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Treatment of *Glycine max* seeds with gibberellins alters root morphology, anatomy, and transcriptional networks

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Abstract

Gibberellins (GAs) regulate diverse aspects of growth and development, but their role in root development and lateral root (LR) formation is poorly understood. In this study, GA₃ was applied to soybean [*Glycine max* (L.) Merr] by seed soaking. The results showed that root length and root surface area were significantly inhibited in early stages after GA₃ treatment. Microscopic examination showed that GA₃ treatment changed the cortex thickness, the pericycle diameter, and cell size in main root. Interestingly, exogenous GA₃ increased the quantity of lateral root primordia (LRP), but LR number decreased in this period. Moreover, the content of GAs, auxin and abscisic acid in root was altered. RNA-seq results revealed that application of GA₃ not only changed the expression of genes in GA biosynthesis pathway, including *GA20ox* and *GA2ox*, but also the GA regulation genes and signalling pathway genes. The changes in expression of gene concerning other hormones were also detected. In addition, GA₃ altered cell wall biogenesis and degradation genes which might be related to the changes of root morphology. In response to increased GA₃, 103 transcription factors were detected. Thus, exogenous GA₃ changed the content of hormones in roots and affected the root development by regulating the expression of respective genes.

Additional key words: gene expression, lateral root, main root, RNA-seq, seed soaking, soybean.

Introduction

Soybean [*Glycine max* (L.) Merr] is an important crop. A taproot-type soybean root system, consists of an embryo-derived main root (MR) and the lateral roots (LRs) which are continuously produced from MR. LRs are the most vigorous and physiologically active part of the soybean root system which is required for crop development, yield, and quality. Because of its significance, the mechanism of

root development and LR formation have been intensively studied (Osmont *et al.* 2007, Slovak *et al.* 2015). The mechanism of root development and LR formation involves complex gene regulatory networks in model plant *Arabidopsis*. However, the detailed gene network mechanism of root development is largely unclarified in soybean. Among the studies related to soybean root, many studies focus on the response of root to biotic and abiotic stresses (Radwan *et al.* 2011, Song *et al.* 2016).

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Abbreviations: ABA - abscisic acid; CKs - cytokinins; DEG - differentially expressed gene; FDR - false discovery rate; FPKM - fragments per kilobase of transcripts per million mapped fragments; GA - gibberellin; GA₃ - gibberellic acid 3; IAA - indole-3-acetic acid; KEGG - Kyoto encyclopedia of genes and genomes; log₂FC - log₂ fold change; LR - lateral root; LRP - lateral root primordium; MR - main root; PEs - pectinesterases; PGs - polygalacturonases; PME - pectin methyl esterase; qPCR - quantitative PCR; TF - transcription factor.

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Gibberellins (GAs), a group of tetracyclic diterpenoid compounds, regulate diverse aspects of plant growth and development (Stamm *et al.* 2017, Manoharlal *et al.* 2018). Previous studies have found that GAs affect the growth and development of plant roots. In *Arabidopsis*, cell production rate and meristem size were unable to increase after germination in GA biosynthetic mutants (*gal-3* and *ga3ox1/ga3ox2*), and exogenous gibberellins relieved this inhibition (Ubeda-Tomás *et al.* 2009). So, GAs regulate root growth by cell elongation and proliferation in *Arabidopsis*. Other studies suggest that GAs can promote MR growth. The MR length of gibberellin-deficient plants was shorter than that of wild type, which elongated after the application of GA. The dose-response experiments show that root growth is regulated by GA in a lower concentration range than is required for shoot growth (Tanimoto 2012). Gibberellins inhibitors delayed the growth of MR (Fu and Harberd 2003, Ueguchi-Tanaka *et al.* 2007). However, exogenous GA₃ dramatically reduces the root growth in carrot, while the shoot growth of carrot is stimulated. It also significantly promotes xylem development in the tuberous root of carrot (Wang *et al.* 2015). In addition, the role of GAs in LR formation is also unclear (Fukaki and Tasaka 2009). Several lines of evidence suggest that GAs may inhibit LR formation. GA-overproducing mutants and GA application in aspen result in suppression of lateral and adventitious root formation (Eriksson *et al.* 2000). The LR proliferation and elongation are found in GA-insensitive (35S: PcGA2ox1) and GA-deficient (35S: rgl1) transgenic poplar (Gou *et al.* 2010). Overexpression of GAs biosynthesis gene in *Arabidopsis* (35S::AtGA20ox1) or the signal transduction gene in hybrid poplar (35S:PttGID1.1, 35S:PttGID1.3) resulted in the decrease of adventitious root number (Gou *et al.* 2010, Mauriat *et al.* 2014). So, the effects of exogenous GAs on root development vary depending on the plant species and experiment (Tanimoto 2012). Their roles in soybean root development and LR formation is poorly understood.

To reveal the regulation mechanism of GAs on root growth during seed germination, different content of GAs in root was established by seed soaking in gibberellic acid (GA₃) of different concentrations. We performed comprehensive analysis of seedling MR and LR morphological and anatomical characteristics, hormone content, and transcript profiles of genes to reveal the effects of GAs on root development. This study provided novel insights into the functions of GAs in soybean root growth during germination and early seedling growth.

Materials and methods

Soybean growth and GA₃ application: The soybean [*Glycine max* (L.) Merr] cultivar Suinong28 was bred by Heilongjiang Academy of Agriculture Sciences, Heilongjiang Province, China. Gibberellic acid 3 (*Sigma-Aldrich*, St. Louis, MO, USA), was dissolved in absolute ethanol and then diluted with water to 1 % (v/v). The concentrations of GA₃ used in this study were 0.43, 0.87, and 1.74 mM corresponding to three different treatments

named GA-15, GA-30, and GA-60. Air dry seeds (100) were soaked with 100 cm³ of GA₃ solution [0.1% ethanol (v/v) was used as a control] for 12 h. Seeds were cultivated in sand as matrix moistened with the Hoagland nutrient solution in plastic containers for 13 d (16-h photoperiod with an irradiance of 300 μmol·m⁻²·s⁻¹ and day/night temperatures of 25/18 °C). The experiments were carried out with three biological replicates with at least twenty plants in each replicate. We investigated the effect of exogenous GA₃ on the soybean root for 13 d after GA₃ treatment. Plant roots were harvested at 60, 72, and 84 h following the treatment to measure the number of LR primordia. Plant roots were harvested after 2, 3, 4, 5, 7, 9, 11, and 13 d for morphological observation. The MR and LR at 4 d were sampled for microscopic analyses. The MR at 1, 3, 5, and 10 d were used to measure the hormone content. Samples of MR treated with 0.87 mM GA₃ and control at 3 d were frozen in liquid nitrogen immediately and used for the RNA-Seq and the real time qPCR.

Observation of root morphology and anatomy: To measure the effects of GA₃ application on root morphology, the root samples were carefully removed from the sand, then washed with sterile water. In each experiment, twenty plants per treatment were chosen. Roots were scanned with an *Epson Perfection V700* photo scanner (*Seiko Epson Corporation*, Nagano, Japan). The main morphological parameters of root system, including the length, number, diameter, surface area, and root volume, were determined using *Image J 1.63* software. To observe the microscopic structure of the maturation zone of the roots, a 1 - 1.5 cm segments were excised from MR and LR tips. Samples were fixed in FAA solution containing 10 cm³ of 37 % (v/v) formaldehyde, 5 cm³ of glacial acetic acid, 50 cm³ of 96 % (v/v) ethanol, and 35 cm³ of distilled water) for 24 h, and then tissue slices were prepared for histological analysis according to Li (2009) and observed with an *Olympus BX61* microscope (*Olympus*, Tokyo, Japan) and quantified using *Image J 1.63* software.

Phytohormone analysis with HPLC-ESI-MS: Fresh mass (1 g) of the main roots was frozen in liquid nitrogen and then stored at a -80 °C freezer until use. Each treatment has three biological replicates and five independent plants were collected in each replicate. The extraction, purification, and determinations of endogenous content of GA₃, indole-3-acetic acid (IAA), and abscisic acid (ABA) were performed with an HPLC-ESI-MS technique as described by Gou *et al.* (2010). The experiment was carried out using a liquid chromatography-mass spectrometry system (*LCQ Deca AMX, HPLC-ESI-MS; Thermo-Finnigan*, San Jose, CA, USA) with *C18 SepPak* and *MCX SPE* columns (*Qasis; Waters*, Milford, MA, USA). The data were analyzed using the software *Xcalibur 2.1* (*Thermo-Finnigan*) and quantified by reference to the internal standards using M⁺ ratios in the equations for isotope dilution analysis.

Sequencing RNA: Ribonucleic acid was isolated from 200 mg of soybean main roots collected at 3 d after treatment

with solution bearing or lacking GA₃. Three biological replicates were used for all RNA-seq experiments from each sample. Further RNA purification, cDNA synthesis and sequencing were performed as previously described (Han *et al.* 2017). Sequencing analysis was performed using a *HiSeq 4000* platform (Illumina, San Diego, CA, USA) for paired end RNA sequencing (read length 2 × 150 bp). The project and sequence data were submitted to the *NCBI Bioproject PRJNA352344* and sequence read archive database under the accession number SRR5028739, SRR5028740, SRR5028741, SRR5028745, SRR5028746, and SRR5028747.

Sequenced RNA data analysis: Clean data without low-quantity reads (more than 50 % of bases with a Q-value ≤ 10) were mapped to a reference genome (*G. max* V1.0) in the *Phytozome* database using *TopHat* (Kim *et al.* 2013). Subsequently, the expression of each transcript was calculated and normalized to obtain fragments per kilobase of transcript per million fragments mapped (FPKM). Then, *P*-values and log₂ fold change (log₂ FC) were calculated. Only the gene with false discovery rate (FDR) ≤ 0.05 and |log₂ FC| ≥ 1 were considered as significant differentially expressed genes (DEGs) between the GA₃ treatment and control samples. For function analysis, DEGs were aligned to *NCBI* non-redundant protein (*Nr*), *Swiss-Prot*,

gene ontology (*GO*, <http://www.geneontology.org/>), clusters of orthologous groups (*COG*), and the Kyoto encyclopedia of genes and genomes (*KEGG*) databases. To predict transcriptional networks in soybean roots after GA₃ treatment, *Arabidopsis* transcription factors (TFs) that were most similar to the soybean TFs and also in the *Arabidopsis* transcriptional regulatory map dataset (*ATRM*, <http://atrm.cbi.pku.edu.cn/>) were selected to generate the transcription networks. *Cytoscape v. 3.2.1* was used to visualize of the transcription networks (Smoot *et al.* 2011).

Real time quantitative PCR analysis: 34 genes involved in GA, auxin, cytokinin, abscisic acid and brassinolide pathways, TFs, and genes related to cell elongation and cell wall modification were selected from *G. max* database in joint genome institute (<http://jgi.doe.gov/>). The PCR primer pairs of all genes are shown in Table 1 Suppl. The *actin* (Glyma18_g52780.1) and eukaryotic elongation factor 1 (*LF1B*, Glyma02g44460.1) gene of soybean was selected as an internal control.

The MR samples of GA-30 and control at 1, 3, and 10 d were used for real time qPCR. The extraction and detection of total RNA were carried out as in RNA-seq experiment. The cDNA was made with *RNA LA PCR kit (AMV ver 1.1; Takara, Dalian, China)* and qPCR was performed using *Takara SYBR Premix Ex Taq* in a total of volume of 20 mm³

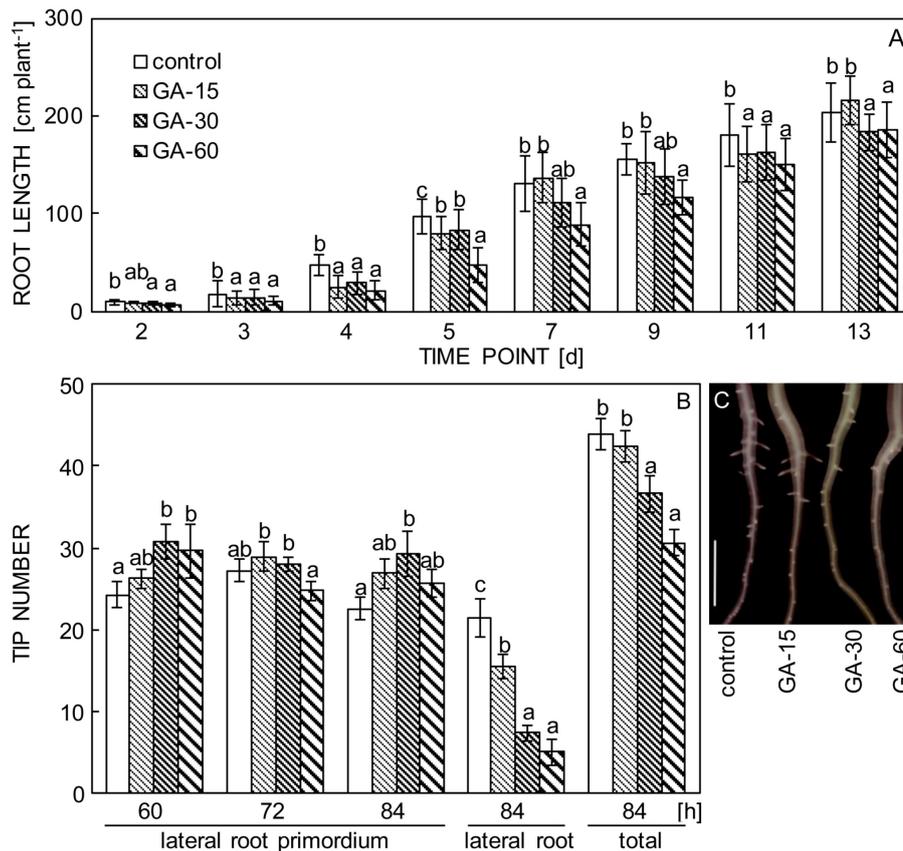


Fig. 1. Effect of gibberellic acid 3 (GA₃) treatment on root length (A) and the number of root tips (B). Soybean seeds soaked in 0.43, 0.87, and 1.74 mM GA₃ corresponding to three different treatments GA-15, GA-30, and GA-60. Means ± SE, *n* ≥ 20, different letters show means which differ significantly (*P* ≤ 0.05) according to one way *ANOVA* combined with the Tukey's multiple comparison posttest. Photographs of representative roots 3 d after treatments with different concentration of GA₃ (C). The bar represents 1 cm.

containing 10 mm³ of *SYBR Premix Ex Taq*, 0.4 mm³ of *ROX* reference dye, 7.0 mm³ of deionized water, 0.8 mm³ of each forward and reverse primer, and 1 mm³ of diluted cDNA strand (100 ×). The qPCR analysis was performed in an *ABI 7500* thermocycler (*Applied Biosystems*, Foster City, CA, USA). PCR cycling was performed at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Data were transformed and analyzed by the $2^{-\Delta\Delta C_t}$ method (Rao *et al.* 2013), and relative expressions of each gene in soybean roots were analyzed using the *Applied Biosystems 7500 SDS V 2.0* qPCR software.

Statistical analysis. One way *ANOVA* with LSD or the

Tukey's multiple comparison posttest was applied to detect differences under different treatments at the 0.05 significance level.

Results

Soybean seeds were treated with three different concentrations of GA₃, and surveyed for 13 d. The root length and root surface area decreased under GA₃ treatments at multiple time periods; GA-60 treatment had the greatest impact (Fig. 1A, Fig. 1 Suppl.). All GA₃ treatments significantly reduced the root volume (Fig. 1

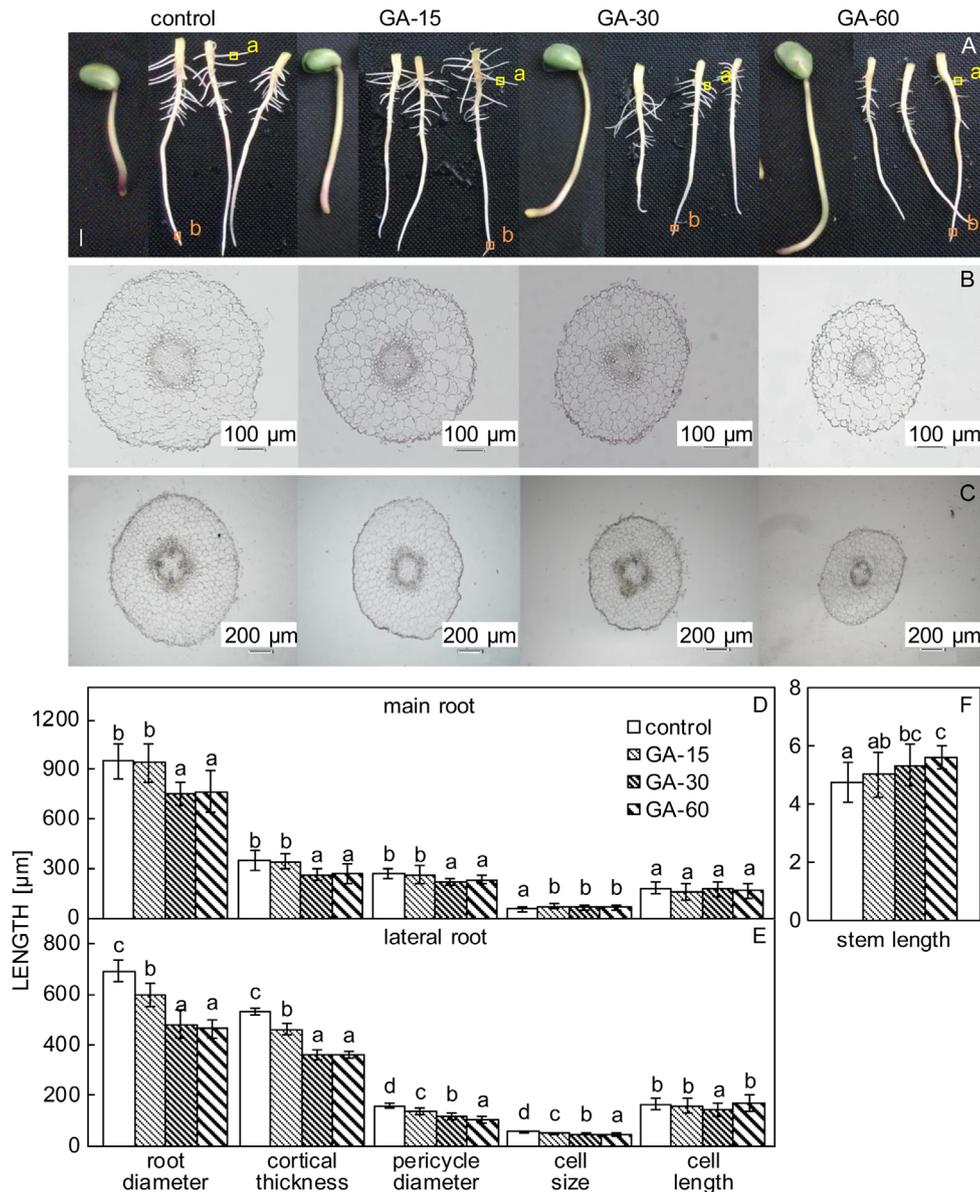


Fig 2. The change of morphology and anatomy of shoots and roots 96 h after gibberellin treatments. *A* - The morphology of shoot and root; the *bar* represents 1 cm; a - sample location in a lateral root, b - sample location in the main root. *B* - The microscopic structures of a segment excised 1 - 1.5 cm from a lateral root tip; the *bar* represents 100 μm . *C* - The microscopic structures of segment excised 1 - 1.5 cm from the main root tip; the *bar* represents 200 μm . *D* - Quantification of different anatomical parameters in the main and lateral roots. Means \pm SEs, $n = 3$, different letters show means which differ significantly ($P \leq 0.05$).

Suppl.). The diameter of MR decreased gradually with the increase of GA₃ concentration at 3, 4, and 5 d, and the diameter of MR in all treatment groups remained lower than that of control at 9 and 11 d. The change of LR diameter was similar with that of MR diameter, and the diameter of LR in all treatment groups was lower than that of control at 3, 4, 7, and 9 d. With the further growing of plant, the effect of GA₃ on soybean root diameter diminished; there was no significant effect after 11 d (Fig. 1 Suppl.).

Tip number, a measure of LR formation, of treated plants was significantly less than that of the control group at 4 d (Fig. 1 Suppl.). The result indicated the LR formation decreased. However, the quantity of lateral root primordia (LRP) of soybean increased after GA₃ treatment. The number of LRP increased in GA-30 and GA-60 treatments at 60 h and GA-30 treatment group at 84 h (Fig. 1B). The quantity of LRP also significantly increased at GA-30 treatment after 84 h. However, many LRP of treated plants did not form LR, as indicated by fewer LR in treated plants as compared with control at 84 h. With increased GA₃ concentration, the number of LR decreased. The number of LR in GA-30 and GA-60 treated plants were significantly less than that of control (Fig. 1B,C). Thus, GA₃ treatments affected the MR and LR development.

Epidermis, cortex, and vascular cylinder comprise the soybean root system, jointly determining the morphology of plant root. GA₃ treatment changed the morphology of soybean root at 4 d. With the increase of GA₃ concentration, the length of hypocotyl increased (Fig. 2A), while the

diameter of LR and MR decreased (Fig. 2D,E). The microscopic observations of transverse sections of LR and MR showed that the decrease in diameter resulted from reduced cortical thickness and pericycle diameter. The size of cortical cell also changed (Fig. 2B,C). However, GA₃ treatment had no obvious effect on root cell length (Fig. 2D).

We assayed the amount of GA₃, IAA, and ABA in root at 1, 3, 5, and 10 d, after GA₃ treatments respectively. As shown in Fig. 3A, the GA₃ content of treated plant roots was greater than that of the control group. The GA₃ content in roots of treatment group and control group gradually decreased over time. GA₃ treatment also affected the content of IAA in root to some extent (Fig. 3B). At 1 d, the content of IAA in GA-30 and GA-60 groups significantly decreased, and the content of IAA in GA-15 group at 3 d, in GA-60 group at 5 d, and in GA-15 group at 10 d significantly increased. As concerns ABA, GA₃ application increased the content of ABA in treatment groups at 1, 3, and 5 d but at 10 d, the content of ABA in GA-60 group decreased (Fig. 3C).

In MR, changes in transcriptome determined 3 d after treatment with solution bearing or lacking GA₃ indicated that reads mapping to the genome sequence made up approximately 85 % of the reads (Table 2 Suppl.). A total of 1 317 DEGs were identified between GA₃ -treated (GA-30) and untreated (control) samples by FPKM, and 940 DEGs were down-regulated, while 377 DEGs were activated (Fig. 2 Suppl.). The number of downregulated

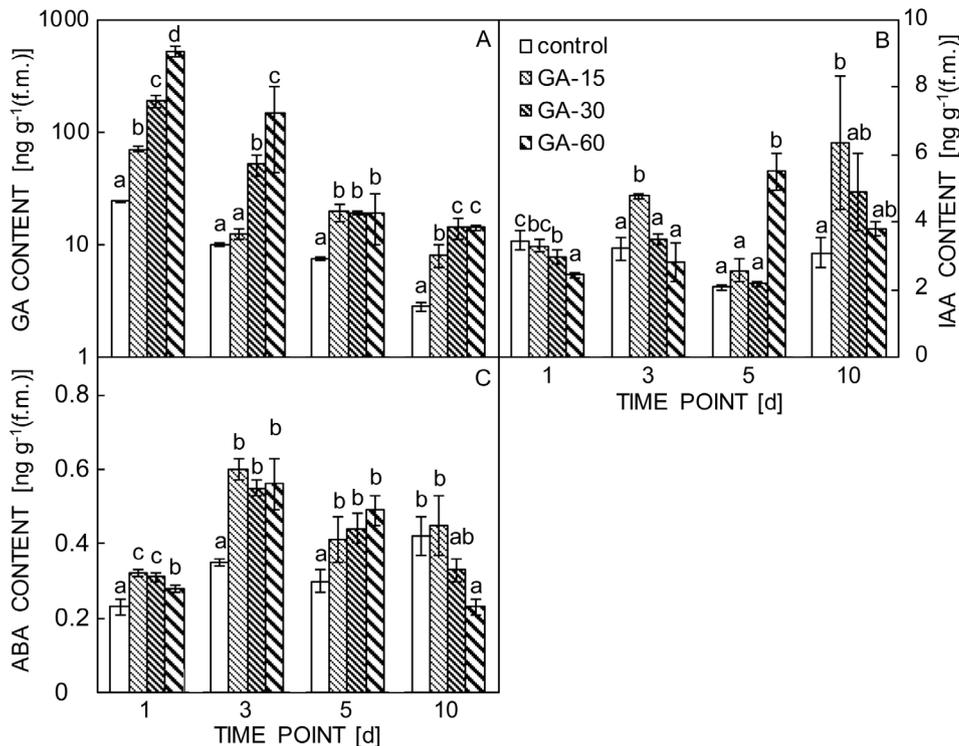


Fig. 3. Content of phytohormones in soybean roots in response to gibberellic acid 3 (GA₃) treatment. The phytohormones content was measured at 24, 72, 120, and 240 h after treatments with different concentrations of GA₃. A - gibberellins (GA) content, B - abscisic acid (ABA) content; C - indole-3-acetic acid (IAA) content. Means ± SEs, *n* = 3, different letters show means which differ significantly (*P* ≤ 0.05).

Table 1. Effects of gibberellic acid 3 (GA₃) on the expressions of genes relating to gibberellin biosyntheses and signaling pathways.

Gene ID (Wm82.a2.v1)	Swissprot_annotation	log ₂ FC
Glyma.03G131200	gibberellin 20 oxidase 2	-1.02
Glyma.04G070500	gibberellin 20 oxidase 1	1.31
Glyma.19G062800	gibberellin 20 oxidase 1	-1.19
Glyma.19G062600	gibberellin 20 oxidase 1	-1.48
Glyma.18G061300	gibberellin 2-beta-dioxygenase 8	-3.61
Glyma.13G259500	gibberellin 2-beta-dioxygenase 1	1.93
Glyma.02G185200	AP2-like ethylene-responsive transcription factor At2g41710	-1.39
Glyma.02G239400	probable acyl-activating enzyme 5, peroxisomal	4.37
Glyma.13G189800	gibberellin receptor GID1C	2.04
Glyma.15G049000	G-box-binding factor 4	1.90
Glyma.16G063700	Myb-related protein 315	1.85
Glyma.02G067600	ethylene-responsive transcription factor LEP	1.20
Glyma.U038900	transcription factor RAX3	1.04
Glyma.17G258200	Snakin-2 (Precursor)	1.13
Glyma.14G219100	Snakin-2 (Precursor)	1.41
Glyma.12G238700	transcription factor bHLH130	-1.03
Glyma.04G101900	transcription factor MYB39	-2.07
Glyma.06G103300	transcription factor MYB39-like	-3.60
Glyma.11G194100	transcription factor RAX2	-1.00
Glyma.06G043400	F-box protein GID2	-1.59
Glyma.13G152900	probable acyl-activating enzyme 12, peroxisomal	-1.88
Glyma.18G044900	probable acyl-activating enzyme 12, peroxisomal	-1.06
Glyma.13G200400	flavin-containing monooxygenase FMO GS-OX5	-1.08
Glyma.13G113100	probable flavin-containing monooxygenase 1-like	-4.12
Glyma.08G021900	probable WRKY transcription factor 53	-1.29
Glyma.09G212500	growth-regulating factor 4	-1.03

genes was 2-times higher than that of upregulation ones. Using $|\log_2 FC| \geq 3$ as a cutoff value, 155 genes in the root showed differential expression patterns between the treatment and the control (Table 3 Suppl.). There were 27 genes with increased transcript abundance and 128 genes with decreased transcript abundances, and the number of downregulated genes were 4.7-times higher than that of upregulated genes. Function analysis of DEGs were conducted in various databases (Fig. 3 Suppl. and Fig. 4 Suppl.).

A large number of DEGs involving in GA biosynthetic and signalling pathways were identified (Table 1). For GA biosynthesis pathway, most of *GA20ox* 2 genes (Glyma.03G131200) and *GA20ox* 1 genes (Glyma.19G062800 and Glyma.19G062600) were down-regulated, while Glyma.04G070500 (*GA20ox* 1) was up-regulated in root after GA₃ application. The genes of *GA20ox* which encode the GA-inactivating enzymes exhibited differential expression after the application of GA₃. Glyma.18G061300 (*GA20ox* 1) was up-regulated by log₂ FC of 1.93. These changes coincided with the feedback mechanism of the responsiveness of tissue to the bioactive GAs influencing GA metabolism (Cheng *et al.* 2015). Glyma.13G259500 (*GA20ox* 8) which hydroxylates the C20-GA GA₁₂ and GA₅₃ (Schomburg *et al.* 2003), was down-regulated by log₂ FC -3.61.

This feedback mechanism also appeared to play a role at the GA perception. For GA signalling pathway, GA₃ negatively regulates the expression of gene *GID2* encoding the gibberellin receptor F-box protein, of gene *GS-OX5* encoding acyl-activating enzyme 12, flavin-containing monooxygenase (FMO), and of some transcription factors, such as *bHLH130*, *MYB39*, *MYB39-like*, *RAX2*, *WRKY53*, *etc.* In contrast, the transcription of probable acyl-activating enzyme 5, gibberellin receptor *GID1C*, G-box-binding factor 4, *Myb15*, *LEP*, *RAX3*, and Snakin-2 exhibited up-regulation following GA₃ treatment (53 % genes belonged TFs among those GA signalling pathway-related genes). The RNA-seq results revealed that GA₃ not only obviously changed expression pattern of the GA biosynthesis genes, but also affected some regulation genes of GA-related pathway.

The TFs play important roles in regulation of gene expression of all kinds of processes including plant development. In DEGs, there are 103 TFs, accounting for 7.8 % of total DEGs. Those TFs belonged to 15 transcription factor families (Fig 4A) classified. The most highly represented TF families were the WRKY (which is a class of DNA-binding proteins that recognize the TTGAC(C/T) W-box elements), bHLH (basic-helix-loop-helix), ERF, MYB (myeloblastosis), and NAC families.

We constructed the transcriptional regulation network

GA₃ increased the transcription of auxin responsive *GH3* gene family and decreased the transcription of small auxin-up RNA (*SAUR*). *GH3* and *SAUR* were auxin-responsive genes which were reported to play a role in cell enlargement and plant growth (Takase *et al.* 2004; Farquharson 2014). As concerns cytokinins, histidine-containing phosphotransfer protein genes (*HPTs*) were down-regulated which were annotated as *AHP4*. *AHP4* in

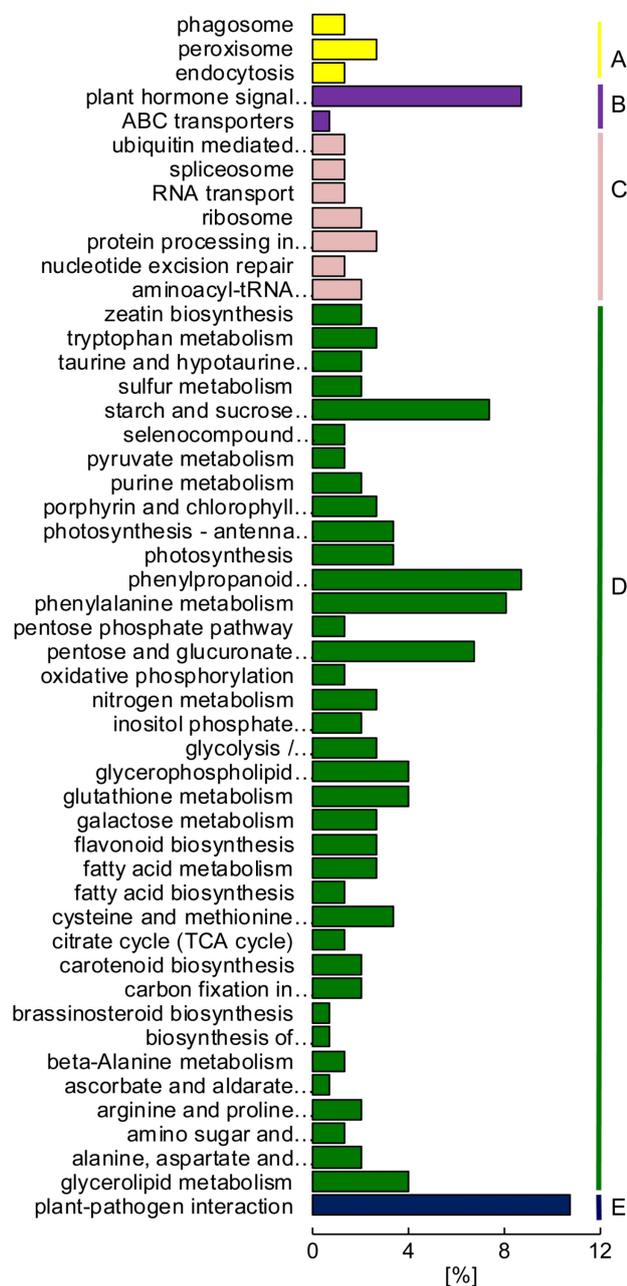


Fig. 5. The Kyoto encyclopedia of genes and genomes (*KEGG*) metabolism pathway categories of assembled unigenes: cellular processes (A), environmental information processing (B), genetic information processing (C); the main functional categories are metabolism (D) and organismal systems (E). Bars represent the numbers of *Glycine max* assignments of unigenes with *BLASTX* matches to each *KEGG* term.

Arabidopsis is a mediator in a multistep phosphorylation pathway for cytokinin signalling and negatively regulated thickening of the secondary cell wall of the anther endothecium (Jung *et al.* 2008). As concerns ABA, GA₃ application increased transcriptions of ABA responsive element binding factor (*ERF1/2*). The transcript of ethylene-responsive transcription factor 1 in ethylene signalling pathway decreased after GA₃ treatment. Probable *WRKY* transcription factor 40 (Glyma.17G222300, log FC -5.29), a negative regulator of ABA-dependent inhibition of seed germination, relocalized from PNBs to the nucleoplasm in the presence of ABA in a dynamic and phosphorylation-dependent manner (Katja and Maik 2015). Brassinosteroid insensitive 1-associated receptor kinase 1 *BAK1* and BR11 kinase inhibitor 1 *BKII*, which acted in brassinosteroid signal pathway, showed decreased expression. The transcript of jasmonate ZIM domain-containing protein JAZ was up-regulated (Fig. 5 Suppl., Table 5 Suppl.) which played versatile roles in multiple aspects of plant development and defense (Yuan *et al.* 2017).

The GA₃ treatment also affect the transcription of some genes in the phenylpropanoid metabolic pathway which is related to the synthesis of many secondary metabolites, such as lignin and flavonoids. This pathway affects plant growth and development together with their adaptation to the environment. DEGs involved in phenylpropanoid pathway were identified in this study (Fig. 6 Suppl.). The transcription of *trans*-cinnamate 4-monooxygenase (Glyma.20G114200) was down-regulated, and the transcription of erulate-5-hydroxylase genes (Glyma.16G131200) were up-regulated. The transcription gene concerning peroxidases were also changed, 13 genes up-regulated and 10 genes down-regulated. Flavonoid pathway is a branch pathway in the common phenylpropanoid pathway. Many genes in this pathway were markedly decreased, including genes of *trans*-cinnamate-4-monooxygenase (Glyma.20G114200), chalcone isomerase (Glyma.20G241700), flavonoid-3'-monooxygenase (Glyma.05G021800), flavanone-4-reductase (Glyma.18G220500, Glyma.09G269500). Many genes in signal pathway of plant-pathogen interaction were identified. In soybean root, transcriptions of most genes were down-regulated by exogenous GA₃ treatment, such as genes of mitogen-activated protein kinase kinase 1 (*MAP2K1*) in MAPK signalling pathway, transcription factor *WRKY29*, *WRKY33*, calcium-binding protein *CML*, and respiratory burst oxidase.

GA-dependent expression of cell wall-related enzymes occurred in several species during seed germination (Ren and Kermode 2000, Chen *et al.* 2001, Ogawa *et al.* 2003). Among GA regulated genes identified in this study, there were some classes of genes implicated in the biogenesis and degradation of cell wall. As shown in Table 6 Suppl., 55 DEGs were classified as genes putatively involved in changing cell-wall composition and properties, which may affect cell-wall development in soybean root. Among cell-wall-related DEGs, 44 genes were down-regulated, including 12 glucosidases, 10 pectinesterases (*PEs*), 5 polygalacturonases (*PGs*), 8 extension (*EXT*), 1 expansins (*EXPs*), 2 β -1,3-glucanase,

1 glycogenin glucosyltransferase, 2 pectatelyases (*PLs*), 1 β -galactosidases, 1 glucan endo-1,3- β -glucanase, 1 acid β -fructofuranosidase, 1 cellulose synthase, and 1 chitinase. After GA_3 treatment, some genes were up-regulated, such as 6 *PGs*, 1 glucan endo-1,3- β -glucosidase 14, 1 endoglucanase, 2 *PEs*, and 1 cellulose synthase. Extensins, hydroxyproline-rich glycoproteins (HRGPnt3), a class of structural proteins of cell wall, play roles in cell wall assembly, cell shape and size, and disease resistance (Fan *et al.* 2017). Treatment with GA_3 down-regulated expression of 8 *EXTs* genes.

In order to verify the reliability of transcriptome data, we selected 36 DEGs for RT-qPCR, which were genes in the hormones related pathways or which we were interested. The quantitative expression analysis of 36 genes were accomplished (Table 7 Suppl.). We detected the expression of 3 *EXTs*, which were the cell wall related genes. The results showed that 3 *EXTs* all down-regulated at 1 d, and expression still kept in low level at 3 d. Until 10 d, its expression recovered (Fig. 6). Two GA biosynthesis genes *GA20OX1-1* and *GA20OX1-2* were down-regulated after GA_3 treatment at the early stage, and the expression recovered or ascended at 10 d to some extent. The expression profile of *GA20OX1* was most correlated with changes in GA_3 increase at the early stage. TF *VNR1* was down-regulated, and kept in a low expression levels. Expression level of *AS* (hydroquinone glucosyltransferase) in sugar metabolism was significantly

up-regulated, and recovered till 10 d. *GH3.1* (indole-3-acetic acid-amido synthetase) was obviously up-regulated, and downed to the normal level at 10 d, which belongs to the auxin responsive *GH3* gene family.

The correlation analysis by Pearson method showed that transcriptome data was positively correlated with the quantitative data, and correlation coefficient was 0.853 (Table 8 Suppl.). These results indicated that the transcriptome data was reliable.

Discussion

The application of plant growth regulators is an important way to improve crop traits. GAs have been widely used to regulate seed germination, plant growth, and fruit yield (Peng 2002). Root system is one of the main factors in maintaining crop yield and quality, and the LR is a vigorous and physiologically active part of the soybean root system. Therefore, the effects of GA_3 on soybean root growth, especially LR growth provides opportunity to gain insight to soybean root system development. In this paper, we performed a detailed analysis of the effect of GA_3 treatment on root development from different aspects: morphological characteristics of roots, root microstructure, hormonal changes, and genes expression profile.

Previous studies have focused on the effects of mutations of GAs synthetic gene on roots. Inhibition

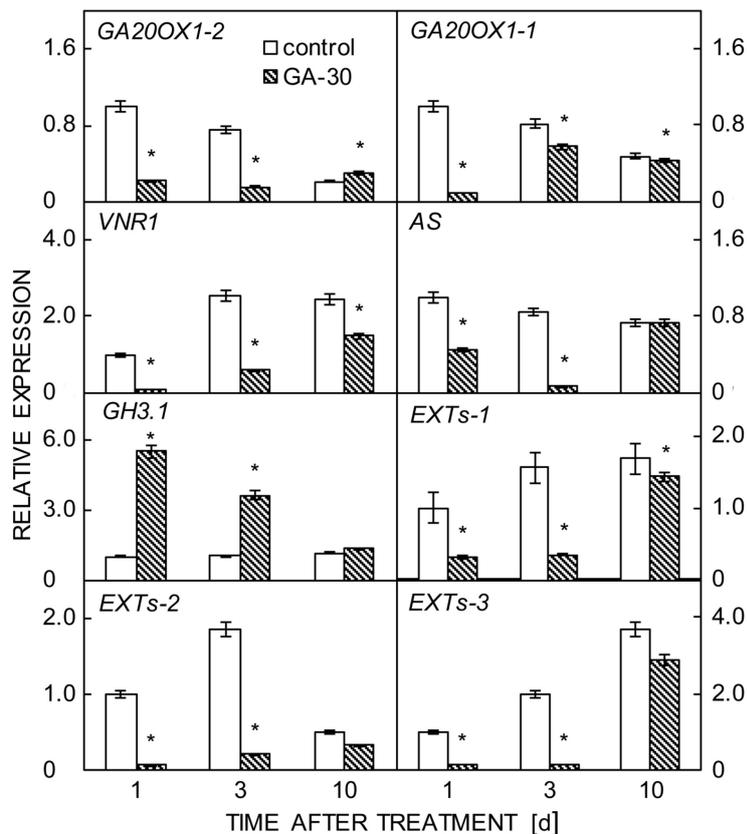


Fig. 6. The reverse transcription quantitative PCR analysis of eight differentially expressed genes (*GA20OX1-1*, *GA20OX1-2*, *VNR1*, *AS*, *GH3.1*, *EXTs-1*, *EXTs-2*, *EXTs-3*) in soybean roots treated with gibberellic acid 3.

effect on root development caused by the mutation of GAS biosynthesis gene showed that GAs have a positive effect on root system (Fu and Harberd 2003, Achard *et al.* 2009, Ubeda-Tomás *et al.* 2009). We investigated the role of GAs by seed soaking with GA₃. Excessive GA₃ did not show positive effect on root, on the contrary it inhibited the breaking of LRP through cortex to form the LR (Fig. 1). Meanwhile, the root diameter, surface area, and volume of root significantly decreased in the early stages. In poplar and carrot, the application of GA₃ also had the negative effect on root growth (Gou *et al.* 2010, Wang *et al.* 2015). Tanimoto (2012) reported GA-suppressed thickening of roots usually observed in the elongation zone of the root and mainly caused by the expansion of cortical cells. Our microscopic examination of anatomical structures showed that GA₃ treatment changed the thickness of the cortex, the diameter of the pericycle, and the cell size (Fig. 2). In our previous study, uniconazole treatment promoted root growth in soybean which indirectly indicated the inhibition effect of GA₃ on root growth (Han *et al.* 2017). The effects of exogenous GAs and GAs inhibitors on root development vary depending on the plant species and experimental conditions (Tanimoto 2012), so more studies in other species will provide further information.

GA₃ treatment altered morphological parameters, anatomical structure, and transcriptional regulatory networks in soybean root. GA₃ treatment restrained root growth that maybe related to the transverse growth of cell. Morphological parameters also showed that GA treatment inhibited the penetration of the LR primordia through the cortex. The GA₃, IAA, and ABA content in soybean root changed after GA₃ application. RNA-seq results suggested that many GA biosynthesis and signalling pathway genes, other hormones signalling pathway genes, TF genes, and cell wall related proteins, especially cell-wall-loosening proteins significantly changed their expression profile, which may be related to the morphogenesis change, the inhibition of LR, and the change of hormone content.

References

- Achard, P., Gusti, A., Cheminant, S., Alioua, M., Dhondt, S., Coppens, F., Beemster, G.T.S., Genschik, P.: Gibberellin signaling controls cell proliferation rate in *Arabidopsis*. - *Curr Biol.* **19**: 1188, 2009.
- Chen, F., Dahal, P., Bradford, K.J.: Two tomato expansin genes show divergent expression and localization in embryos during seed development and germination. - *Plant Physiol.* **127**: 928-936, 2001.
- Cheng, C., Jiao, C., Singer, S.D., Gao, M., Xu, X., Zhou, Y., Li, Z., Fei, Z., Wang, Y., Wang, X.: Gibberellin-induced changes in the transcriptome of grapevine (*Vitis labrusca* × *V. vinifera*) cv. Kyoho flowers. - *BMC Genomics* **16**: 128, 2015.
- Eriksson, M. E., Israelsson, M., Olsson, O., Moritz T.: Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. - *Nat Biotechnol.* **18**: 784-788, 2000.
- Fan, C., Li, Y., Hu, Z., Hu, H., Wang, G., Li, A., Wang, Y., Tu, Y., Xia, T., Peng, L.: Ectopic expression of a novel *OsExtensin-like* gene consistently enhances plant lodging resistance by regulating cell elongation and cell wall thickening in rice. - *Plant Biotechnol. J.* **16**: 254-263, 2017.
- Farquharson, K.L.: SAUR19 links auxin and plasma membrane H⁺-ATPases in cell expansion. - *Plant Cell* **26**: 1835, 2014.
- Friedrichsen, D.M., Nemhauser, J., Muramitsu, T., Maloof, J.N., Alonso, J., Ecker, J.R., Furuya, M., Chory, J.: Three redundant brassinosteroid early response genes encode putative bHLH transcription factors required for normal growth. - *Genetics* **162**: 1445, 2002.
- Fu, X., Harberd, N.P.: Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. - *Nature* **421**: 740, 2003.
- Fukaki, H., Tasaka, M.: Hormone interactions during lateral root formation. - *Plant mol. Biol.* **69**: 437, 2009.
- Gou, J., Strauss, S. H., Tsai, C.J., Fang, K., Chen, Y., Jiang, X., Busov, V.B.: Gibberellins regulate lateral root formation in *Populus* through interactions with auxin and other hormones. - *Plant Cell* **22**: 623-639, 2010.
- Han, Y., Gao, Y., Shi, Y., Du, J., Zheng, D., Liu, G.: Genome-wide transcriptome profiling reveals the mechanism of the effects of uniconazole on root development in *Glycine max*. - *J. Plant Biol.* **60**: 387-403, 2017.
- Jung, K.W., Oh, S.I., Kim, Y.Y., Yoo, K.S., Cui, M.H., Shin, J.S.: *Arabidopsis* histidine-containing phosphotransfer factor 4 (AHP4) negatively regulates secondary wall thickening of the anther endothecium during flowering. - *Mol. Cells* **25**: 294-300, 2008.
- Katja, G., Maik, B.H.: Dynamic subnuclear relocalization of WRKY40, a potential new mechanism of ABA-dependent transcription factor regulation. - *Plant Signal Behav.* **10**: e1106659, 2015.
- Kim, D., Perteza, G., Trapnell, C., Pimentel, H., Kelley, R., Salzberg, S.L.: TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. - *Genome Biol.* **14**: R36, 2013.
- Li, H. (ed.): Botanical Microtechnique. - Science Press, Beijing 2009.
- Mauriat, M., Petterle, A., Bellini, C., Moritz, T.: Gibberellins inhibit adventitious rooting in hybrid aspen and *Arabidopsis* by affecting auxin transport. - *Plant J.* **78**: 372-384, 2014.
- Manoharlal, R., Saiprasad, G.V.S., Thambrahalli, A., Madhavakrishna K.: Dissecting the transcriptional networks underlying the gibberellin response in *Nicotiana tabacum*. - *Biol Plant.* **62**: 647, 2018.
- Noh, S.A., Choi, Y.I., Cho, J.S., Lee, H.: The poplar basic helix-loop-helix transcription factor *BEE3-Like* gene affects biomass production by enhancing proliferation of xylem cells in poplar. - *Biochem. biophys. Res Commun.* **462**: 64-70, 2015.
- Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y., Yamaguchi, S.: Gibberellin biosynthesis and response during *Arabidopsis* seed germination. - *Plant Cell* **15**: 1591-1604, 2003.
- Osmont, K. S., Sibout, R., Hardtke, C.S.: Hidden branches: developments in root system architecture. - *Annu. Rev. Plant Biol.* **58**: 93-113, 2007.
- Peng, J.: The role of GA-mediated signalling in the control of seed germination. - *Curr. Opin. Plant Biol.* **5**: 376-381, 2002.
- Radwan, O., Liu, Y., Clough, S. J. Transcriptional analysis of soybean root response to *Fusarium virguliforme*, the causal agent of sudden death syndrome. - *Mol. Plant Microbe Interact.* **24**: 958-972, 2011.
- Rao, X., Huang, X., Zhou, Z., Lin, X.: An improvement of the 2^{-ΔΔCt} method for quantitative real-time polymerase chain reaction data analysis. - *Biostat. Bioinform. Biomath* **3**: 71-85, 2013.
- Ren, C., Kermodé, A.R.: An increase in pectin methyl esterase

- activity accompanies dormancy breakage and germination of yellow cedar seeds. - *Plant Physiol.* **124**: 231-242, 2000.
- Schomburg, F.M., Bizzell, C.M., Lee, D.J., Zeevaart, J.A., Amasino, R.M.: Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. - *Plant Cell* **15**: 151, 2003.
- Slovak, R., Ogura, T., Satbhai, S.B., Ristova, D., Busch, W.: Genetic control of root growth: from genes to networks. - *Ann. Bot.* **117**: 9, 2015.
- Smoot, M.E., Ono, K., Ruschinski, J., Wang, P.L., Ideker, T.: Cytoscape 2.8: new features for data integration and network visualization. - *Bioinformatics* **27**: 431-432, 2011.
- Song, L., Prince, S., Valliyodan, B., Joshi, T., Maldonado dos Santos, J.V., Wang, J., Lin, L., Wan, J., Wang, Y., Xu, D., Nguyen, H.T.: Genome-wide transcriptome analysis of soybean primary root under varying water-deficit conditions. - *BMC Genomics* **17**: 57, 2016.
- Stamm, P., Topham, A.T., Mukhtar, N.K., Jackson, M.D., Tomé, D.F., Beynon, J.L., Bassel, G.W.: The transcription factor *ATHB5* affects GA-mediated plasticity in hypocotyl cell growth during seed germination. - *Plant Physiol.* **173**: 907, 2017.
- Takase, T., Nakazawa, M., Ishikawa, A., Kawashima, M., Ichikawa, T., Takahashi, N., Shimada, H., Manabe, K., Matsui, M.: *yd1-D*, an auxin-responsive GA3 mutant that is involved in hypocotyl and root elongation. - *Plant J.* **37**: 471-483, 2004.
- Tanimoto, E.: Tall or short? Slender or thick? A plant strategy for regulating elongation growth of roots by low concentrations of gibberellin. - *Ann. Bot.* **110**: 373-381, 2012.
- Ubeda-Tomás, S., Federici, F., Casimiro, I., Beemster, G. T., Bhalerao, R., Swarup, R., Doerner, P., Haseloff, J., Bennett, M. J.: Gibberellin signaling in the endodermis controls Arabidopsis root meristem size. - *Curr. Biol.* **19**: 1194-1199, 2009.
- Ueguchi-Tanaka, M., Nakajima, M., Katoh, E., Ohmiya, H., Asano, K., Saji, S., Hongyu, X., Ashikari, M., Kitano, H., Yamaguchi, I., Matsuoka, M.: Molecular interactions of a soluble gibberellin receptor, *GID1*, with a rice DELLA protein, *SLR1*, and gibberellin. - *Plant Cell* **19**: 2140-2155, 2007.
- Wang, G.L., Que, F., Xu, Z.S., Wang, F., Xiong, A.S.: Exogenous gibberellin altered morphology, anatomic and transcriptional regulatory networks of hormones in carrot root and shoot. - *BMC Plant Biol.* **15**: 290, 2015.
- Yuan, L.B., Dai, Y.S., Xie, L.J., Yu, L.J., Zhou, Y., Lai, Y.X., Yang, Y.C., Xu, L., Chen, Q.F., Xiao, S.: Jasmonate regulates plant responses to postsubmergence reoxygenation through transcriptional activation of antioxidant synthesis. - *Plant Physiol.* **173**: 1864-1880, 2017.