíregyháza, Hungary. pp. 5–22. ISBN 978-963-473-704-9
- Dobránszki J, Teixeira da Silva JA (2010) Micropropagation of apple—A review. *Biotechnol Adv* 28:462–488. <https://doi.org/10.1016/j.biotechadv.2010.02.008>
- Dreher KA, Brown J, Saw RE, Callis J (2006) The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness. *Plant Cell* 18(3):699–714. <https://doi.org/10.1105/tpc.105.039172>
- Elliott A, Shaw SL (2018) Update: plant cortical microtubule arrays. *Plant Physiol* 176:94–105. <https://doi.org/10.1104/pp.17.01329>
- Erland LAE, Saxena P (2018) Auxin driven indoleamine biosynthesis and the role of Tryptophan as an inductive signal in hypericum perforatum (L). *PLoS ONE* 14(10):e0223878. <https://doi.org/10.1371/journal.pone.0223878>
- Erland LA, Murch SJ, Reiter RJ, Saxena PK (2015) A new balancing act: the many roles of melatonin and serotonin in plant growth and development. *Plant Signal Behav* 10:e1096469. <https://doi.org/10.1080/15592324.2015.1096469>
- Feller A, Machemer K, Braun EL, Grotewold E (2011) Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. *Plant J* 66(1):94–116. <https://doi.org/10.1111/j.1365-3113.X.2010.04459.x>
- Ferretti G, Turco I, Bacchetti T (2014) Apple as a source of dietary phytonutrients: bioavailability and evidence of protective effects against human cardiovascular disease. *Food Nutr Sci* 5:1234–1246. <https://doi.org/10.4236/fns.2014.513134>
- Francis D (2011) A commentary on the G2/M transition of the plant cell cycle. *Ann Bot* 107:1065–1070. <https://doi.org/10.1093/aob/mcr055>
- Frank M, Schmülling T (1999) Cytokinin cycles cells. *Trends Plant Sci* 4:243–244. [https://doi.org/10.1016/S1360-1385\(99\)01425-9](https://doi.org/10.1016/S1360-1385(99)01425-9)
- Frugis G, Chua NH (2002) Ubiquitin-mediated proteolysis in plant hormone signal transduction. *Trends Cell Biol* 12(7):308–311. [https://doi.org/10.1016/s0962-8924\(02\)02308-5](https://doi.org/10.1016/s0962-8924(02)02308-5)
- Fukazawa J, Teramura H, Murakoshi S, Nasuno K, Nishida N, Ito T, Yoshida M, Kamiya Y, Yamaguchi S, Takahashi Y (2014) DELLAs function as coactivators of GAI-ASSOCIATED FACTOR1 in regulation of Gibberellin homeostasis and signaling in Arabidopsis. *Plant Cell* 26(7):2920–2938. <https://doi.org/10.1105/tpc.114.125690>
- Hammes UZ, Murphy AS, Schwechheimer C (2022) Auxin Transporters—A biochemical view. *Cold Spring Harb Perspect Biol* 14(2):a039875. <https://doi.org/10.1101/cshperspect.a039875>
- Hare PD, van Staden J (1997) The molecular basis of cytokinin action. *Plant Growth Reg* 23:41–78. <https://doi.org/10.1023/A:1005902508249>
- Hashimoto T (2015) Microtubules in plants. *Arabidopsis Book* 13:e0179. <https://doi.org/10.1199/tab.0179>
- Hazarika BN (2006) Morpho-physiological disorders in in vitro culture of plants. *Sci Hortic* 108(2):105–120. <https://doi.org/10.1016/j.scienta.2006.01.038>
- Hong JC (2016) General aspects of plant transcription factor families. In: Gonzelez, D. ed. *Plant transcription factors*, Academic Press. pp. 35–56 <https://doi.org/10.1016/b978-0-12-800854-6.00003-8>
- Hu Y, Shani E (2023) Cytokinin activity - transport and homeostasis at the whole plant, cell, and subcellular levels. *New Phytol* 239(5):1603–1608. <https://doi.org/10.1111/nph.19001>
- Huetteman CA, Preece JE (1993) Thidiazuron: a potent cytokinin for Woody plant tissue culture. *Plant Cell Tiss Organ Cult* 33:105–119. <https://doi.org/10.1007/BF01983223>
- Itana DD, Habib ZY (2022) In vitro micropropagation of Apple (*Malus domestica*) through axillary buds and shoot apices culture. *Int J Zool Appl Biosci* 7(3):1–7. <https://doi.org/10.55126/ijzab.2022.v07.i03.001>
- Jang G, Choi YD (2018) Drought stress promotes xylem differentiation by modulating the interaction between cytokinin and jasmonic acid. *Plant Sign Behav* 13(3):e1451707. <https://doi.org/10.1080/15592324.2018.1451707>
- Kaur A, Singh D, Varsha Gupta N, Kumar A (2018) In vitro propagation of important rootstocks of Apple for rapid cloning and improvement. In: Gosal S, Wani S (eds) *Biotechnologies of crop improvement*, vol 1. Springer, Cham. https://doi.org/10.1007/978-3-319-78283-6_6
- Kazan K, Manners JM (2013) MYC2: the master in action. *Mol Plant* 6(3):686–703 Epub 2012 Nov 9. PMID: 23142764. <https://doi.org/10.1093/mp/sss128>
- Kerstens MHL, Schranz ME, Bouwmeester K (2020) Phylogenomic analysis of the APETALA2 transcription factor subfamily across angiosperms reveals both deep conservation and lineage-specific patterns. *Plant J* 103(4):1516–1524. <https://doi.org/10.1111/tpj.14843>
- Kumar A, Bhuj BD, Dhar S, Dixit KMJ, Singh SP (2023) Micropropagation of fruit crops: A review. *Adv Crop Sci Technol* 11:595. <https://doi.org/10.14719/pst.1891>
- Li SB, Xie ZZ, Hu CG, Zhang JZ (2016) A review of auxin response factors (ARFs) in plants. *Front Plant Sci* 7:47. <https://doi.org/10.3389/fpls.2016.00047>
- Li C, Yan C, Sun Q, Wang J, Yuan C, Mou Y, Shan S, Zhao X (2021) The bHLH transcription factor AhbHLH112 improves the drought tolerance of peanut. *BMC Plant Biol* 21. <https://doi.org/10.1186/s12870-021-03318-6>
- Li W, Lin YCJ, Chen YL, Zhou C, Li S, De Ridder N, Oliveira DM, Zhang L, Zhang B, Wang JP, Xu C (2024) Woody plant cell walls: fundamentals and utilization. *Mol Plant* 17(1):112–140. <https://doi.org/10.1016/j.molp.2023.12.008>
- Licausi F, Giorgi FM, Zenoni S, Osti F, Pezzotti M, Perata P (2010) Genomic and transcriptomic analysis of the AP2/ERF superfamily in *vitis vinifera*. *BMC Genomics* 11:1–16. <https://doi.org/10.1186/1471-2164-11-719>
- Lipavská H, Mašková P, Vojvodová P (2011) Regulatory dephosphorylation of CDK at G2/M in plants: yeast mitotic phosphatase cdc25 induces cytokinin-like effects in Transgenic tobacco morphogenesis. *Ann Bot* 107:1071–1086. <https://doi.org/10.1093/aob/mcr016>
- Locascio A, Blázquez MA, Alabadi D (2013) Dynamic regulation of cortical microtubule organization through prefoldin-DELLA interaction. *Curr Biol* 23(9):804–809. <https://doi.org/10.1016/j.cub.2013.03.053>
- Lucas J, Shaw SL (2008) Cortical microtubule arrays in the Arabidopsis seedling. *Curr Opin Plant Biol* 11:94–98. <https://doi.org/10.1016/j.pbi.2007.12.001>
- Magyar-Tábori K, Dobránszki J, Teixeira da Silva JA, Bulley SM, Hudák I (2010) The role of cytokinins in shoot organogenesis in Apple. *Plant Cell Tissue Organ Cult* 101:251–267. <https://doi.org/10.1007/s11240-010-9696-6>
- Meents MJ, Watanabe Y, Samuels AL (2018) The cell biology of secondary cell wall biosynthesis. *Ann Bot* 121(6):1107–1125. <https://doi.org/10.1093/aob/mcy005>
- Menges M, Samland AK, Planchais S, Murray JA (2006) The D-type Cyclin CYCD3;1 is limiting for the G1-to-S-phase transition in

- Arabidopsis. *Plant Cell* 18(4):893–906. <https://doi.org/10.1105/pc.105.039636>
- Mierczak K, Garus-Pakowska A (2024) An overview of Apple varieties and the importance of Apple consumption in the prevention of Non-Communicable Diseases-A narrative review. *Nutrients* 16(19):3307 PMID: 39408274; PMCID: PMC11478947. <https://doi.org/10.3390/nu16193307>
- Montesinos JC, Abuzeineh A, Kopf A, Juanes-Garcia A, Ötvös K, Petrášek J, Sixt M, Benková E (2020) Phytohormone cytokinin guides microtubule dynamics during cell progression from proliferative to differentiated stage. *EMBO J* 39:e104238. <https://doi.org/10.15252/embj.2019104238>
- Motyka V, Vaňková R, Čapková V, Petrášek J, Kamínek M, Schmülling T (2003) Cytokinin-induced upregulation of cytokinin oxidase activity in tobacco includes changes in enzyme glycosylation and secretion. *Physiol Plant* 117:11–21. <https://doi.org/10.1034/j.1399-3054.2003.1170102.x>
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15(3):473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- Murre C, McCaw PS, Baltimore D (1989) A new DNA binding and dimerization motif in Immunoglobulin enhancer binding, daughterless, MyoD, and Myc proteins. *Cell* 56(5):777–783. [https://doi.org/10.1016/0092-8674\(89\)90682-X](https://doi.org/10.1016/0092-8674(89)90682-X)
- Nikolai B, Maria H, Sergiy L (2014) Transcriptional regulation of cuticle biosynthesis. *Biotech Adv* 32(2):526–540 ISSN 0734–9750. <https://doi.org/10.1016/j.biotechadv.2014.01.005>
- Oyenihi AB, Belay ZA, Mditshwa A, Caleb OJ (2022) An Apple a day keeps the Doctor away: the potentials of Apple bioactive constituents for chronic disease prevention. *J Food Sci* 87(6):2291–2309. <https://doi.org/10.1111/1750-3841.16155>
- Polivanova OB, Bedarev VA (2022) Hyperhydricity in plant tissue culture. *Plants* 11(23):3313. <https://doi.org/10.3390/plants11233313>
- Posmyk MM, Janas KM (2009) Melatonin in plants. *Acta Physiol Plant* 31:1. <https://doi.org/10.1007/s11738-008-0213-z>
- Rinaldi R, Robin J, Matthew T, Clough JR, Marco K, Pieter CAB, Bert MW (2016) Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. *Angew Chem Int Ed* 55(29):8164–8215. <https://doi.org/10.1002/anie.201510351>
- Riou-Khamlichi C, Huntley R, Jacqmar A, Murray JA (1999) Cytokinin activation of Arabidopsis cell division through a D-type Cyclin. *Science* 283:1541–1544. <https://doi.org/10.1126/science.283.5407.1541>
- Schmülling T (2004) Cytokinin. In: Lennarz W, Lane MD (eds) *Encyclopedia of biological chemistry*. Academic Press/Elsevier Science, pp 1–7
- Schmülling T, Werner T, Riefler M, Krupková E, Bartrina y Manns I (2003) Structure and function of cytokinin oxidase/dehydrogenase genes of maize, rice, Arabidopsis and other species. *J Plant Res* 116(3):241–252. <https://doi.org/10.1007/s10265-003-0096-4>
- Shi JL, Dong ZD, Song CH, Xie BY, Zheng XB, Song SW, Jiao J, Wang MM, Bai TH (2021) Establishment of an efficient micro-propagation system in enhancing rooting efficiency via stem cuttings of Apple rootstock M9T337. *Hort Sci* 48:63–72. <https://doi.org/10.17221/106/2020-HORTSCI>
- Soni R, Carmichael JP, Shah ZH, Murray JAH (1995) A family of Cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. *Plant Cell* 7:85–103. <https://doi.org/10.1105/tpc.7.1.85>
- Staswick PE, Serban B, Rowe M, Tiryaki I, Maldonado MT, Maldonado MC, Suza W (2005) Characterization of an Arabidopsis enzyme family that conjugates amino acids to indole-3-acetic acid. *Plant Cell* 17(2):616–627. <https://doi.org/10.1105/tpc.104.026690>
- Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Beisson F (2005) Cuticular lipid composition, surface structure, and gene expression in Arabidopsis stem epidermis. *Plant Physiol* 139(4):1649–1665 Epub 2005 Nov 18. PMID: 16299169; PMCID: PMC1310549. <https://doi.org/10.1104/pp.105.070805>
- Teixeira da Silva JA, Gulyás A, Magyar-Tábori K, Wang MR, Wang QC, Dobránszki J (2019) In vitro tissue culture of Apple and other Malus species: recent advances and applications. *Planta* 249:975–1006. <https://doi.org/10.1007/s00425-019-03100-x>
- To JP, Deruère J, Maxwell BB, Morris VF, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ (2007) Cytokinin regulates type-A Arabidopsis response regulator activity and protein stability via two-component phosphorelay. *Plant Cell* 19(12):3901–3914. <https://doi.org/10.1105/tpc.107.052662>
- Vylčilová H, Bryksová M, Matušková V, Doležal K, Plíhalová L, Strnad M (2020) Naturally occurring and artificial N9-Cytokinin conjugates: from synthesis to biological activity and back. *Biomolecules* 10(6):832. <https://doi.org/10.3390/biom10060832>
- Wani SH, Anand S, Singh B, Bohra A, Joshi R (2021) WRKY transcription factors and plant defense responses: latest discoveries and future prospects. *Plant Cell Rep* 40:1071–1085. <https://doi.org/10.1007/s00299-021-02691-8>
- Wu X, Xia M, Su P, Zhang Y, Tu L, Zhao H, Gao W, Huang L, Hu Y (2024) MYB transcription factors in plants: A comprehensive review of their discovery, structure, classification, functional diversity and regulatory mechanism. *Int J Biol Macromol* 282(2):136652. <https://doi.org/10.1016/j.ijbiomac.2024.136652>
- Xiao R, Zhang C, Guo X, Li H, Lu H (2021) MYB transcription factors and its regulation in secondary cell wall formation and lignin biosynthesis during xylem development. *Int J Mol Sci* 22(7):3560. <https://doi.org/10.3390/ijms22073560>
- Zhai Y, Zhang L, Xia C, Fu S, Zhao G, Jia J, Kong X (2016) The wheat transcription factor, TabHLH39, improves tolerance to multiple abiotic stressors in Transgenic plants. *Biochem Biophys Res Commun* 473(4):1321–1327 Epub 2016 Apr 16. PMID: <https://doi.org/10.1016/j.bbrc.2016.04.071>
- Zhang H, Yu Z, Yao X, Chen J, Chen X, Zhou H, Lou Y, Ming F, Jin Y (2021) Genome-wide identification and characterization of small auxin-up RNA (SAUR) gene family in plants: evolution and expression profiles during normal growth and stress response. *BMC Plant Biol* 21:1–14. <https://doi.org/10.1186/s12870-020-02781-x>