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Characteristics of galacturonate reductase (*GalUR*) genes in garlic (*Allium sativum* L.) and changes in their expression in response to abiotic stressors

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Abstract. In plants, the synthesis of L-ascorbic acid (Aa), in addition to the main L-galactose pathway, is carried out by three known alternative pathways. One of them, the D-galacturonic acid pathway, is thought to be specific for tissues with excess D-galacturonate, the substrate of D-galacturonate reductase (GalUR), which belongs to the Aldo-Keto Reductase (AKR) superfamily. In this study, the *AKR* gene family of garlic *Allium sativum* L. was identified and seven genes, *AsGalUR1–7*, presumably encoding GalUR enzymes, were determined. The structure and phylogeny of the *AsGalUR1–7* genes and the proteins they encode, as well as the *AsGalUR1–7* expression pattern in different organs of the garlic plant (*in silico* and qRT-PCR), were characterized. Based on the obtained data, the genes were conditionally divided into root (*AsGalUR1–4*) and leaf (*AsGalUR5–7*) groups depending on the highest expression level in the underground and aboveground parts of the plant, respectively. The *AsGalUR* expression in leaves and roots was analyzed in response to drought, salt and cold stresses, as well as exogenous phytohormones (abscisic acid, methyl jasmonate), accompanied by the AsA content measurement. It was shown that hormone treatment suppresses the expression of all analyzed genes in both organ types. Cold conditions stimulate the expression of root group genes and suppress that of leaf group genes in roots, and have the opposite effect in leaves. Osmotic stressors (NaCl, PEG) suppress the transcription of all genes in leaves, but do not change (NaCl) or stimulate (PEG) it in roots, which is accompanied by an increase in AsA accumulation in organs of both types. A positive correlation between the expression of the *AsGalUR1* and *4* genes and the AsA content is found in leaves under stress conditions. The data obtained can form the basis for further study of the mechanisms regulating AsA synthesis in garlic and other *Allium* species.

Key words: garlic; *Allium sativum* L.; L-ascorbic acid biosynthesis; D-galacturonic pathway; D-galacturonate reductase; GalUR

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Характеристика генов галактуронатредуктаз (*GalUR*) чеснока (*Allium sativum* L.) и изменение их экспрессии в ответ на абиотические стрессоры

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Аннотация. Помимо основного L-галактозного пути синтеза L-аскорбиновой кислоты (АсК) в растениях, известно три альтернативных пути. Один из них, D-галактуроновый, считается специфичным для тканей с избытком D-галактуроната – субстрата D-галактуронатредуктазы (GalUR), относящейся к семейству альдегидкеторедуктаз (AKR). В настоящей работе идентифицировано семейство генов *AKR* чеснока *Allium sativum* L. и определено семь генов, *AsGalUR1–7*, предположительно кодирующих ферменты GalUR. Охарактеризованы структура и филогения генов *AsGalUR1–7* и кодируемых ими белков, а также профиль экспрессии генов *AsGalUR* в различных органах растения чеснока (*in silico* и ПЦР-РВ). На основе полученных данных гены условно разделены на корневую

(*AsGalUR1–4*) и листовую (*AsGalUR5–7*) группы по признаку наибольшей экспрессии в подземной и надземной частях растения соответственно. Проведен анализ экспрессии генов *AsGalUR* в листьях и корнях в ответ на воздействие засухи, солевого и холодового стрессоров, а также экзогенных фитогормонов (абсцизовая кислота, метилжасмонат), вкупе с измерением содержания АсК. Показано, что обработка гормонами подавляет экспрессию всех анализируемых генов в обоих типах органов. Холодовые условия в корнях стимулируют экспрессию корневых генов и подавляют – листовых и оказывают противоположный эффект в листьях. Осмотические стрессоры (NaCl, PEG) подавляют транскрипцию всех генов в листьях, но не меняют (NaCl) или стимулируют (PEG) ее в корнях, что сопровождается ростом накопления АсК. В листьях в стрессовых условиях выявлена положительная корреляция между экспрессией генов *AsGalUR1* и *4* и содержанием АсК. Полученные данные могут стать основой для дальнейшего изучения механизмов регуляции синтеза АсК у чеснока и других видов *Allium*.

Ключевые слова: чеснок; *Allium sativum* L.; биосинтез L-аскорбиновой кислоты; D-галактуроновый путь; D-галактуронатредуктаза; GalUR

Introduction

L-ascorbic acid (AsA, ascorbate) is a major component of the non-enzymatic antioxidant system in plants (Smirnoff and Wheeler, 2024). The required level of ascorbate in cells is maintained through a balance between the biosynthesis, degradation, and recycling (reduction of oxidized forms) of AsA, as well as ascorbate transport (Smirnoff, 2018). AsA is synthesized primarily in leaves (Franceschi, Tarlyn, 2002; Badejo et al., 2012) and transported via the phloem to various plant parts in the form of stable and oxidation-protected ascorbyl glycosides (Richardson et al., 2021; Huang et al., 2025).

In plants, AsA biosynthesis occurs primarily via the Smirnoff–Wheeler L-galactose pathway. However, there are three alternative pathways – D-galacturonic, L-gulose, and myo-inositol (Broad et al., 2020; Smirnoff, Wheeler, 2024), which also significantly contribute to ascorbate accumulation in some organs or at certain stages of plant development. For example, in strawberry (*Fragaria* × *ananassa* Duchesne ex Rozier), the L-galactose pathway is characteristic of leaves, while the D-galacturonic pathway is characteristic of ripe berries (Agius et al., 2003; Cruz-Rus et al., 2011; Liu et al., 2022). Similarly, in tomato (*Solanum lycopersicum* L.), leaves and growing fruits synthesize AsA via the L-galactose pathway, whereas ripe fruits, via the D-galacturonic pathway (Badejo et al., 2012). The transition from the main AsA biosynthetic pathway to an alternative variant in ripe fruits is associated with the active degradation of cell wall pectin, formed mainly by galacturonic acid residues, and the conversion of its main monomer, D-galacturonate, to AsA (Peltonen, Richard, 2022).

Of the alternative pathways, the D-galacturonic acid pathway is the best studied. It begins, as mentioned above, with the breakdown of pectin, resulting in the formation of D-galacturonic acid, which is converted to D-galacturonate (D-GalUA), followed by reduction to L-galactonate catalyzed by D-galacturonate reductase. L-galactonate is converted by aldonolactonase to L-galactono-1,4-lactone, which is oxidized to AsA by L-galactono-1,4-lactone dehydrogenase (Ishikawa et al., 2008; Peltonen, Richard, 2022).

Galacturonate reductases (EC 1.1.1.365; GalUR) structurally belong to the Aldo-Keto-Reductase (AKR) superfamily (Duan et al., 2020), one of 16 subfamilies of which, AKR4, includes AKR proteins of plant origin (Penning, 2015). The number of *AKR* genes identified in higher plants (Sengupta et al., 2015) varies significantly depending on the species. For example, the genome of *Arabidopsis thaliana* L. contains 22 *AKR* genes, that of *S. lycopersicum*, 25 genes, and that of *F.* × *ananassa*, 80 genes (Duan et al., 2020; Liu et al., 2022), and the functions of their protein products are not limited to participation in the synthesis of AsA. Plant AKRs also control the formation of other secondary metabolites and osmolytes, including vitamin B6, sorbitol, isoflavones, phytoestrogens, and others (Sengupta et al., 2015; Ha et al., 2019). Such functional differentiation of AKR proteins is considered to enhance plant adaptation to various environmental conditions, including stress factors. Regarding the AsA synthesis, it should be noted that the AKR family includes not only the GalUR enzymes of the D-galacturonic pathway, but also L-galactose dehydrogenase (L-GalDH) of the main L-galactose pathway (Vargas et al., 2022).

Garlic (*Allium sativum* L.) is an economically significant vegetable crop with an annual production of approximately 30 million tons (FAO; http://www.fao.org). The ascorbate content in leaves and bulbs has been extensively studied in various garlic varieties and accessions (Skoczylas et al., 2023; Šnirc et al., 2023; Popa et al., 2024; Yenealem et al., 2025). However, currently available information on the molecular regulation of AsA metabolism in *A. sativum* and equally economically valuable related *Allium* species is limited to research data from our laboratory. Namely, in *A. sativum*, individual genes of monodehydroascorbate reductases (MDHAR) of the ascorbate recycling pathway were identified and characterized (Anisimova et al., 2022), and changes in the expression of AsA biosynthesis and recycling genes in response to infection of garlic plants with the pathogenic fungus *Fusarium proliferatum* were shown (Shchennikova et al., 2025). In leek (*Allium porrum* L.), the variability of the GDP-L-galactose phosphorylase gene *GGP1* of the L-galactose pathway was determined (Anisimova et al., 2021a), and possible dependences of the AsA content on the expression level of the *MDHAR* genes (Anisimova et al., 2021b; Filyushin et al., 2021) and some other genes of the L-galactose and AsA recycling pathways (Filyushin et al., 2025) were identified.

The present study was aimed to identify and characterize D-galacturonate reductase (*GalUR*) genes in the genome of garlic *A. sativum* and to determine their expression pattern in various plant organs, as well as in response to abiotic stress factors and exogenous phytohormones.

Materials and methods

Identification and structural characterization of garlic *AsGalUR* genes. The D-galacturonate reductase gene sequences were searched for in the genomic and transcriptomic data of garlic *A. sativum* cv. Ershuizao (PRJNA606385, assembly Garlic.V2.fa) available in the AlliumDB database (https://allium.qau.edu.cn/). The *GalUR* sequences of tomato (LOC101256763 and LOC101250974 (=SlAKR4B)) (Suekawa et al., 2016) and strawberry (*FaGalUR*; AF039182.1) (Agius et al., 2003) were used as reference.

Sequence alignment and analysis were performed using MEGA 7.0 (https://www.megasoftware.net/). For the AsGalUR proteins, conserved domains and motifs (NCBI-CDD, http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; MEME 5.5.7, http://meme-suite.org/tools/meme), molecular weight (Mw) and isoelectric point (pI) (ExPASy, https://web.expasy.org/protparam/), molecular function and cellular localization (in terms of Gene Ontology, GO, PANNZER, http://ekhidna2.biocenter.helsinki.fi/sanspanz/) were determined. Phylogenetic analysis was performed using AKR sequences of *A. sativum*, *S. lycopersicum* and *A. thaliana* (MEGA 7.0, Neighbor-Joining method, bootstrap based on 1,000 replicates).

Analysis of *AsGalUR* gene expression in different garlic organs. The organ-specific expression pattern of the identified *AsGalUR* genes was determined using two methods: *in silico* and real-time PCR (qRT-PCR).

In silico analysis of *AsGalUR* gene expression was performed using available transcriptome data of different organs of the garlic cv. Ershuizao (roots, leaves, stems, seedlings, buds, flowers, and bulbs at developmental stages 1–8) (Sun et al., 2020). The data were visualized as a heatmap (Heatmapper, http://www.heatmapper.ca/expression/), where expression levels were presented as fragments per kilobase per million mapped reads (FPKM).

Using qRT-PCR, the expression of *AsGalUR* genes was determined in the roots, base, bulb, pseudostem, and leaves of garlic cv. Scorpion plants grown in soil under greenhouse conditions (ECCF, Federal Research Center of Biotechnology, Russian Academy of Sciences; 16-h photoperiod (light phase from 8:00); day/night – 22/16 °C; lighting intensity 190 μM/m^2/s). The plant material was ground in liquid nitrogen and used for the isolation of total RNA with purification from DNA impurities (RNeasy Plant Mini Kit and RNase-free DNasy set; QIAGEN, Germany) and cDNA synthesis (GoScript™ Reverse Transcription System, Promega, USA).

Based on the identified *AsGalUR* sequences, specific primers for qRT-PCR were developed (Table 1). *GAPDH* (MZ171220.1) and *UBQ* (MZ171222.1) were used as reference genes. The reaction mixture included 3 ng of cDNA and the "Reaction mixture for qRT-PCR in the presence of SYBR Green I and ROX" kit (Synthol LLC, Russia). The reaction was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA), in six technical replicates of two biological replicates under the following conditions: 95 °C for 5 min; 40 cycles (95 °C for 15 s, 62 °C for 50 s). Data were statistically processed using one-way ANOVA with Bonferroni correction ("multiple comparisons, corrected with Bonferroni test") and visualized in GraphPad Prism v. 8 (https://www.graphpad.com).

Table 1. Primer sequences for qRT-PCR

Gene	Primer sequences (5′→3′)
AsGalUR1	TGCCATCCGAAGGAGTCTTGT AGCATTTGGCACTCCTCCATC
AsGalUR2	TGCCATCCGAAGGAGTCTTGT AGCATTTGGCACTCCTCCATG
AsGalUR3	GAGGAGTGCCAAATGCTTGGAT CGTCCACCCAAAGGAGAGTG
AsGalUR4	GTGTAACGATGCCCATCCTGAT CTACTGGAAATGGGTGTGATCC
AsGalUR5	CTGCTGTTAATCAGGTGGAGT ACACTTCCCCACGGAGCTC
AsGalUR6	CTGCTGTTAATCAGGTGGAGC ACACTTCCCCACGGAGCTC
AsGalUR7	TCGGTGTGAGCAATTTCTCATC ACACTTCCCCACGGAGCTC
GAPDH	CCATGTTTGTTGTTGGTGTGAATGAG TGGTGCAGCTAGCGTTGGAGAC
UBQ	AAGCCAAGATACAGGACAAG GCATACCACCTCTCAATCTC

Analysis of the *AsGalUR* gene expression dynamics in garlic seedlings exposed to various stressors (drought, salinity, cold) and exogenous phytohormones. Garlic cv. Scorpion plants were grown in transparent glass beakers in water until 15-day-old seedlings were obtained; cloves were fixed in a porous polyethylene substrate so that only the lower part of the clove (the root zone) was submerged in water. At 9:00 and 15:00 of the light growth phase, leaf and root samples were collected (stored at –80 °C) for subsequent analysis (qRT-PCR) of *AsGalUR* gene expression.

To imitate stressful conditions, experimental plants were transferred to the corresponding aqueous solutions for 24 h (100 mM NaCl for salinity; 10 % PEG-6000 for drought; 100 μM abscisic acid (ABA) and 100 μM methyl jasmonate (MeJA) for exogenous phytohotmones). Control plants remained in water. For cold exposure, experimental plants were placed in a climate chamber (+4 °C, without light), while the control plants were maintained in the dark at 22 °C. After 6 h and 24 h of exposure to the stressor/hormone, roots and leaves were collected from plants in the experimental and control groups and stored at –80 °C.

The collected plant material was ground in liquid nitrogen and used to obtain RNA/cDNA preparations and perform qRT-PCR as described above.

Determination of ascorbate content in plant tissues. The AsA content (mg/g fresh weight) was measured in the roots and leaves of garlic plants subjected to stress and treatment with phytohormones. Analysis was performed using the L-Ascorbic acid kit (R-Biopharm AG, Germany), and absorption spectra were recorded on an Eppendorf BioSpectrometer® basic spectrophotometer (Eppendorf, Germany). Regression analysis of the data (search for correlations between the expression level of *AsGalUR* genes and ascorbate content) was performed using GraphPad Prism v. 8 (https://www.graphpad.com).

Results

Identification and structural characterization of garlic D-galacturonate reductase genes

Twenty-seven genes encoding AKR family proteins were identified in the *A. sativum* genome, seven of which were homologous to *GalUR* in *S. lycopersicum* (LOC101256763 and LOC101250974) and *F.* × *ananassa* (AF039182.1) and annotated in AlliumDB as "KEGG pathway: D-galacturonate reductase". These seven garlic genes were numbered *AsGalUR1–7* in the order of their localization on chromosomes 5, 6, and 7. The genes differed in the number of exons – 3 (*AsGalUR5–7*) or 4 (*AsGalUR1–4*), and in size – from 1,105 bp (*AsGalUR5*) to 4,085 bp (*AsGalUR7*). The genes were similar in terms of the length of the coding sequence (CDS) and the predicted physicochemical properties of the encoded proteins (Table 2).

To construct a phylogenetic dendrogram, the amino acid sequences of all 27 identified AKRs from *A. sativum* were compared with AKR homologs from *S. lycopersicum* and *A. thaliana*. In the resulting dendrogram, the proteins were divided into two subfamilies, AKR4A and AKR4B (Fig. 1).

Table 2. Characteristics of *AsGalUR* genes

Gene	Gene ID / Transcript ID	Genome localization	Gene, bp	Exon number	CDS, bp	Protein, aa	Mw, kDa	pI
AsGalUR1	Asa5G02985/ Asa3G05397.1	chr5: 764719171..764720824	1,654	4	945	314	35.0	5.73
AsGalUR2	Asa5G05896/ Asa5G00222.1	chr5: 1607364860..1607366708	1,849	4	969	322	36.2	5.48
AsGalUR3	Asa6G06508/ Asa1G06086.1	chr6: 1808233236..1808234881	1,646	4	945	314	35.1	5.73
AsGalUR4	Asa6G06509/ Asa1G06085.1	chr6: 1808295357..1808297099	1,743	4	951	316	35.5	5.21
AsGalUR5	Asa7G05242.1/ Asa5G04903.1	chr7: 1457744393..1457745497	1,105	3	963	320	36.1	6.39
AsGalUR6	Asa7G05243.1/ Asa5G04902.1	chr7: 1457746950..1457748486	1,537	3	975	324	36.7	5.96
AsGalUR7	Asa7G05244.1/ Asa4G05086.1	chr7: 1458214446..1458218530	4,085	3	963	320	36.1	6.18

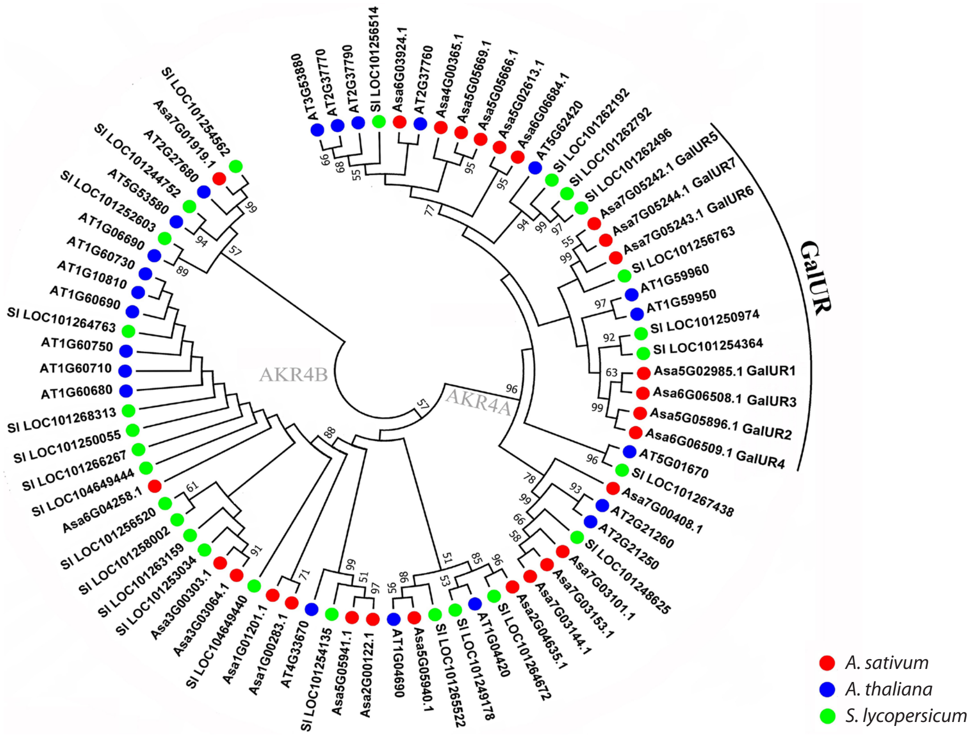


Fig. 1. Dendrogram constructed based on the amino acid sequences of AKR family proteins from garlic (*A. sativum*), Thale cress (*A. thaliana*), and tomato (*S. lycopersicum*) (MEGA 7.0, Neighbor-Joining method, 1,000 bootstrap replicates, significant bootstrap values (%) are indicated at the base of the branches).

The AsGalUR1–7 sequences formed a separate clade within AKR4A, dividing into subclades I (AsGalUR1–4) and II (AsGalUR5–7). The first (I) turned out to be homologs of D-galacturonate reductases from *A. thaliana* (AT1G59950 and AT1G59960 (Duan et al., 2020)) and *S. lycopersicum* SlAKR4B (LOC101250974 (Suekawa et al., 2016)), as well as of an uncharacterized *S. lycopersicum* AKR (LOC101254364). The second (II) clustered with known *S. lycopersicum* GalUR (LOC101256763 (Suekawa et al., 2016)) (Fig. 1). The homology of AsGalUR sequences was 86–99 % (I) and 89–95 % (II) within subclades, and 48–52 % between subclades I and II.

All the AsGalUR1–7 proteins were found to contain the conserved AKR domain PF00248.18 (the domain position was determined by comparison with homologs from *S. lycopersicum*, *Vitis vinifera* L., *F.* × *ananassa*, and *A. thaliana*) and, according to (Suekawa et al. 2016), 10 functionally important amino acid residues (aa), including the plant AKR cofactor binding sites (Fig. 2).

In the same proteins, conserved motifs were also identified (Fig. 3). The composition and position of the motifs were similar within the group of analyzed proteins, with the exception of slightly different C-terminal consensuses, which is associated with variability in the extra-domain region. Namely, individual proteins lacked motif 7 (AT1G59950 and AT1G59960) or 8 (Sl_LOC101256763, FaGalUR, and VvGalUR), or both motifs (AsGalUR2).

Analysis of AsGalUR1–7 sequences in PANNZER predicted that all seven proteins possess oxidoreductase activity (GO:0016616) and are localized in the cytosol (GO:0005829).

Determination of the *AsGalUR1–7* gene expression pattern in garlic plants

Using *A. sativum* cv. Ershuizao transcriptome data (Sun et al., 2020), the expression profiles of the *AsGalUR1–7* genes were determined in various garlic organs (including stages 1–8 of bulb growth and development) (Fig. 4). It was found that the *AsGalUR1*, *3*, and *4* genes (subclade I) were expressed in all analyzed organs, with the highest levels in roots (all three genes), leaves (*AsGalUR3* and *4*), stems (*AsGalUR1* and *3*), and buds (*AsGalUR3*). Transcripts of the fourth gene of subclade I, *AsGalUR2*, were not detected in any of the organs analyzed.

Transcripts of the *AsGalUR5–7* genes (subclade II) were present predominantly in aboveground organs (except flowers), with a maximum in seedlings (all three genes). *AsGalUR5* and *7* (but not *AsGalUR6*) were also highly expressed in leaves,

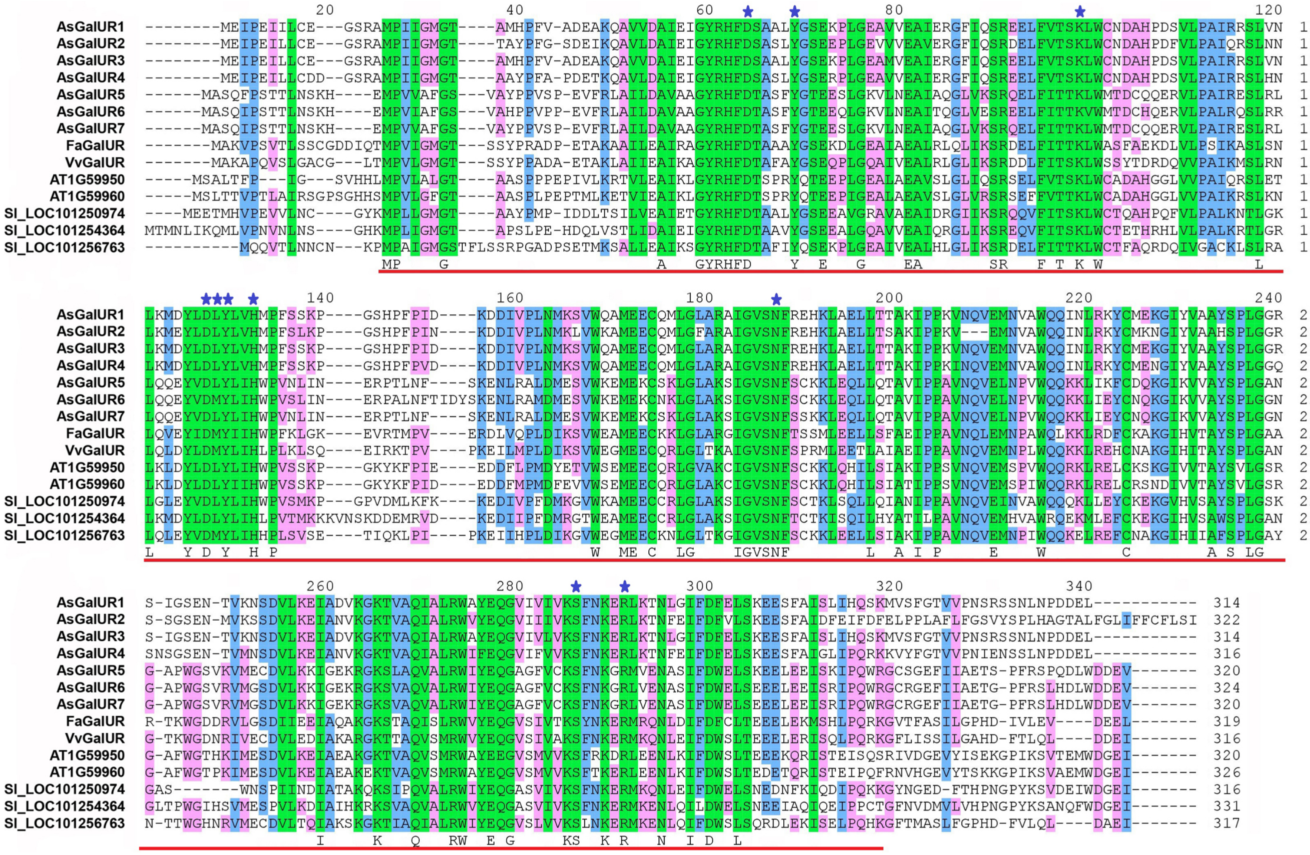
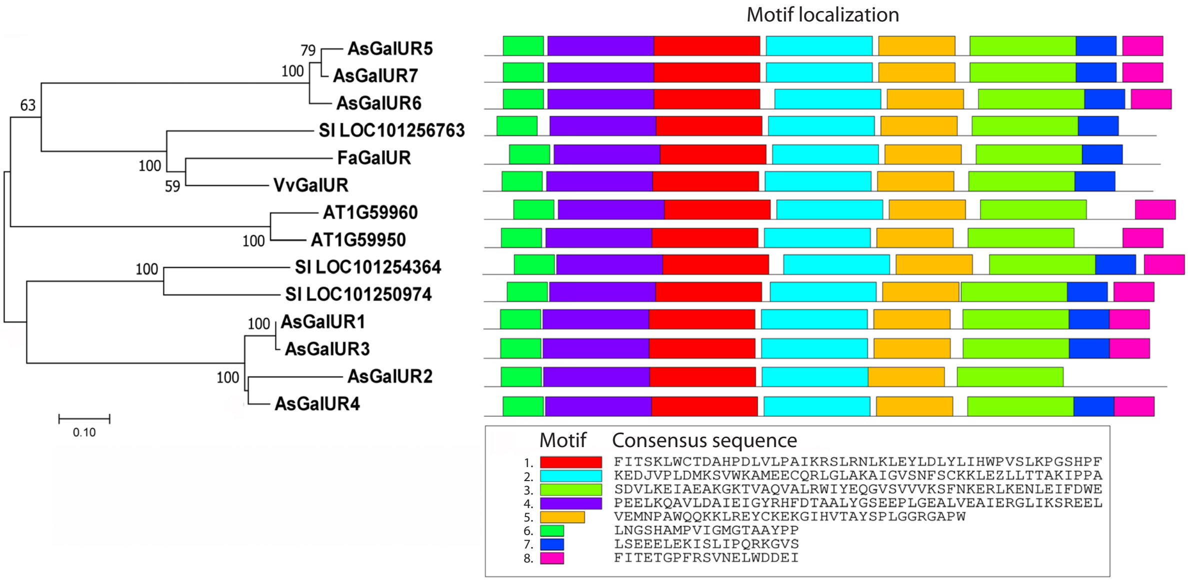


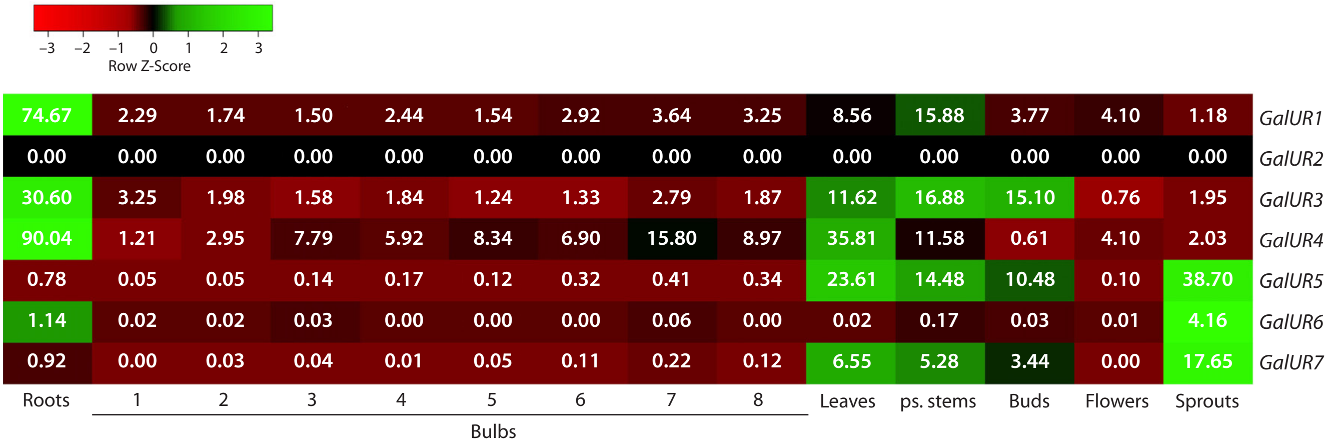
Fig. 2. Alignment of the amino acid sequences of D-galacturonate reductases from *A. sativum* (As), *F.* × *ananassa* (Fa), *V. vinifera* (Vv), *A. thaliana* (AT), and *S. lycopersicum* (Sl).

The background color corresponds to the level of amino acid conservation in the analyzed proteins (green – 100 %, blue – 80 %, pink – 60 %). The position of the AKR domain PF00248.18 is indicated by red underlining. Blue asterisks indicate active sites and cofactor-binding sites of plant AKRs (according to (Suekawa et al., 2016)).



Motif		Consensus sequence
1.	🟥	FITSKLWCTDAHPDLVLPAIKRSLRNLKLEYLDLYLIHWPVSLKPGSHPF
2.	🟦	KEDJVPLDMKSVWKAMEECQRLGLAKAIGVSNFSCKKLEZLLTTAKIPPA
3.	🟩	SDVLKEIAEAKGKTVAQVALRWIYEQGVSVVVKSFNKERLKENLEIFDWE
4.	🟪	PEELKQAVLDAIEIGYRHFDTAALYGSEEPLGEALVEAIERGLIKSREEL
5.	🟨	VEMNPAWQQKKLREYCKEKGIHVTAYSPLGGRGAPW
6.	🟩	LNGSHAMPVIGMGTAAYPP
7.	🟦	LSEEELEKISLIPQRKGVS
8.	🟪	FITETGPFRSVNELWDDEI

Fig. 3. Phylogenetic relationships and comparative profiling of conserved motifs of D-galacturonate reductases from *A. sativum* (As), *F. × ananassa* (Fa), *V. vinifera* (Vv), *A. thaliana* (AT) и *S. lycopersicum* (Sl).



Roots	1	2	3	4	5	6	7	8	Leaves	ps. stems	Buds	Flowers	Sprouts	
74.67	2.29	1.74	1.50	2.44	1.54	2.92	3.64	3.25	8.56	15.88	3.77	4.10	1.18	*GalUR1*
0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	*GalUR2*
30.60	3.25	1.98	1.58	1.84	1.24	1.33	2.79	1.87	11.62	16.88	15.10	0.76	1.95	*GalUR3*
90.04	1.21	2.95	7.79	5.92	8.34	6.90	15.80	8.97	35.81	11.58	0.61	4.10	2.03	*GalUR4*
0.78	0.05	0.05	0.14	0.17	0.12	0.32	0.41	0.34	23.61	14.48	10.48	0.10	38.70	*GalUR5*
1.14	0.02	0.02	0.03	0.00	0.00	0.00	0.06	0.00	0.02	0.17	0.03	0.01	4.16	*GalUR6*
0.92	0.00	0.03	0.04	0.01	0.05	0.11	0.22	0.12	6.55	5.28	3.44	0.00	17.65	*GalUR7*

Bulbs (columns 1–8)

Fig. 4. Heatmap of *AsGalUR1–7* expression in different organs of garlic *A. sativum* cv. Ershuizao based on transcriptome data (Sun et al., 2020).

Numbers in boxes indicate the average FPKM values for three biological replicates. For bulbs, stages 1–8 (corresponding to 192, 197, 202, 207, 212, 217, 222, and 227 days of development) are shown.

stems, and buds. In bulbs and roots, the expression level of the *AsGalUR5–7* genes was low relative to the aboveground parts of the plant (Fig. 4).

Overall, based on the preferred expression profile (Fig. 4), the genes were conditionally assigned to the root (*AsGalUR1*, *3*, and *4*, despite a significant number of transcripts also being expressed in leaves/stems) and leaf (*AsGalUR5–7*) groups, which corresponded to their distribution among phylogenetic subclades I and II (Fig. 1). The exception was the *AsGalUR2* gene of subclade I (Fig. 1), for which no expression was detected in garlic organs (Fig. 4).

In the organs of garlic cv. Scorpion plants, *AsGalUR1–7* gene expression was determined using qRT-PCR (Fig. 5). In contrast to the transcriptome data (Fig. 4), transcripts of the *AsGalUR2* gene were detected everywhere, while *AsGalUR6* was not expressed. Subclade I genes (*AsGalUR1–4*) were expressed in all analyzed organs, with a maximum in the roots and clove base. Transcripts of subclade II genes (*AsGalUR5* and *7*) were detected only in the leaves (maximum) and pseudostem (Fig. 5).

Thus, the results of qRT-PCR (Fig. 5) made adjustments to the *in silico* expression profile of the *AsGalUR2* and *6* genes (Fig. 4) and confirmed the possibility of dividing the genes of subclades I and II into genes with predominant expression in the underground (I) and aboveground (II) parts of the plant.

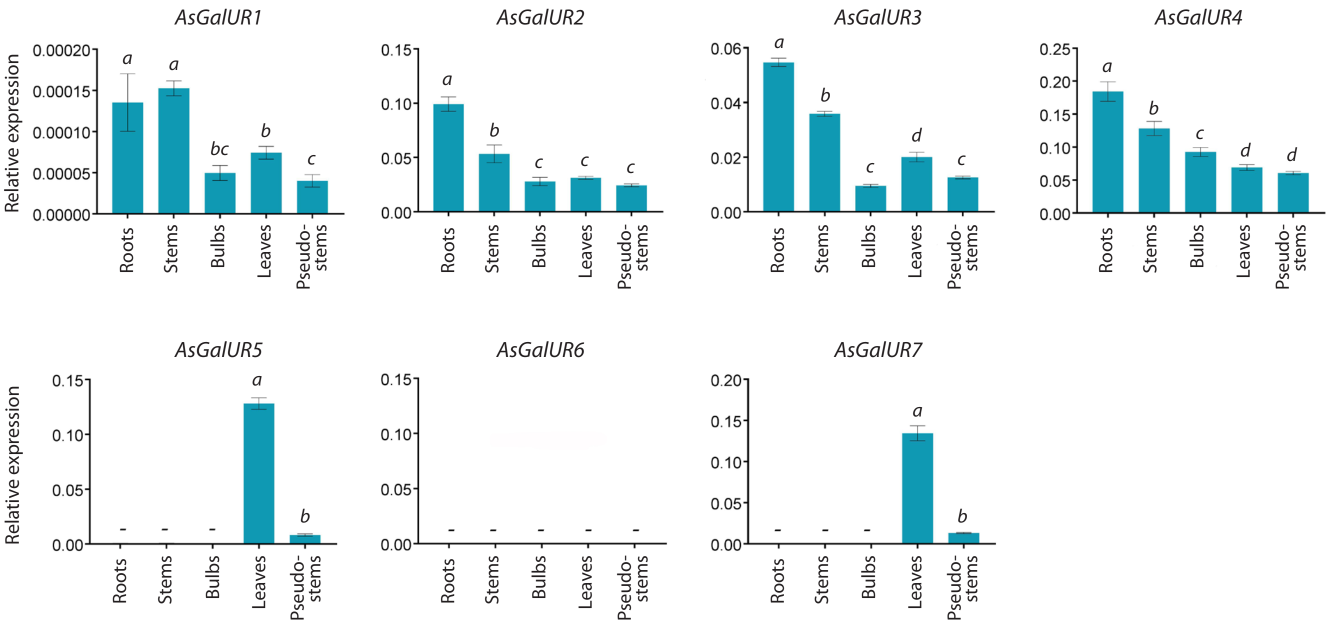
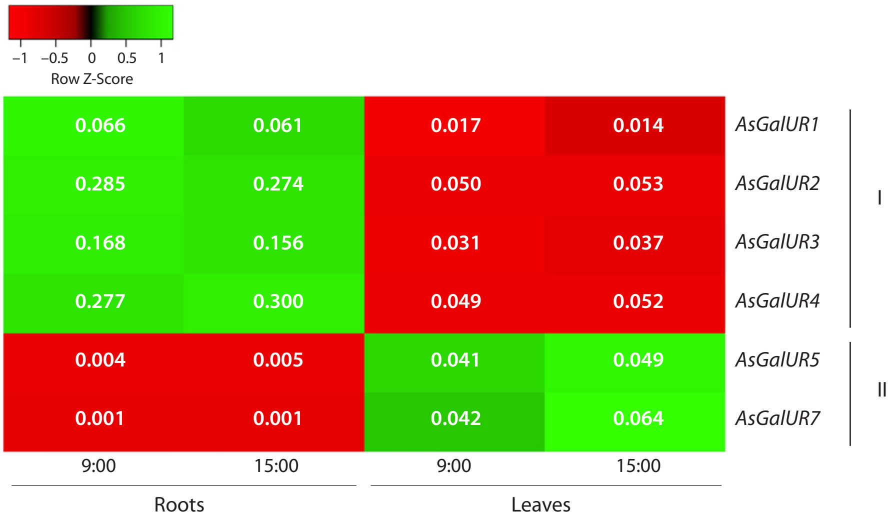


Fig. 5. Expression profile (qRT-PCR) of the *AsGalUR1–7* genes in cv. Scorpion garlic plants.

Significant differences in gene expression levels between different organs at $^{a-d}p < 0.05$.



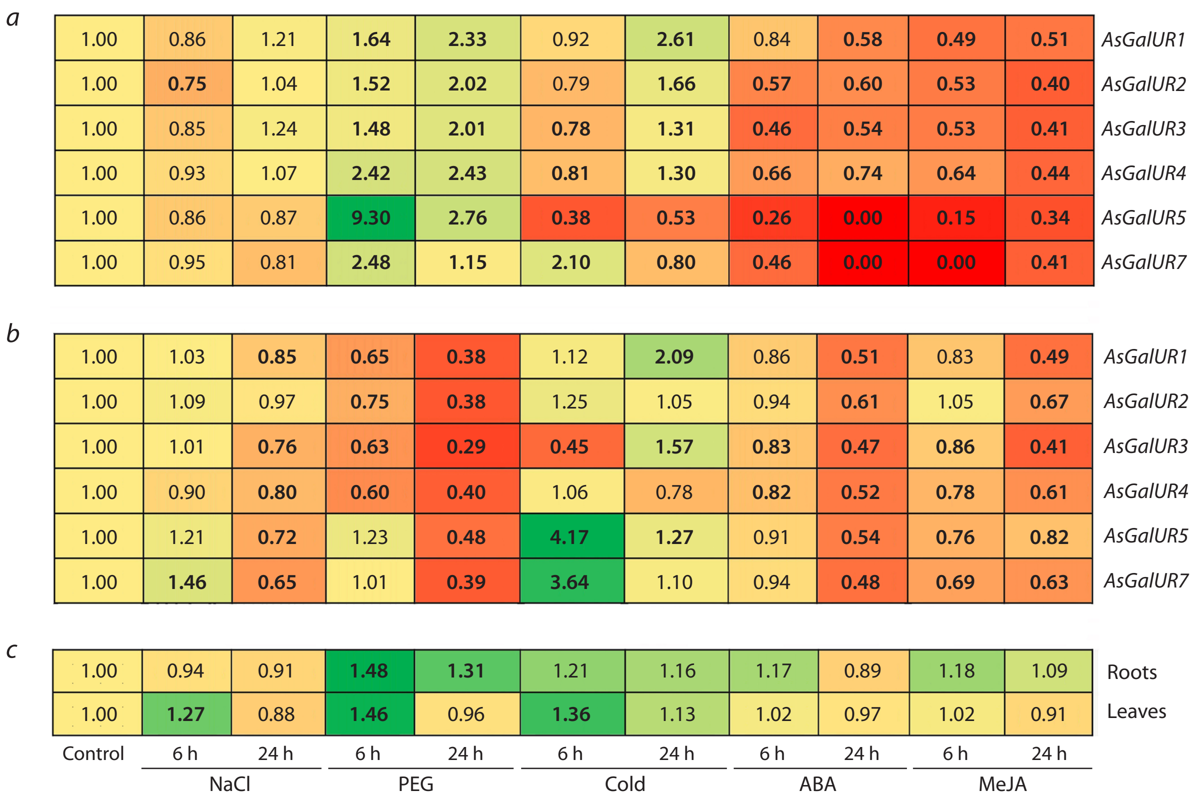
Roots 9:00	Roots 15:00	Leaves 9:00	Leaves 15:00		
0.066	0.061	0.017	0.014	*AsGalUR1*	I
0.285	0.274	0.050	0.053	*AsGalUR2*	
0.168	0.156	0.031	0.037	*AsGalUR3*	
0.277	0.300	0.049	0.052	*AsGalUR4*	
0.004	0.005	0.041	0.049	*AsGalUR5*	II
0.001	0.001	0.042	0.064	*AsGalUR7*	

Fig. 6. Heatmap of the expression (based on qRT-PCR results) of the *AsGalUR1–5* and *7* genes in the roots and leaves of 15-day-old garlic cv. Scorpion seedlings at two time points of the plant growth light phase – 9:00 and 15:00.

The numbers in boxes indicate the average values of the *AsGalUR* gene expression levels, normalized to the expression of the reference genes *GAPDH* and *UBQ*.

Determination of the expression pattern of the *AsGalUR1–7* genes in garlic seedlings: 6-h dynamics during the light phase of plant growth and response to stress factors and exogenous phytohormones

The *AsGalUR1-5* and *7* gene expression levels were determined in roots and leaves of 15-day-old garlic cv. Scorpion seedlings at two time points (9:00 and 15:00) of the light growth phase (Fig. 6). It was confirmed that the genes of root subclade I (*AsGalUR1-4*) are most active in roots, while the genes of leaf subclade II (*AsGalUR5* and *7*) are most active in leaves. Moreover, over the 6-h light phase of plant growth, the gene expression level changed insignificantly, maintaining organ specificity. Interestingly, the transcripts of the "leaf" genes *AsGalUR5* and *7*, which in adult plants were present only in aboveground organs (Fig. 5), were also detected in the roots of seedlings, although with a significantly lower (20–25 times) expression level than in the leaves (Fig. 6).



a

Control	NaCl 6 h	NaCl 24 h	PEG 6 h	PEG 24 h	Cold 6 h	Cold 24 h	ABA 6 h	ABA 24 h	MeJA 6 h	MeJA 24 h	
1.00	0.86	1.21	**1.64**	**2.33**	0.92	**2.61**	0.84	**0.58**	**0.49**	**0.51**	*AsGalUR1*
1.00	**0.75**	1.04	**1.52**	**2.02**	0.79	**1.66**	**0.57**	**0.60**	**0.53**	**0.40**	*AsGalUR2*
1.00	0.85	1.24	**1.48**	**2.01**	**0.78**	**1.31**	**0.46**	**0.54**	**0.53**	**0.41**	*AsGalUR3*
1.00	0.93	1.07	**2.42**	**2.43**	**0.81**	**1.30**	**0.66**	**0.74**	**0.64**	**0.44**	*AsGalUR4*
1.00	0.86	0.87	**9.30**	**2.76**	**0.38**	**0.53**	**0.26**	**0.00**	**0.15**	**0.34**	*AsGalUR5*
1.00	0.95	0.81	**2.48**	**1.15**	**2.10**	**0.80**	**0.46**	**0.00**	**0.00**	**0.41**	*AsGalUR7*

b

Control	NaCl 6 h	NaCl 24 h	PEG 6 h	PEG 24 h	Cold 6 h	Cold 24 h	ABA 6 h	ABA 24 h	MeJA 6 h	MeJA 24 h	
1.00	1.03	**0.85**	**0.65**	**0.38**	1.12	**2.09**	0.86	**0.51**	0.83	**0.49**	*AsGalUR1*
1.00	1.09	0.97	**0.75**	**0.38**	1.25	1.05	0.94	**0.61**	1.05	**0.67**	*AsGalUR2*
1.00	1.01	**0.76**	**0.63**	**0.29**	**0.45**	**1.57**	**0.83**	**0.47**	**0.86**	**0.41**	*AsGalUR3*
1.00	0.90	**0.80**	**0.60**	**0.40**	1.06	0.78	**0.82**	**0.52**	**0.78**	**0.61**	*AsGalUR4*
1.00	1.21	**0.72**	1.23	**0.48**	**4.17**	**1.27**	0.91	**0.54**	**0.76**	**0.82**	*AsGalUR5*
1.00	**1.46**	**0.65**	1.01	**0.39**	**3.64**	1.10	0.94	**0.48**	**0.69**	**0.63**	*AsGalUR7*

c

Control	NaCl 6 h	NaCl 24 h	PEG 6 h	PEG 24 h	Cold 6 h	Cold 24 h	ABA 6 h	ABA 24 h	MeJA 6 h	MeJA 24 h	
1.00	0.94	0.91	**1.48**	**1.31**	1.21	1.16	1.17	0.89	1.18	1.09	Roots
1.00	**1.27**	0.88	**1.46**	0.96	**1.36**	1.13	1.02	0.97	1.02	0.91	Leaves

Fig. 7. Dynamics of *AsGalUR* gene expression in roots (*a*) and leaves (*b*) of garlic seedlings in response to abiotic stressors (salinity, 100 mM NaCl; drought, 10 % PEG-6000; cold stress, +4 °C) and exogenous phytohormones (100 μM ABA; 100 μM MeJa). Changes in the AsA content (*c*) in the roots and leaves of the same garlic seedlings in response to abiotic stressors and phytohormones.

Values significantly ($p < 0.05$) different from the control are shown in bold. Gradient coloring of cells corresponds to changes in gene expression level towards a decrease (shades of red) or an increase (shades of green) in relation to the control (yellow).

To determine the involvement of D-galacturonate reductases in the stress response of garlic plants, 15-day-old cv. Scorpion seedlings were exposed to various stressors (salinity, drought, cold, and the exogenous phytohormones ABA and MeJA). After 6 h and 24 h of exposure, gene expression of *AsGalUR1–5* and *7* was analyzed in the roots and leaves (Fig. 7).

In roots, it was shown that excess NaCl either did not change (*AsGalUR1*, *3–5*, and *7*) or suppressed (*AsGalUR2*, 6 h under stress conditions) gene expression. Dehydration (PEG) significantly stimulated gene expression (by 1.5–9.3 times) at both time points, with the exception of *AsGalUR7*, the transcript levels of which approached control values after 24 h. Six hours of cold exposure affected individual genes, decreasing (*AsGalUR3*, *4*, and *5*) or increasing (*AsGalUR7*) their expression. However, after 24 h, the expression of all genes changed in a subclade-specific manner: it increased (*AsGalUR1–4*, subclade I) or decreased (*AsGalUR5* and *7*, subclade II) compared to the control level. Phytohormone treatment significantly reduced the expression of all genes, reaching zero for *AsGalUR5* and *7* (Fig. 7*a*).

In leaves, 6 h of exposure to salt stress stimulated the expression of *AsGalUR7* (by 1.5 times), while 24 h of exposure suppressed the activity of all genes (except *AsGalUR2*). Six hours of drought did not change (*AsGalUR5* and *7*) or led to a decrease (*AsGalUR1–4*) in gene expression; after 24 h, the transcript levels of *AsGalUR1–5* and *7* decreased by 2.5–3.4 times. In response to 6 h of cold conditions, gene expression increased (*AsGalUR5* and *7*), decreased (*AsGalUR3*), or did not change (*AsGalUR1*, *2*, and *4*); 24 h of exposure led to an increase in the expression of *AsGalUR1*, *3*, and *5*. Treatment with ABA and MeJA, as in roots, negatively affected the expression of the *AsGalUR1–5* and *7* genes (Fig. 7*b*).

In garlic seedlings exposed to stress factors and exogenous phytohormones, the ascorbate content was determined to be 1.4–2.2 and 12.1–24.3 mg/100 g in roots and leaves, respectively (Fig. 7*c*). In roots, AsA content increased at both time points (6 h and 24 h of stress) in response to drought (PEG), whereas it did not change under the influence of other stressors and phytohormones. In leaves, increased ascorbate accumulation was observed after 6 h of exposure to NaCl, drought, and cold stress; at 24 h, the AsA content returned to control values (Fig. 7*c*).

In the leaves of garlic seedlings, the correlation analysis revealed a significant ($p < 0.05$) dependence of the ascorbate content on the expression level of the *AsGalUR1* (correlation coefficient $r = 0.63$) and *AsGalUR4* ($r = 0.66$) genes under stress conditions.

Discussion

The D-galacturonic pathway for L-ascorbic acid biosynthesis in plants was first discovered in the 1950s, and the enzyme D-galacturonate reductase was discovered at the same time. It catalyzes the reduction of D-galacturonic acid methyl ester to L-galactonic acid (Isherwood, Mapson, 1956), which is converted in several stages to AsA (Ishikawa et al., 2008; Peltonen, Richard, 2022). In 2003, the first gene of the *GalUR* family encoding D-galacturonate reductase was cloned in strawberry *F.* × *ananassa* (*FaGalUR*), in which ripe berries ascorbate is synthesized predominantly via the D-galacturonic pathway (Agius et al., 2003; Cruz-Rus et al., 2011).

In this study, the *AKR4* gene family (Fig. 1) was identified for the first time in the genome of garlic *A. sativum*. Seventeen of the 27 identified *AsAKR* genes belonged to the *AKR4A* subfamily, which was consistent with the higher number of *AKR4A* genes compared to *AKR4B* in higher plants (Duan et al., 2020). Seven *AKR4A* genes, *AsGalUR1–7* (Fig. 1, Table 2), encoded homologs of known GalUR proteins from *S. lycopersicum* (Suekawa et al., 2016), *A. thaliana* (Duan et al., 2020), and *F.* × *ananassa* (Agius et al., 2003).

Based on structural homology, it was suggested that AsGalUR1–7 proteins may function as D-galacturonate reductases in garlic plants. The presence of functionally important sites and a conserved domain specific for AKR in all AsGalUR1-7 proteins (Fig. 2), along with the results of GO analysis, also indicates the presence of oxidoreductase activity characteristic of AKR in AsGalUR1–7 when localized in the cell cytosol. The predicted localization of AsGalUR1–7 was consistent with the fact that the two-step conversion of D-galacturonate to L-galactono-1,4-lactone involving GalUR occurs precisely in the cytosol (Smirnoff et al., 2001).

Based on the number of exons, the genes were divided into two groups: *AsGalUR1–4* (4 exons) and *AsGalUR5–7* (3 exons) (Table 2), which suggested the existence of functional differences between them, possibly associated with the specificity of expression for individual organs/tissues/developmental stages and/or with the adaptive reactions of the garlic plant. This division was confirmed by phylogenetic analysis. In the dendrogram, the AsGalUR1–7 proteins formed two subclades: I (AsGalUR1-4) and II (AsGalUR5-7) (Fig. 1), although the set of conserved motifs looked identical, with the exception of the C-terminal part of AsGalUR2 (Fig. 3). The variability of the AsGalUR2 C-terminal sequence may indicate the presence of individual functional properties in this protein, but does not exclude its involvement in the synthesis of AsA. The different numbers of subclade I and II *GalUR* genes in *A. sativum* and, for example, *S. lycopersicum*, *A. thaliana* or *Brassica rapa* L. (Duan et al., 2020) (Fig. 1) suggests the emergence of precursors of these genes before the separation of the monocot and dicot classes and subsequent species-specific duplication evolution of the corresponding gene families.

As mentioned above, activation of D-galacturonic acid pathway of AsA synthesis as an alternative to the main L-galactose pathway occurs in ripe, juicy fruits, which is associated with the degradation of cell wall pectin in the pulp with the release of D-galacturonate (a substrate for GalUR) (Cruz-Rus et al., 2011; Badejo et al., 2012). Growing and ripening fruits obtain the required amount of ascorbate through internal synthesis via the main pathway and translocation of AsA from the leaves (Badejo et al., 2012). Softening of ripe fruits is accompanied by the destruction of cell walls. During this, pectin, which makes up ~35 % of the primary walls in dicotyledons and non-cereal monocots (including garlic) (Mohnen, 2008) and consists of ~70 % galacturonic acid residues (Mølhøj et al., 2004; Cruz-Rus et al., 2011; Badejo et al., 2012), is degraded. There is a significant increase in the amount of substrate for GalUR, which stimulates a switch to an alternative pathway for AsA synthesis in fruits (Peltonen, Richard, 2022).

Thus, to activate the D-galacturonic acid pathway, plants require D-galacturonate as a substrate for GalUR (Peltonen, Richard, 2022). However, in vegetative tissues, as well as in growing storage organs, this compound is strictly necessary for the synthesis of cell wall pectin, since plant growth and development are accompanied by active cell division (Mohnen, 2008). Nevertheless, the constant presence of high concentrations of D-galacturonate in the cytosol of vegetative tissue cells suggests that D-galacturonic acid pathway of AsA synthesis may also occur there as a minor addition to the main L-galactose pathway. This is indirectly confirmed by the positive correlation between the content of AsA and the expression level of D-galacturonate reductase genes found in the leaves of *B. rapa* (Duan et al., 2020) and tea bush (*Camellia sinensis* (L.) O. Kuntze) (Li et al., 2017).

Based on the above, in this study, we analyzed the expression of the *AsGalUR1–7* genes in various organs of the *A. sativum* cv. Ershuizao plant (*in silico*, using transcriptome data (Sun et al., 2020)) (Fig. 4), as well as in the leaves and roots (qRT-PCR) of the garlic cv. Scorpion, including in response to abiotic stressors and exogenous phytohormones (Fig. 5–7). In addition to vegetative organs, bulb samples (as a storage organ) at several stages of growth and maturation were included in the *in silico* analysis (Sun et al., 2020).

The analysis confirmed that the groups of *AsGalUR* genes belonging to subclades I and II differ in their expression profiles and, presumably, in their functional specialization. So, the transcript levels of genes *AsGalUR1–4* (I) were significantly higher in the underground part of the garlic plant compared to the aboveground organs, while the opposite pattern was observed for genes *AsGalUR5–7* (II) (Figs. 4–6). This suggests organ-specific involvement of these genes in the D-galacturonic pathway; subclade I and II genes were provisionally named as root (*AsGalUR1–4*) and leaf (*AsGalUR5–7*) genes. A question remains regarding the *AsGalUR2* and *6* genes, which showed different expression patterns depending on the type of analysis, but based on the totality of the data (Fig. 4 and 5), these genes correspond to the characteristics of their groups, although they require further study. In our further work, we relied on the results of qRT-PCR, that is, we excluded *AsGalUR6* from the study.

As expected, in the bulb, the root genes *AsGalUR1–4* were expressed significantly higher than the leaf genes; however, no significant trends in transcript level changes were observed as the bulbs approached maturity (Fig. 4). This may be due to differences in the definition of full ripeness between juicy fruits and root vegetables. While in fruits biological ripeness is accompanied by softening of the pulp (Badejo et al., 2012), in root vegetables it means enlargement and vacuolization of cells for nutrient accumulation and the onset of physiological dormancy (Teper-Bamnolker et al., 2012). Softening of root tissue may be a sign of dormancy release, wilting, or rotting.

Consistent with the fact that 12 *GalUR* genes of *B. rapa* exhibit obvious divergence of expression under different stress conditions (Duan et al., 2020), in our study, the *AsGalUR1–5* and *7* genes also responded diversely and organ-dependently to both abiotic stressors and exogenous ABA and MeJA (Fig. 7*a*, *b*). Despite the conditional division of genes into root and leaf groups, in most cases their expression in response to stress changed in both roots and leaves (Fig. 7*a*, *b*). This indicates the involvement of all *AsGalUR1–5* and *7* genes in AsA synthesis via an alternative pathway under stress conditions throughout the plant, but with predominant specificity in the underground or aboveground parts.

Exogenous phytohormones suppressed the expression of genes of both group in both tissue types (Fig. 7*a*, *b*), which is consistent with the role of ABA and MeJA as plant growth regulators and cell division stimulators (Fattorini et al., 2009; Xie et al., 2020). It is possible that hormone treatment accelerates cell division, which requires increased pectin synthesis, reduces the amount of substrate for GalUR and, as a result, downregulates the expression of *AsGalUR* genes. On the other hand, exogenous ABA has been shown to stimulate AsA synthesis (Xu et al., 2022); however, this most likely relates to the main ascorbate synthesis pathway. In addition, we did not observe an increase in ascorbate content in response to phytohormones (Fig. 7*c*), which may suggest the absence of an effect or a delayed stimulatory effect of ABA on AsA synthesis.

The effects of abiotic stressors on gene expression were more diverse than those of hormones (Fig. 7*a*, *b*). The main difference between the reaction of root (*AsGalUR1–4*) and leaf (*AsGalUR5*, *7*) genes was the opposite expression dynamics under low temperature exposure. In roots (as expected from conditional gene specialization), after 24 h of treatment, the expression of root genes increased, while that of leaf genes decreased (Fig. 7*a*). In leaves, on the contrary, after 6 h of exposure, the expression of leaf *AsGalUR5* and *7* sharply increased, while the expression of root *AsGalUR1–4* decreased or remained unchanged (Fig. 7*b*). This is consistent with the positive dependence of plant cold resistance on AsA content (Fu et al., 2023) and emphasizes the organ specificity of the increase in *AsGalUR* gene expression in response to cold, which led to a significant (leaves) or weaker (roots) increase in ascorbate content (Fig. 7*c*).

Ascorbic acid promotes plant tolerance to salinity and drought (Younis et al., 2010). Accordingly, the expression of *AsGalUR* genes changed in response to osmotic/ionic stress (PEG, NaCl) with a more pronounced effect in the roots, which is associated with the specificity of stress conditions created (roots immersed in a PEG or NaCl solution). However, no functional differences were found between leaf and root *AsGalUR* genes: in leaves, the expression level of all genes decreased, while in roots it did not change (NaCl) or increased (PEG) (Fig. 7*a*, *b*), which was accompanied by an increase in AsA accumulation in organs of both types (Fig. 7*c*). It should be noted that the correlation between the gene expression level and the ascorbate content under stress conditions was found only in leaves and only for the *AsGalUR1* and *4* genes assigned to the root group, which confirms the conventionality of the functional division of *AsGalUR* genes in the adaptive responses of garlic plants.

The data we obtained are fully consistent with the activation of alternative pathways for ascorbate synthesis in plants under stressful conditions shown in other studies (Xu et al., 2012; Ruggieri et al., 2016). In particular, this may be due to a decrease in the flux of the main L-galactose pathway in response to stressors (due to the active involvement of this pathway precursor, D-glucose, in plant stress responses), which was demonstrated in the analysis of the *vtc1* and *vtc2* mutants of *A. thaliana* (Quiñones et al., 2024).

Conclusion

In this study, we identified the *A. sativum AKR* gene family and defined seven *AsGalUR1–7* genes, which presumably encode D-galacturonate reductases – key enzymes in the alternative D-galacturonic acid pathway of L-ascorbic acid biosynthesis. We characterized the structure and phylogeny of the genes and encoded proteins, as well as the organ-specific expression profile of the *AsGalUR1–7* genes. Based on this, the genes were conditionally divided into root (*AsGalUR1–4*) and leaf (*AsGalUR5–7*) groups. We analyzed gene expression in response to abiotic stress factors (salinity, drought, and cold) and exogenous phytohormones (ABA, MeJA), which revealed additional functional features of the genes in determining garlic plant stress tolerance.

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