



## Article

# Garlic (*Allium sativum* L.) Invertase Genes: Genome-Wide Identification and Expression in Response to Abiotic Stresses and Phytohormones

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**Abstract:** Invertases are involved in plant growth, development, and stress adaptation; however, invertase-encoding genes have not yet been reported in *Allium* species. In this study, we identified 23 invertase genes in garlic (*Allium sativum* L.): 11 encoding putative neutral/alkaline (AsN/AINV1–11) and 12 acid (6 cell-wall—AsCWINV1–6 and 6 vacuolar—AsVINV1–6) enzymes. Among them, AsN/AINV1, 3, 8–10, AsCWINV2–5, and AsVINV2–6 showed significant transcription in garlic organs (roots, bulbs, pseudostems, leaves, sprouts, and reproductive parts) in a tissue-specific manner, whereas the AsN/AINV4–6, 11, AsCWINV1, 6, and AsVINV1 genes had weak or no detectable expression. Gene promoters contained nine, nine, and sixteen hormone-, stress-, and light-responsive *cis*-regulatory elements, respectively, and fifteen sites related to transcription factor binding and plant development. Expression analysis revealed that 12 invertase genes strongly transcribed in the roots of *A. sativum* cv. Ershuizao showed differential expression in the roots and leaves of *A. sativum* cv. Sarmat exposed to abiotic stresses (low temperature, high salinity, and drought) and phytohormones (abscisic acid and methyl jasmonate), which was significantly correlated with glucose, fructose, and sucrose contents. Our results should further functional analysis of invertases from *Allium* crops and contribute to the breeding of stress-tolerant varieties.



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**Keywords:** garlic; *Allium sativum* L.; invertase genes; gene structure; gene expression; abiotic stress response

## 1. Introduction

Sucrose, a glucose–fructose disaccharide, is a primary metabolite in plants, a source of energy and carbon atoms for growth and development [1,2]. In agricultural crops, the contents of sucrose and its constituents are essential for yield, quality, and stress resistance [3–6]. Sucrose, as the final product of photosynthesis, is transported from photosynthetic to storage (sink) organs, where it undergoes reversible hydrolysis by sucrose synthase (EC 2.4.1.13) or irreversible cleavage by invertases (EC 3.2.1.26, also known as  $\beta$ -fructofuranosidases) to glucose and fructose [1,7]. The activity of sucrose synthase is considered important in the biosynthesis of cellulose and cell wall development [8], whereas the roles of invertases are more diverse, including carbon partitioning, sink development, sugar signaling, and stress response [9,10].

Plant invertases are classified into neutral/alkaline (N/AINV, optimum pH 6.5–6.8/8.0–9.0), which can be localized in the cytosol, mitochondria, plastids, and nucleus, and acid; the latter are subdivided into soluble vacuole-located (VINV, optimum pH 4.5–5.5) and insoluble cell wall-associated (CWINV, optimum pH 3.5–5.0) [11]. N/AINVs belong to the glycoside hydrolase (GH) 100 family and CWINVs and VINVs belong to the GH32 family and share some enzymatic and biochemical properties [12,13].

Invertase gene families have been identified in the genome of many plant species, including model organism *Arabidopsis thaliana*, dicots such as *Solanum tuberosum*, *Camellia*

*sinensis*, *Malus domestica*, *Glycine max*, and *Pisum sativum*, and monocots such as *Oryza sativa*, *Zea mays*, and *Triticum aestivum* [12,14–17]. The importance of invertases in plant development is emphasized by phylogenetic studies of cytosolic and organelle enzymes from algae to seed plants, which link the evolution of invertases to terrestrial colonization by land plants [18]. The facts that N/AINV and VIN/CWIN are simultaneously present in plants and have similarity with the invertases of cyanobacteria and respiratory eukaryotes, respectively, and that VIN/CWIN are more diverse than N/AINV are consistent with the origin of phototrophic eukaryotes through endosymbiosis between cyanobacteria and non-photosynthetic respiratory eukaryotes [15].

Both N/AINV and VIN/CWIN have been reported to primarily participate in the development of leaves, roots, and reproductive organs (flower, fruit, and seed/grain), where they exert positive effects, and in the regulation of plant resistance to biotic and abiotic stresses [12]. It has been shown that the activity of acid invertases can be affected by pathogens, wounding, cold, osmotic stress, and phytohormones (gibberellic and abscisic acids, auxins, etc.) [14]; thus, in *S. tuberosum* tubers, VINs are shown to be responsible for cold-inducing sweetening (negative agricultural trait) [15]. Cytosolic invertases are known to affect sugar homeostasis and stress tolerance of male meiocytes in *Z. mays* [19] and vegetative growth, flowering, fruit set, and yield in *Solanum lycopersicum* [20].

Despite the close involvement of invertases in plant growth and development, including agriculturally valuable traits, little is known about these enzymes in the *Allium* genus comprising well-known crops. Garlic (*Allium sativum* L.) is a popular spice plant with considerable medicinal properties due to the presence of a large number of biologically active compounds with beneficial health effects [21]; it is also used to improve soil quality through intercropping and crop rotation [22]. Garlic is cultivated all over the world, which, given its non-sexual reproduction, suggests high adaptive plasticity of the genome and a well-developed stress response system [23–25]; it is known to be sensitive to various abiotic stresses, including low temperatures [26], soil salinity [27,28], and drought [29]. Transcriptomic and metabolomic studies have revealed that stresses affect the metabolism of sucrose and fructans, which contribute to garlic yield and quality [26–29]. Thus, cold exposure results in an increase in the content of fructans, the main reserve carbohydrates in *Allium* species, and in the upregulation of key genes controlling fructan metabolism, including *FEH*, which encodes fructan exohydrolase [26].

Since the accumulation of sucrose and fructans increases plant stress resistance, the regulation of their metabolism is an important part of the plant stress defense mechanism [3–6,30]. Fructans are polymers of D-fructose and as such represent sucrose derivatives [31]. Similar to sucrose, fructans can act as osmoregulators and phloem-mobile signaling compounds under stress and may contribute to the overall homeostasis of cellular reactive oxygen species [31]. As fructan synthesis depends on D-fructose content, it is regulated by the activity of sucrose-hydrolyzing enzymes sucrose synthase and invertases. The degradation of fructans is catalyzed by FEHs, which hypothetically are derived from ancestral invertases—VINs or CWINs [32]. FEHs cleave only terminal bonds between fructose residues and lack invertase activity because of a mutation that changes substrate specificity [33].

The release of the high-quality genome and transcriptome sequence of *A. sativum* cultivar (cv.) Ershuizao [34] has facilitated the identification and characterization of garlic gene families [35–38]. In this study, we identified 23 genes of the invertase family in the *A. sativum* genome and performed their characterization in terms of structure, phylogeny, chromosomal location, tissue expression patterns, and abiotic stress responses. The results should be useful for further investigation of the physiological role of invertases in the development of garlic and onions in general and can be used in garlic breeding to obtain stress-resistant accessions.

## 2. Materials and Methods

### 2.1. Identification and Structure Analysis of the *A. sativum* Invertase Gene Family In Silico and In Vitro

The gene sequences annotated as invertases were searched in the *A. sativum* (cv. Ershuizao) genome (PRJNA606385, assembly Garlic.V2.fa) and transcriptomes (PRJNA607255) [34]. Sequences without start and stop codons were removed during analysis. All comparative sequence alignments were carried out in MEGA 7.0.26 [39]. The identified genes were characterized in terms of chromosomal localization (visualized in MG2C v2.1, [http://mg2c.iask.in/mg2c\\_v2.1/](http://mg2c.iask.in/mg2c_v2.1/); accessed on 3 December 2023), exon–intron structure (GSDS v2.0 [40]), and promoter-specific (~1.0 kb) *cis*-regulatory elements (PlantCARE, <http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>; accessed on 3 December 2023). The putative protein products were characterized in terms of domain structure (Conserved Domain Database; <https://www.ncbi.nlm.nih.gov/cdd>; accessed on 3 December 2023), consensus motifs (MEME 5.5.5; <http://meme-suite.org/tools/meme>; accessed on 20 March 2024), molecular weight (MW) and isoelectric point (pI) (ExPASy ProtParam; <https://www.expasy.org/resources/protparam>; accessed on 3 December 2023), grand average of hydropathy (GRAVY) value (Protein GRAVY; [https://www.genecorner.ugent.be/protein\\_gravy.html](https://www.genecorner.ugent.be/protein_gravy.html); accessed on 3 December 2023), phylogenetic relationships (MEGA 7.0.26: Neighbor-Joining method, the JTT matrix-based model, bootstrap 1000), subcellular localization prediction (WoLF PSORT; <https://wolfpsort.hgc.jp/>; accessed on 3 December 2023), prediction of signal peptide and transmembrane helices (DeepTMHMM; <https://dtu.biolib.com/DeepTMHMM>; accessed on 3 December 2023), and Gene Ontology (GO) functional annotation (PANNZER2 server; <http://ekhidna2.biocenter.helsinki.fi/sanspanz/>; accessed on 3 December 2023).

VINV coding sequences (CDSs) were amplified by PCR using mixed cDNA preparations from garlic cv. Sarmat seedlings and cDNA-specific primers based on the cv. Ershuizao data (Supplementary Table S1). RNA extraction and cDNA synthesis are described in Section 2.4. The amplicons were sequenced and analyzed using MEGA 7.0.26.

### 2.2. In Silico Profiling of *A. sativum* Invertase Gene Expression

Expression patterns of the invertase genes were determined in silico by analyzing the transcriptome of seven tissues of *A. sativum* cv. Ershuizao (PRJNA607255): roots, bulbs (at 192, 197, 202, 207, 212, 217, 222, and 227 days after sowing [DAS]), leaves, pseudostems, buds, sprouts (at 217 DAS), and flowers (at 217 DAS) [34]. The expression data were visualized as a heatmap using Heatmapper [41]; the reliability of gene transcription was determined according to the fragments per kilobase of transcripts per million reads mapped (FPKM) value  $\geq 10$ .

### 2.3. Garlic Plants and Abiotic Stress Simulation

Winter garlic cv. Sarmat used in this study was kindly provided by the Federal Scientific Vegetable Center (Moscow region, Russia). Peeled garlic bulb cloves of a uniform size and without external signs of disease were selected, treated with 70% ethanol for 5 min, washed three times with water, and left to germinate in water until active growth of roots and sprouts (~3 weeks at 23–25 °C and 16 h light/8 h dark cycle).

The effects of each stress factor were tested using two biological and three technical replicates; untreated plants grown under normal (pre-stress) conditions were used as a control. Seedlings of 12–15 cm were exposed to cold (4 °C), drought (10% polyethylene glycol-6000), high salinity (250 mM NaCl), abscisic acid (ABA, 100  $\mu$ M), or methyl jasmonate (MeJA, 100  $\mu$ M) for 2, 4, 6, and 24 h; after 24 h, some experimental plants were returned to normal conditions for another 24 h (post-stress period). Leaves and roots were collected at each time point, frozen in liquid nitrogen, and stored at –80 °C until further analyses.

### 2.4. Invertase Gene Expression Analysis

Invertase gene expression was analyzed by quantitative (q) Real-Time (RT)-PCR. Total RNA was extracted from ~0.1 g of tissue using the RNeasy Plant Mini Kit and RNase free

DNase set (QIAGEN, Hilden, Germany), and cDNA was synthesized using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). The concentrations of RNA and cDNA were measured using Qubit® Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Amplification was performed in a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) at the following cycling conditions: 95 °C for 5 min and 40 cycles of 95 °C for 15 s and 60 °C for 40 s. Reactions contained SYBR Green RT-PCR mixture (Syntol, Moscow, Russia), 3.0 ng of cDNA, and gene-specific primers, which were selected in cDNA variable regions and were separated by at least one intron according to the cv. Ershuizao genome and transcriptome [34] (Supplementary Table S1). Additional verification of primer specificity was performed using BLASTn (<https://blast.ncbi.nlm.nih.gov/>; accessed on 3 December 2023) and Primer3 (<http://frodo.wi.mit.edu/primer3/>; accessed on 3 December 2023).

The expression of the invertase genes was normalized to that of two stably expressed reference genes encoding ubiquitin (*UBQ*; NCBI Gene ID MZ171222.1) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; MZ171220.1); such combination increases the accuracy of qRT-PCR results compared to the single reference assay [42] and has been successfully used to test garlic gene expression [35–38].

### 2.5. Soluble Sugar Content

Garlic tissue samples were analyzed for the content (mg/100 g of fresh weight) of soluble sugars (glucose, fructose, and sucrose) using the Enzytec™ Liquid D-Glucose/D-Fructose and Enzytec™ Liquid Sucrose/D-Glucose kits (R-Biopharm AG, Darmstadt, Germany) according to the manufacturer's instructions.

### 2.6. Statistical Analysis

Biochemical and qRT-PCR data were statistically processed using GraphPad Prism v. 9 (<https://www.graphpad.com/scientific-software/prism/>; accessed on 30 January 2024) and visualized as heatmaps using Excel. The data were compared based on One-Way ANOVA (multiple comparisons corrected with Bonferroni test);  $p < 0.05$  was considered to indicate statistically significant difference.

## 3. Results

### 3.1. Invertase Gene Family in *A. sativum*

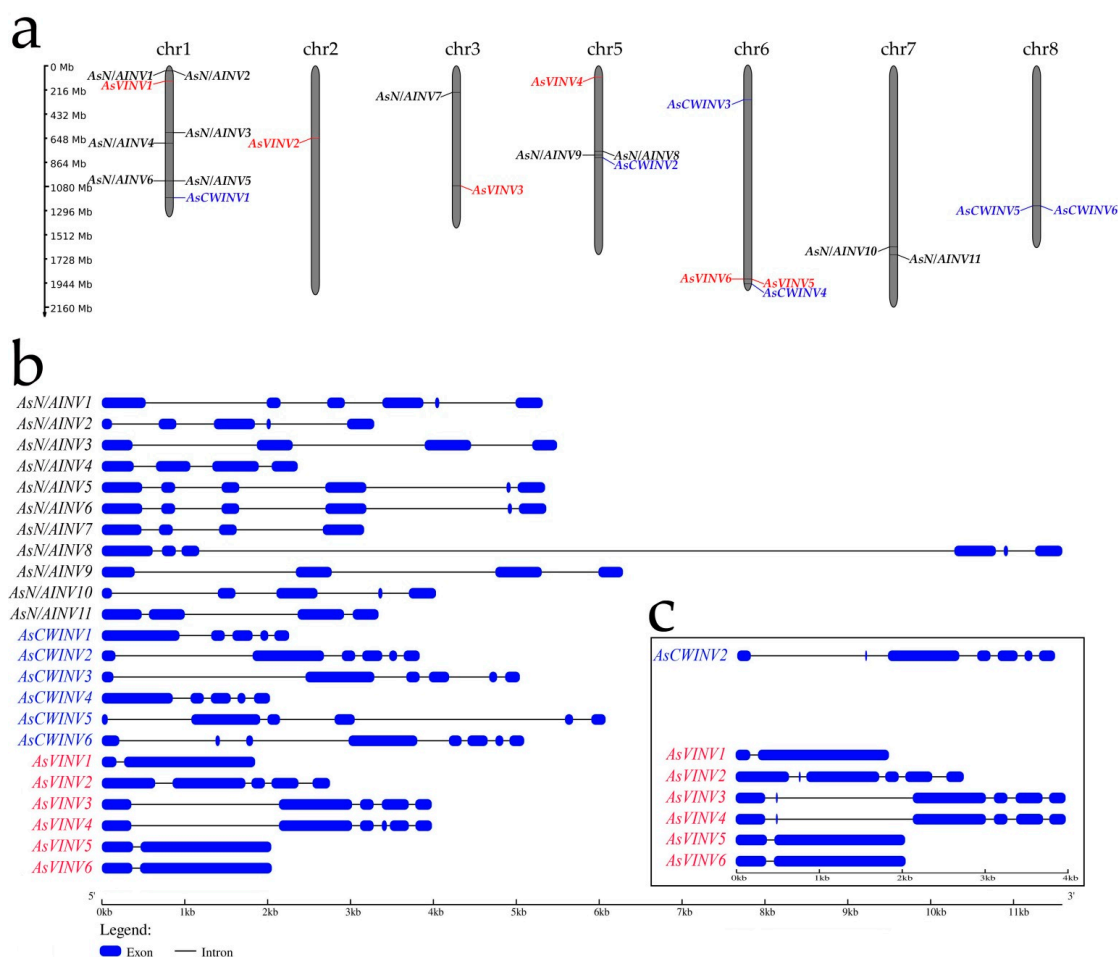
Twenty-three putative full-length invertase genes were detected through comparative analysis of the annotated *A. sativum* cv. Ershuizao genomic and transcriptomic data; among the genes, eleven, six, and six were identified as *N/AINV*-like, *CWINV*-like, and *VINV*-like and named as *A. sativum* (*As*) *N/AINV1–11*, *AsCWINV1–6*, and *AsVINV1–6*, respectively (the numbering was based on the order of gene location in the *A. sativum* chromosomes) (Table 1, Figure 1a). Gene sequences contained 4–6 (*AsN/AINVs*), 5–8 (*AsCWINVs*), or 2–6 (*AsVINV*s) exons (Figure 1b); no splice variants or intronless sequences were found.

Six *AsN/AINV* genes were located as three tandems of two genes on chromosome 1, two and two were located in tandems on chromosomes 5 and 7, respectively, and one gene was detected on chromosome 3 (Figure 1a). The exon–intron structures of tandem genes were similar for *AsN/AINV1–AsN/AINV2* and *AsN/AINV5–AsN/AINV6* but differed for *AsN/AINV3–AsN/AINV4*, *AsN/AINV8–AsN/AINV9*, and *AsN/AINV10–AsN/AINV11*; the structure of *AsN/AINV7* located alone on chromosome 3 was similar to that of tandem genes *AsN/AINV5–AsN/AINV6* (Figure 1b).

Among the *AsCWINV* genes, one, one, and two were located on chromosomes 1, 5, and 6, respectively, and two formed a tandem on chromosome 8 (Figure 1a); it should be noted that the two tandemly clustered genes (*AsCWINV5* and 6) significantly differed in the exon–intron structure (Figure 1b).

**Table 1.** Characteristics of the *A. sativum* cv. Ershuizao invertase genes.

| Gene              | Gene ID/Transcript ID [34] | Genomic Localization        | Gene, bp | CDS, bp | Protein, aa | MW, kDa | pI   | GRAVY  |
|-------------------|----------------------------|-----------------------------|----------|---------|-------------|---------|------|--------|
| <i>AsN/AINV1</i>  | Asa1G00182.1/Asa7G01205.1  | chr1: 40208894-40214212     | 5319     | 1776    | 591         | 66.78   | 5.88 | −0.313 |
| <i>AsN/AINV2</i>  | Asa1G00196.1/Asa7G01204.1  | chr1: 45988527-45991812     | 3286     | 1203    | 400         | 45.55   | 5.53 | −0.303 |
| <i>AsN/AINV3</i>  | Asa1G02231.1/Asa2G02607.1  | chr1: 597271753-597277244   | 5492     | 1659    | 552         | 62.96   | 6.11 | −0.188 |
| <i>AsN/AINV4</i>  | Asa1G02550.1/Asa2G02247.1  | chr1: 692918625-692920987   | 2363     | 1671    | 556         | 62.74   | 6.32 | −0.147 |
| <i>AsN/AINV5</i>  | Asa1G03777.1/Asa2G00901.1  | chr1: 1030642218-1030647564 | 5347     | 1737    | 578         | 66.01   | 6.39 | −0.277 |
| <i>AsN/AINV6</i>  | Asa1G03784.1/Asa2G00903.1  | chr1: 1031491320-1031496681 | 5362     | 1737    | 578         | 66.01   | 6.39 | −0.277 |
| <i>AsN/AINV7</i>  | Asa3G00854.1/Asa8G04269.1  | chr3: 235042209-235045372   | 3164     | 1353    | 450         | 50.99   | 6.11 | −0.212 |
| <i>AsN/AINV8</i>  | Asa5G02977.1/Asa6G02773.1  | chr5: 760809575-760821162   | 11,588   | 1866    | 621         | 69.83   | 6.61 | −0.286 |
| <i>AsN/AINV9</i>  | Asa5G03124.1/Asa3G04501.1  | chr5: 803147316-803153602   | 6287     | 1683    | 560         | 63.46   | 6.51 | −0.189 |
| <i>AsN/AINV10</i> | Asa7G05846.1/Asa5G05408.1  | chr7: 1623050841-1623054871 | 4031     | 1203    | 400         | 45.81   | 5.76 | −0.285 |
| <i>AsN/AINV11</i> | Asa7G06162.1/Asa5G05675.1  | chr7: 1691491430-1691494767 | 3338     | 1782    | 593         | 67.39   | 8.73 | −0.378 |
| <i>AsCWINV1</i>   | Asa1G04355.1/Asa2G00250.1  | chr1: 1178706763-1178709021 | 2259     | 1608    | 535         | 60.55   | 5.86 | −0.321 |
| <i>AsCWINV2</i>   | Asa5G03190.1/Asa6G03410.1  | chr5: 820997521-821001354   | 3834     | 1707    | 568         | 64.43   | 7.72 | −0.407 |
| <i>AsCWINV3</i>   | Asa6G01167.1/Asa6G07032.1  | chr6: 308416872-308421914   | 5043     | 1641    | 546         | 62.42   | 5.72 | −0.315 |
| <i>AsCWINV4</i>   | Asa6G07063.1/Asa1G04317.1  | chr6: 1957444508-1957446535 | 2028     | 1539    | 512         | 58.27   | 8.48 | −0.505 |
| <i>AsCWINV5</i>   | Asa8G04697.1/Asa7G04854.1  | chr8: 1251531812-1251537888 | 6077     | 1569    | 522         | 58.96   | 5.86 | −0.319 |
| <i>AsCWINV6</i>   | Asa8G04714.1/Asa7G04858.1  | chr8: 1255046760-1255051854 | 5095     | 1836    | 611         | 69.34   | 6.35 | −0.391 |
| <i>AsVINV1</i>    | Asa1G00540.1/Asa0G04097.1  | chr1: 133485285-133487133   | 1849     | 1755    | 584         | 64.87   | 4.9  | −0.279 |
| <i>AsVINV2</i>    | Asa2G02355.1/Asa3G02390.1  | chr2: 650863353-650866104   | 2752     | 2217    | 738         | 81.18   | 5.16 | −0.253 |
| <i>AsVINV3</i>    | Asa3G03921.1/Asa5G01574.1  | chr3: 1070971636-1070975615 | 3980     | 1920    | 639         | 70.28   | 4.94 | −0.135 |
| <i>AsVINV4</i>    | Asa5G00414.1/Asa6G00588.1  | chr5: 101522664-101526645   | 3982     | 1884    | 627         | 69.04   | 4.93 | −0.137 |
| <i>AsVINV5</i>    | Asa6G06910.1/Asa1G04114.1  | chr6: 1914547560-1914549603 | 2044     | 1953    | 650         | 71.53   | 5.00 | −0.221 |
| <i>AsVINV6</i>    | Asa6G06912.1/Asa1G04116.1  | chr6: 1915034543-1915036592 | 2050     | 1956    | 651         | 72.4    | 4.98 | −0.197 |

**Figure 1.** Chromosomal localization and exon–intron structure of the *A. sativum* invertase genes. (a) Location of the *AsN/AINV* (black), *AsCWINV* (blue), and *AsVINV* (red) genes. Chromosome (chr) size (Mb, megabase) according to the *A. sativum* cv. Ershuizao genome [34] is indicated by the scale on the left. (b,c) Exon–intron structure of the cv. Ershuizao (b) and cv. Sarmat (c) invertase genes; (c) gene structures corrected according to our data are shown.

The *VINV* genes were located on chromosomes 1 (*AsVINV1*), 2 (*AsVINV2*), 3 (*AsVINV3*), and 5 (*AsVINV4*) as single genes and clustered in tandem on chromosome 6 (*AsVINV5/6*) (Figure 1a); the latter had the same exon–intron structure (Figure 1b).

No invertase genes were found on garlic chromosome 4 (Figure 1a).

We also searched the garlic genome for *FEH* genes and found eight *FEH*-annotated sequences (*Asa1G02142.1*, *Asa3G02847.1*, *Asa3G04718.1*, *Asa4G02029.1*, *Asa4G04915.1*, *Asa7G00583.1*, *Asa7G06871.1*, and *Asa7G07634.1*) on chromosomes 1, 3, 4, and 7; however, they were incomplete and were not transcribed, as evidenced by transcriptomic analysis. These *FEH*-like sequences started with a methionine codon, and the putative translated regions (67–197 aa; Supplementary Table S2) were homologous to the C-terminal domain of acid invertases. It can be suggested that the observed truncation of the detected *FEH*-like sequences could be a result of inaccurate genome assembly; additional experimental verification is required. The presence of incomplete *FEH*-like sequences may also indicate that in addition to the 23 invertase genes found, there are also family members that were lost during genome assembly.

Next, we validated the assembly of the invertase CDSs using the *VINV* genes as an example. The *AsVINV1*–6 CDSs of *A. sativum* cv. Sarmat were amplified and sequenced (Supplementary Table S3). Sequence alignment with the *AsVINV1*–6 transcripts from cv. Ershuizao (PRJNA607255) verified the correct assembly of *AsVINV1*, 5, 6, whereas the *AsVINV2*–4 sequences were corrected, because it was found that these genes contained 9-bp mini-exon II lost during transcript assembly, and in case of *AsVINV1*, 5, 6, this 9 bp region was a 5'-part of exon II. We also found that the *AsVINV4* cDNA included sequence gttgttgagtatgaatgcataaaagcagcggtgcag in exon V, which in the *AsVINV4* gene of cv. Ershuizao was determined as the intron between exons VI and V because of the erroneous 1 bp insertion [34]. Thus, the verified *AsVINV4* sequence contained 9 bp mini-exon II as well as exon V, which is the combination of ex-exons VI and V (Figure 1b,c).

Analysis of the *AsCWINV2* gene sequence also suggested the presence of a similar 9 bp mini-exon missed in the annotation of Sun et al. [34].

### 3.2. Putative *A. sativum* Invertase Proteins

Putative *AsN/AINV*, *AsCWINV*, and *AsVINV* proteins contained 400–621, 512–611 and 584–738 amino acids (aa), respectively; their MW and pI values are shown in Table 1. The updated characteristics of *AsVINVs* in cv. Sarmat are given in Supplementary Table S3.

GO analysis indicated that garlic neutral/alkaline and acid invertases could be involved in sucrose catabolism (GO:0005987) and carbohydrate metabolism (GO:0005975). Thus, *AsN/AINVs* could have sucrose alpha-glucosidase (GO:0004575) and glycopeptide alpha-N-acetylgalactosaminidase (GO:0033926) activities, *AsCWINVs*—hydrolase activity (GO:0004553, GO:0016798), and *AsVINVs*—beta-fructofuranosidase activity (GO:0004564) (Supplementary Table S4).

The secondary structure of *AsN/AINVs* was characterized by the presence of the Glyco\_hydro\_100 domain (pfam12899), pointing to their possible membership in the GH100 family, whereas *AsCWINVs* and *AsVINVs* contained the Glyco\_32 domain (smart00640) characteristic for the GH32 family (Supplementary Table S4).

In addition, *AsVINVs* (except *AsVINV5*) included an N-terminal DUF3357 domain (cl13304), which, according to [43], may consist of a signal peptide for vacuolar sorting and a propeptide associated with protein folding, targeting, and/or control of enzymatic activity. A transmembrane helix predicted in *AsVINV2*–6 and a signal peptide predicted in *AsVINV1* (Supplementary Table S4) may suggest a role of the N-terminal region in facilitating the transport of *AsVINVs* through the tonoplast into the vacuole.

Consistent with the sequence annotation, WoLF PSORT analysis indicated the localization of *AsN/AINVs* in the cytoplasm (*AsN/AINV2*–4, 9–11) or chloroplasts (*AsN/AINV1*, 5–8) and *AsVINV2*–6 in the vacuoles; however, *AsVINV1* was mapped to the cytoplasm (Supplementary Table S4). Unexpectedly, for *AsCWINVs*, the predicted subcellular localization was not the cell wall but the cytoplasm (*AsCWINV1*, 3–5), chloroplasts (*AsCWINV2*), and nucleus (*AsCWINV6*) (Supplementary Table S4). Such a discrepancy could be at-

tributed to possible errors in the gene/transcript assemblage or to insufficient accuracy of WoLF PSORT, whose sensitivity and specificity are around 70% for some sites (such as the nucleus, mitochondria, cytosol, plasma membrane, chloroplast, and extracellular space) but very low for the other [44]. Nevertheless, the localization of garlic invertases in various subcellular organelles suggests a diversity of their roles associated with the spatiotemporal pattern of sucrose catabolism during plant growth, development, and adaptation to the environment.

The predicted pI values for garlic invertases were consistent with the data for plant invertases. Thus, vacuolar AsVINV1–6 had acidic pI (4.9–5.16), which is in agreement with their location in the vacuoles known for acidic pH. The cell wall enzymes had basic (7.72–8.48; AsCWINV2, 4) or slightly acidic (5.86–6.35; AsCWINV1, 3, 5, 6) pI values and most neutral/alkaline invertases had slightly acidic pI (5.53–6.61), except for AsN/AINV11 (8.73) (Table 1); acidic pI has also previously been observed for neutral/alkaline invertases in other plant species [16].

The hallmarks of acid invertases include the Cys residue in the WECXDF motif near the C-terminus and the NDPNG pentapeptide near the N-terminus [43]. Our analysis indicated that all acid invertases found in garlic contained the WECV(P/I)DF motif and most of them had the N(T)DPNG(A) pentapeptide, with the exception of AsCWINV2 and AsVINV2–4, which contained only NG but not DPN (Supplementary Figure S1). In this respect, it should be noted that in the cDNAs of the *AsVINV2–4* and *AsCWINV2* genes of cv. Sarmat, the DPN motif was encoded by the 9 bp exon II, which is lost or mapped as an intronic sequence during the assembly of these genes in cv. Ershuizao [34]. This fact suggests that the absence of the DPN consensus in the cv. Ershuizao invertases could be due to errors in gene assembly.

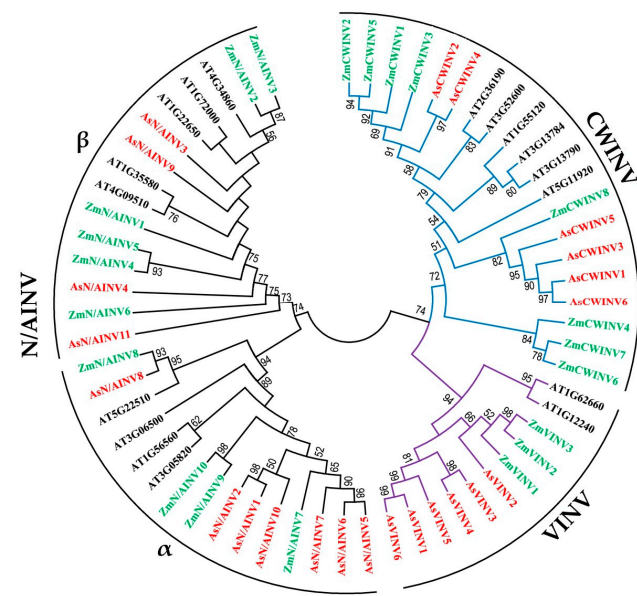
Analysis of the AsVINV and AsCWINV sequences for the analogue of Asp239, which is responsible for the functional difference between acid invertases and FEHs [33], revealed a corresponding Asp residue in all AsVINVs (Asp277, 431, 336, 336, 345, and 341 in AsVINV1–6, respectively) and in most AsCWINVs (Asp264, 246, 208, 223, and 316 in AsCWINV2–6, respectively), except AsCWINV1, which did not contain the target Asp because of the incomplete N-terminal sequence. Thus, we can conclude that all AsVINV and AsCWINV genes encode acid invertases and not FEHs.

Phylogenetic analysis confirmed the evolutionary division of garlic invertases into neutral/alkaline and acid, which in turn are branched into  $\alpha/\beta$  N/AINVs and CWINVs/VINVs, respectively (Figure 2). Comparison with the invertases of *A. thaliana* (dicot) and *Z. mays* (monocot) revealed that the *Arabidopsis* proteins mostly formed separate clusters, whereas maize invertases showed closer homology with garlic isozymes, which is consistent with the monocot nature of both plants (Figure 2). The high level of similarity between garlic invertases and their maize orthologs suggests similar functional activities.

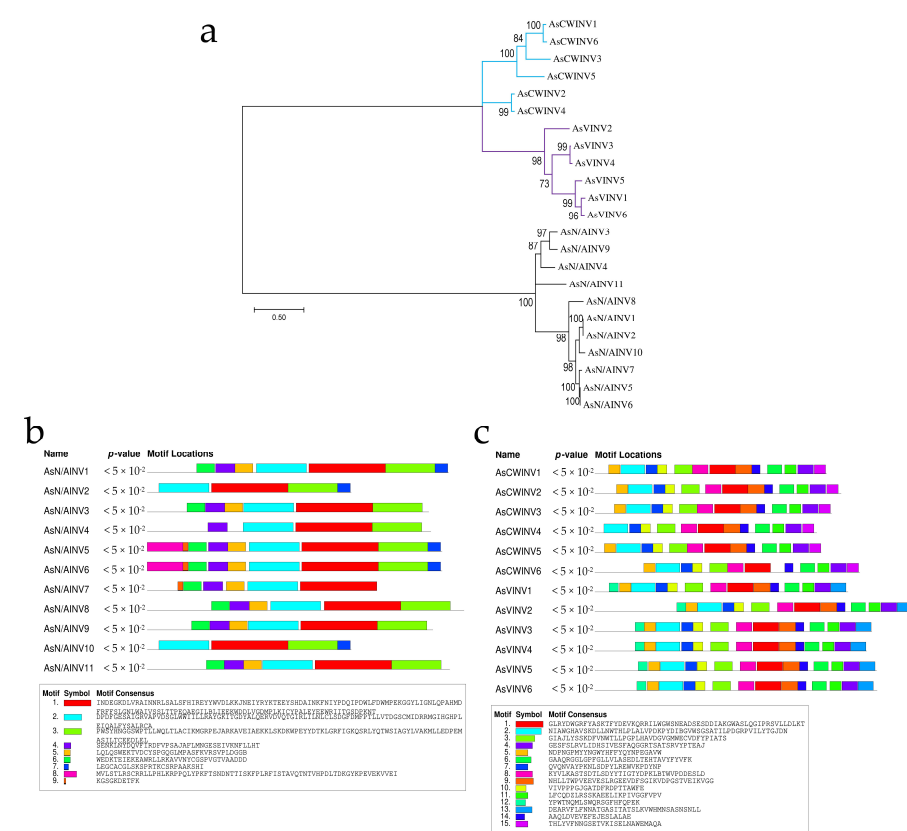
The demonstrated phylogenetic division was supported by MEME-based analysis, which revealed nine and fifteen conserved motifs in AsN/AINVs and AsVINVs/AsCWINVs, respectively (Figure 3). Thus, in AsN/AINVs, sequential motifs 6–4–5–2–1–3 constituted the Glyco\_hydro\_100 (pfam12899) domain (Figure 3b, Supplementary Table S4). The absence of some of these motifs (6–4–5 in AsN/AINV1, 10; 6 in AsN/AINV4, and 3 in AsN/AINV7) or the presence of unusual motifs (8 and/or 9 in AsN/AINV5–7) may be due to incomplete or incorrectly assembled gene sequences.

In AsVINVs/CWINVs, sequential motifs 5–2–7–10–3–8–1–9–14–6–11–4 represented the Glyco\_32 (smart00640) domain. N-terminal motif 12 (which corresponds to the DUF3357 (cl13304) domain in the AsVINVs) and C-terminal motifs 13 and 15 define the difference between AsCWINVs and AsVINVs (Figure 3c, Supplementary Table S4).

Thus, both neutral/alkaline and acid invertases of garlic are characterized by highly conserved group-specific motif arrangements, which point on the functional similarity of the enzymes within each group.



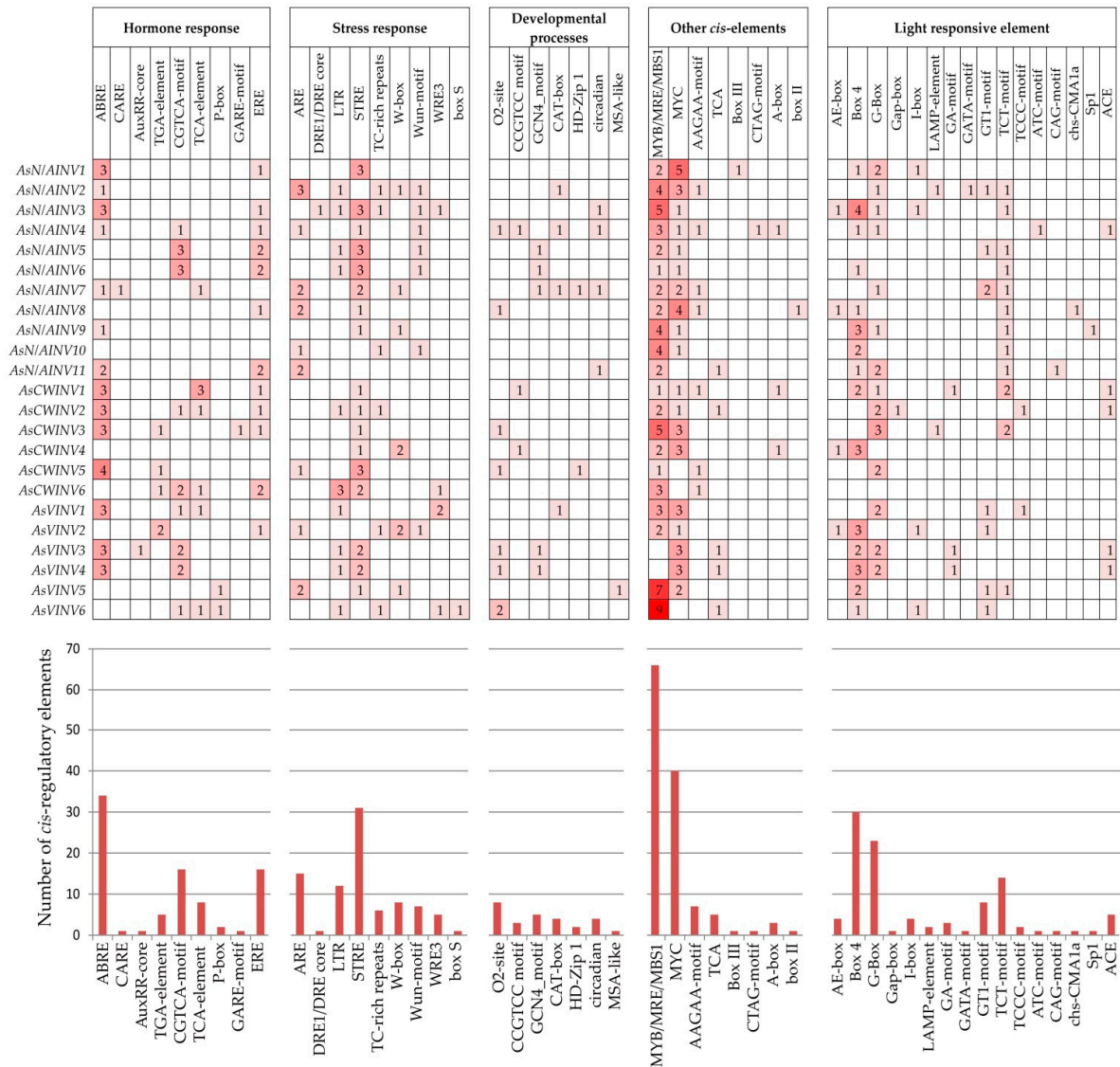
**Figure 2.** Evolutionary relationships between *A. sativum* (red), *Z. mays* (green), and *A. thaliana* (black) invertases. NCBI IDs and the corresponding protein sequences are provided in Supplementary Table S5. The unrooted dendrogram was constructed in MEGA 7.0.26 using the Neighbor-Joining method, JTT model (bootstrap with 1000 replicates).



**Figure 3.** Phylogenetic relationships of garlic invertases (a) and motif distribution in AsN/AINV (b) and AsVINV/CWINV (c) putative proteins. The dendrogram was constructed in MEGA 7.0.26 using the Neighbor-Joining method, JTT model (bootstrap with 1000 replicates). Motif consensus sequences were detected using MEME 5.5.5. The p-value ( $<0.05$ ) is the combined value based on the individual p-values (in a range  $1.0 \times 10^{-401}$ – $5.6 \times 10^{-12}$ ) for each motif found.

3.3. Analysis of Promoter Regions in the *A. sativum* Invertase Genes

Considering that transcriptional regulation of sucrose metabolism-related genes plays an important role in plant stress response, we searched for the corresponding *cis*-acting regulatory elements in the putative invertase gene promoter regions (~1 kb upstream of the start codon). As a result, we detected nine sites associated with phytohormone signaling, nine—with stress responses, sixteen—with light response, and fifteen—with developmental processes and transcription factor (TF) binding (Figure 4).



**Figure 4.** *Cis*-regulatory elements found in the *A. sativum* invertase gene promoters (~1000 bp). Analysis was performed using PlantCARE. Color intensity (pale to dark) corresponds to the number of *cis*-elements (low to high). Details about the associations of *cis*-elements are provided in Supplementary Table S6. Briefly, factors and the associated elements (in brackets) were ABA (ABRE, CARE), auxin (AuxRR-core, TGA-element), JA (CGTCA-motif, TCA-element), gibberellin (P-box, GARE-element), ethylene (ERE), anaerobic stress (ARE), drought (DRE1/DRE core), cold (LTR), heat, low pH, nutrient starvation (STRE), any stress (TC-rich repeats), wounding and pathogens (W-box, Wun-motif, WRE3, box S, AAGAA-motif), nitrogen (O2-site, GCN4-motif), meristem-specific (CCGTTC-motif) and endosperm-specific (O2-site, GCN4-motif) gene expression, regulation of cell-cycle (MSA-like) and circadian rhythm (circadian), binding to WRKY (W-box, Wun-motif, box S, WRE3), HD-Zip (HD-Zip 1), MYB (MYB/MRE/MBS1), bZIP (A-box) TFs and plant nuclear factor GT-1 (box II, box III); unknown (CTAG-motif, CAT-box, TCA).

Among the elements, the most represented were those associated with the responses to hormones ABA (ABRE, CARE), JA (CGTCA-motif), and ethylene (ERE), stresses such as heat, osmotic, low pH, nutrient starvation, and hypoxia (STRE, ARE), light (Box 4, C-Box), and binding to MYB and MYC TFs. Among all genes, only two (*AsN/AINV10* and *AsVINV4*) did not have hormone response elements and six (*AsN/AINV1*, 9, 10; *AsCWINV2*, 6, and *AsVINV2*) lacked developmental process-associated sites. Although all genes except *AsCWINV6* had light-responsive elements in the promoter, only four (*AsN/AINV3*, 4, 7, 11) had elements associated with circadian rhythms.

Rare elements such as those associated with responses to auxin (AuxRR-core or TGA-motif) or gibberellin (P-box or GARE-motif) were found in five (*AsCWINV3*, 5, 6 and *AsVINV2*, 3) and three (*AsCWINV3* and *AsVINV5*, 6) genes, respectively.

In line with the important and complex role of phytohormones in stress responses [45], invertase promoters that differed in the content of phytohormone-related sites also differed in abiotic and biotic stress-responsive elements. Besides the most represented STRE and ARE mentioned above, we found sites associated with the response to low temperature (LTR) in 10 genes (*AsN/AINV2*, 3, 5, 6, *AsCWINV2*, 6 and *AsVINV1*, 3, 4, 6), biotic stress (W-box, Wun-motif, WRE3, box S, AAGAA-motif) in 13 genes (*AsN/AINV2–10* and *AsCWINV1*, 4–6), and nitrogen (O2-site, GCN4-motif; these elements are also related to endosperm-specific gene expression) in 10 genes (*AsN/AINV4–8*, *AsCWINV3*, 5 and *AsVINV3*, 4, 6). A drought-responsive element (DRE1/DRE core) was found in one gene (*AsN/AINV3*).

Considering the paramount role of various TFs in the regulation of plant stress response [46], it was not surprising to find *cis*-elements for TF binding in the promoters of most invertase genes. Thus, sites for MYB TFs were detected in all genes except *AsVINV4*, 6, those for MYC TFs—in nineteen genes, for WRKY TFs—in fourteen, for bZIP TFs—in three, and for HD-Zip I TFs—in two. In addition, the binding sites for nuclear factor GT-1, a key enzyme for carbon fixation in higher plants [47], were found in the promoters of *AsN/AINV1*, 8.

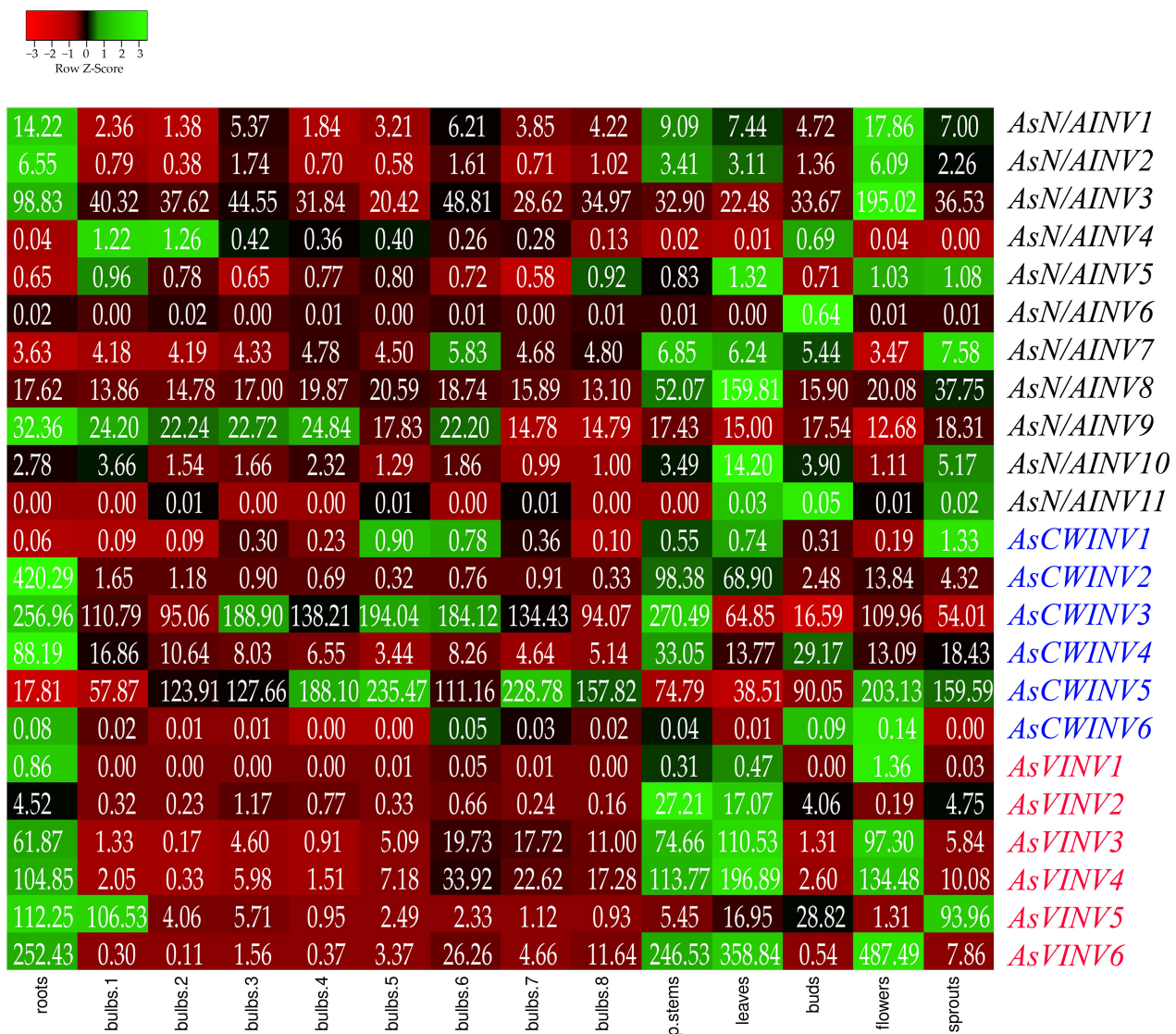
Sites associated with the specificity of gene expression to endosperm (*AsN/AINV4–8*, *AsCWINV3*, 5, and *AsVINV3*, 4, 6), meristem (*AsN/AINV4* and *AsCWINV1*, 4), or cell cycle stage (*AsVINV5*) were also detected.

Comparison of the promoters in neutral/alkaline and acid invertase genes revealed that they mostly differed in the profiles of light-sensitive elements; furthermore, there were no auxin- and gibberellin-associated sites in the *AsN/AINV* genes (Figure 4).

### 3.4. Organ-Specific Expression Pattern of the Invertase Genes in Garlic

The expression of invertase genes was analyzed based on the *A. sativum* cv. Ershuizao transcriptome (PRJNA607255). Overall, the highest transcription levels were most frequently observed in the roots, pseudostems, leaves, and flowers (up to 420, 270, 358, and 487 FPKM, respectively).

Seven genes (*AsN/AINV4–6*, 11, *AsCWINV1*, 6, and *AsVINV1*) had very weak or no expression in garlic tissues and are likely to be inactive. The remaining 16 genes showed organ-specific expression patterns; *AsN/AINV2*, 9, *AsCWINV2*, 4, and *AsVINV5* had maximal activity in the roots, *AsCWINV5*—in stage 5 bulbs, *AsN/AINV8*, 10 and *AsVINV3*, 4—in the leaves, *AsCWINV3* and *AsVINV2*—in the pseudostems, *AsN/AINV7*—in the sprouts, and *AsN/AINV1*, 3 and *AsVINV6*—in the flowers (Figure 5). Relatively high expression levels were also observed for *AsN/AINV1*, 3, 8, *AsCWINV3*, 5, and *AsVINV2–4*, 6 in the roots, *AsN/AINV3*, 7–9, *AsCWINV3*, 4, and *AsVINV3–6* in the bulbs, *AsN/AINV1*, 3, 7–9, *AsCWINV2*, 4, 5, and *AsVINV3*, 4, 6 in the pseudostems, *AsN/AINV1*, 3, 7, 9, *AsCWINV2–5*, and *AsVINV2*, 5, 6 in the leaves, *AsN/AINV1*, 3, 8–10 *AsCWINV3–5*, and *AsVINV5* in the sprouts, *AsN/AINV3*, 7–9, *AsCWINV3–5*, and *AsVINV5* in the buds, and *AsN/AINV2*, 7–9, *AsCWINV2–5*, and *AsVINV3*, 4 in the flowers (Figure 5).



**Figure 5.** Heatmap of invertase gene mRNA levels in *A. sativum* cv. Ershuizao (PRJNA607255). Roots, bulbs 1–8 (192-, 197-, 202-, 207-, 212-, 217-, 222-, and 227-day-old, respectively), leaves, pseudostems (p. stems), buds, flowers, and sprouts were analyzed. Numbers in the heatmap indicate FPKM values rounded to the second decimal place (all unrounded values are available in Supplementary Table S7).

During the development of buds into flowers, some genes were significantly upregulated (*AsN/AINV1–3*, *AsCWINV2*, 3, 5, and *AsVINV3*, 4, 6) or downregulated (*AsN/AINV4*, 6, 10, *AsCWINV4*, and *AsVINV2*, 5) (Figure 5).

During bulb growth over 36 days (stages 1–8), the strongest expression was observed for *AsCWINV3*, 5, with a tendency to increase towards middle stages and decrease towards the final stage (Figure 5).

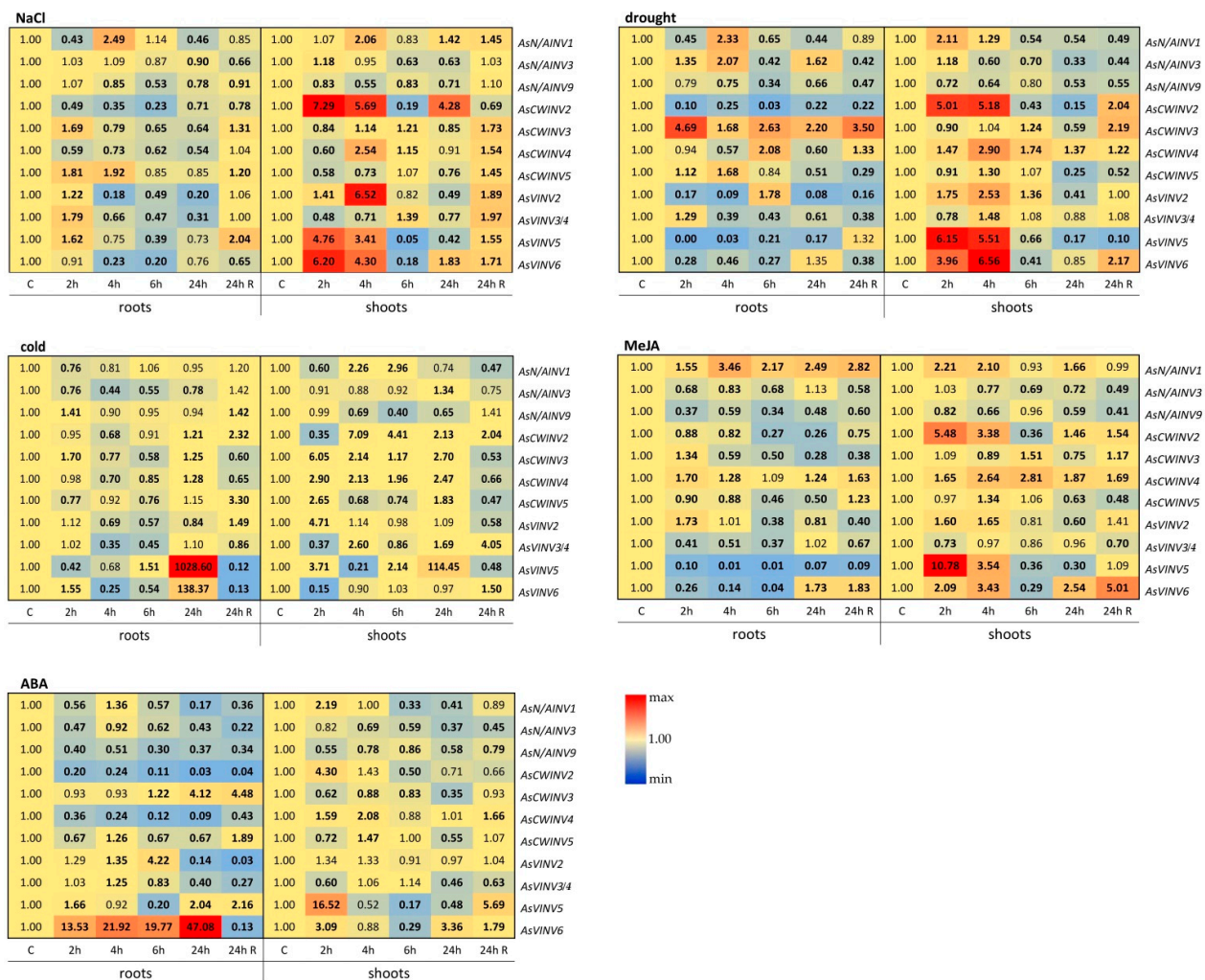
Analysis of the correlation between gene expression and the degree of structural similarity provided both positive and negative examples. Thus, genes *AsVINV3*, 4, which had a high degree of similarity in the gene structure and composition of *cis*-elements in the promoter, showed similar expression profiles and genes *AsVINV5*, 6, which had similar structures but differed in the *cis*-element content, also differed in organ-specific expression. However, genes *AsCWINV5*, 6 with similar structures and promoter compositions did not show similarity in the expression patterns (Figures 1b, 4 and 5).

### 3.5. Expression of Invertase Genes in cv. Sarmat Seedlings in Response to High Salinity, Drought, Cold, and Phytohormones

Next, we addressed transcriptional responses to abiotic stresses of 12 genes that showed distinct expression patterns in *A. sativum* cv. Ershuizao: *AsN/AINV1*, 3, 9, *AsCWINV2–5*, and *AsVINV2*, 3/4, 5, 6.

The CDSs of *AsVINV3* and 4 are highly homologous and are expressed in a very similar manner in cv. Ershuizao (Figure 5). As it was not possible to select primers specific for each of these genes, the final qRT-PCR primers (Supplementary Table S1) revealed the total expression level of *AsVINV3* and 4.

Transcriptional analysis of garlic cv. Sarmat plants after exposure to high salinity, drought, cold, and phytohormones revealed that stresses had different effects on the activity of the 12 invertase genes in the roots and shoots (Figure 6, Supplementary Figure S2).



**Figure 6.** Heatmap of time-dependent invertase gene expression in response to abiotic stresses and phytohormones. *A. sativum* cv. Sarmat seedlings were exposed to the indicated factors for 2, 4, 6, and 24 h and then returned to normal conditions for 24 h (24 h R). The number in the box is the ratio of the experimental and control qRT-PCR values, obtained by dividing the value for the stressed plant by the value for the unstressed plant. The number shows how many times the level of gene expression in a stressed plant is lower (<1) or higher (>1) than in a control (non-stressed plant) at each specific time point. The color gradient from blue to red indicates changes in the expression levels from decrease to increase, respectively, relative to control.

### 3.5.1. Response to High Salinity

Thus, in the roots, the salinity stress caused a rather suppressive effect on the expression of the analyzed genes, with the exception of an increase in the expression of *AsN/AINV1*, *AsCWINV3*, 5 and *AsVINV3/4* at the beginning of treatment. By the end of the stress period (24 h), gene expression returned to control values, remained decreased (*AsN/AINV3*, *AsVINV6*) or increased (*AsCWINV3*, 5, *AsVINV5*). In the shoots, the transcriptional response to salt stress was characterized by sharp upregulation of *AsCWINV2*, 4 and *AsVINV2*, 5, 6 at 2 and 4 h and their downregulation at 6 h. By the end of the stress period (24 h), gene expression returned to control values, or was upregulated (*AsCWINV3*–5, *AsVINV2*–5) (Figure 6, Supplementary Figure S2).

### 3.5.2. Response to Drought

During the drought stress, the strongest transcriptional response in the roots was observed for *AsN/AINV1*, *AsCWINV2*, and *AsVINV2*, 5, 6, which were sharply downregulated, and *AsN/AINV3*, *AsCWINV3*, which was sharply upregulated at 2 h. At 24 h, the mRNA level of *AsN/AINV3* and *AsCWINV3* remained high and *AsVINV6* became equal to control, whereas the other genes remained downregulated. After 24 h of post-stress treatment, most genes were downregulated, except for *AsN/AINV1* and *AsVINV5*, which were equal to control, and *AsCWINV3*, 4 which were upregulated (Figure 6, Supplementary Figure S2).

In the shoots, the transcriptional response to drought stress was noticeably different from that in the roots. Thus, after 2–4 h, the expression of *AsN/AINV1*, *AsCWINV2*, 4, and *AsVINV2*, 5, 6 increased, whereas that of *AsN/AINV9* and *AsVINV3/4* decreased. By 24 h, all genes except *AsCWINV4* and *AsVINV3*, 6 were significantly downregulated, and after 24 h in post-stress conditions, *AsN/AINV1*, 3, 9, *AsCWINV5*, and *AsVINV5* remained downregulated, *AsVINV2*, 3/4, 6 levels were recovered to the pre-stress values, and *AsCWINV2*–4 were upregulated (Figure 6, Supplementary Figure S2).

### 3.5.3. Response to Cold

The response to cold in the roots was rather weak in the first hours; gene expression mostly remained at the normal level or changed slightly, except for *AsN/AINV9* and *AsCWINV3* (upregulation). However, at the end (24 h), the *AsVINV5*, 6 genes were sharply upregulated (by ~1000 and ~138 times, respectively), but after 24 h post-stress period, both were downregulated to an almost zero level. At the same time, *AsN/AINV9*, *AsCWINV2*, 5 and *AsVINV2* were upregulated, and *AsCWINV3*, 4 and *AsVINV3/4*—downregulated (Figure 6, Supplementary Figure S2).

In the shoots, 2 h cold stress decreased the expression of *AsN/AINV1*, *AsCWINV2*, and *AsVINV3/4*, 6 and increased that of *AsCWINV3*–5 and *AsVINV2*, 5. After 4 h, the situation changed to the opposite; *AsN/AINV1*, *AsCWINV2* and *AsVINV3/4*, 6 were upregulated, whereas *AsCWINV5* and *AsVINV2*, 5 were downregulated. By the end of the stress period (24 h), the expression of most genes was increased, especially that of *AsVINV5* (by ~114 times); however, the latter was downregulated after 24 h post-stress exposure (~2 times below the control value). Overall, after 24 h in post-stress conditions, most genes were downregulated below the control level, except for *AsN/AINV9*, *AsCWINV2*, and *AsVINV3/4*, 6, which were upregulated (Figure 6, Supplementary Figure S2).

### 3.5.4. Response to Phytohormones

The response of garlic seedlings to phytohormones MeJA and ABA varied significantly.

In the roots, the expression of all genes after MeJA treatment was mostly downregulated, with few exceptions (*AsN/AINV1*, *AsCWINV4*). In contrast, in the shoots, several genes were significantly upregulated at the early points (2–4 h): *AsN/AINV1* by ~2 times, *AsCWINV2* by ~5–3 times, *AsCWINV4* by ~1.5–1.7 times, *AsVINV2* by ~1.5–2 times, *AsVINV5* by ~11–3.5 times, and *AsVINV6* by ~2–3 times. By 24 h, *AsN/AINV1*, *AsCWINV2*, 4, and *AsVINV6* were upregulated, whereas the other genes were downregulated. After 24 h

of post-stress treatment, the expression of AsCWINV2–4 and AsVINV6 was increased, that of AsN/AINV1, and AsVINV2, 5 returned to normal, and that of the remaining genes was decreased (Figure 6, Supplementary Figure S2).

In the ABA-treated roots, the transcription of AsN/AINV1, 3, 9, AsCWINV2, 4, and AsVINV3/4 was mostly decreased, whereas that of AsCWINV3 and AsVINV6 was increased. The expression of AsVINV2 was upregulated by ~4 times after 6 h stress but was significantly downregulated, similar to that of AsVINV3/4, after 24 h stress and 24 h post-stress. The AsCWINV5 mRNA level decreased at 2, 6, 24 h of stress but increased at 4 h of stress and 24 h post-stress. The most interesting response was detected in AsVINV6, which was significantly upregulated during stress (by 13–47 times) but was sharply downregulated (to an almost zero level) after stress (Figure 6, Supplementary Figure S2).

In the shoots, the most remarkable effect was observed for AsVINV5, which was upregulated (16.5-fold) at 2 h, significantly downregulated at 4–24 h, and again upregulated (~5-fold) after 24 h of post-stress treatment. No effect was found for the gene AsVINV2. The AsN/AINV1, AsCWINV2, AsVINV6 genes were upregulated after 2 h stress, and then were downregulated at 6 h; at 24 h post-stress, AsVINV6 was upregulated, and AsN/AINV1 and AsCWINV2 returned to the control level (Figure 6, Supplementary Figure S2).

Overall, these results revealed that the expression of all 12 analyzed genes was affected (sometimes in an organ-dependent manner) by tested abiotic stresses and phytohormone treatments, suggesting the role of invertases in the regulation of defense responses in garlic.

### 3.6. Sucrose, Glucose, and Fructose Content in *cv. Sarmat* Seedlings in Response to Cold, Drought, High Salinity, and Phytohormones

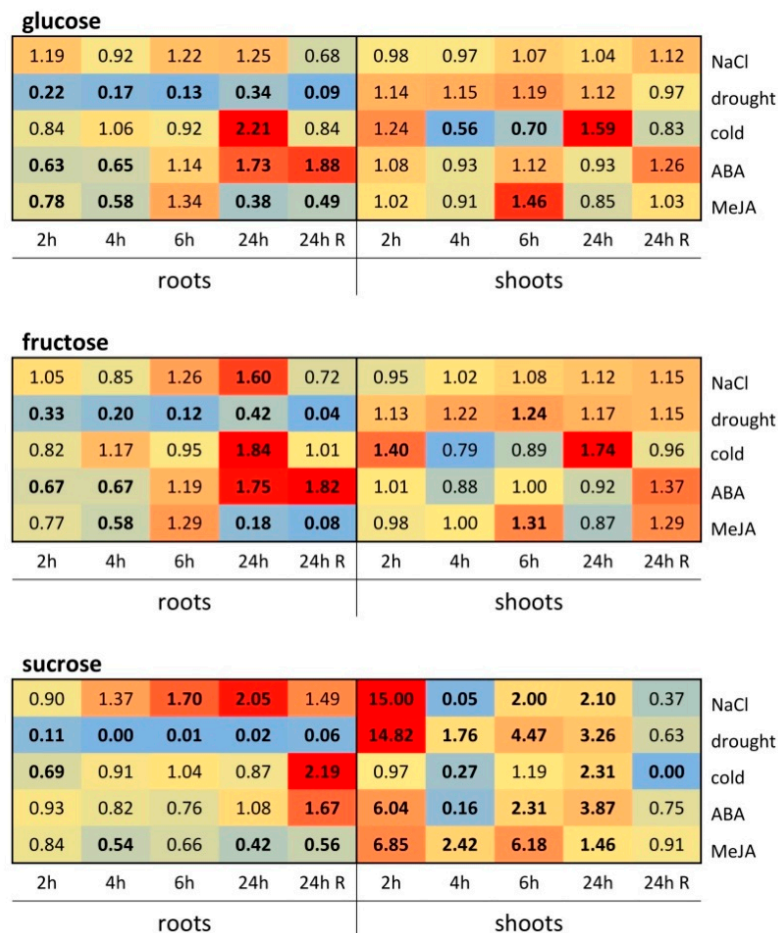
The stress-exposed and phytohormone-treated garlic seedlings were also analyzed for the content of soluble sugars: sucrose, glucose, and fructose.

The results showed that the concentration of all sugars in the roots was increased in response to salt stress (2–24 h); at 24 h post-stress, the content of monosaccharides was decreased, whereas that of sucrose was increased. In the shoots, the levels of monosaccharides slightly differed from the control, whereas that of sucrose was increased by ~15 times at 2 h and further dynamics at each point were opposite to the control (Figure 7, Supplementary Figure S3).

During and after drought exposure, the content of all sugars in the roots was decreased to trace levels, whereas in the shoots, the contents of glucose and fructose were slightly increased and that of sucrose was increased by ~15 times (2 h), remained higher than the control throughout the stress, and after stress decreased and became equal to the control (24 h post-stress) (Figure 7, Supplementary Figure S3).

In cold-affected roots, the content of monosaccharides was stable at early time points, increased by 2 times after 24 h of stress, and became equal to the control 24 h after stress period. The amount of sucrose decreased by 1.5 times after 2 h, returned to the control level at 4–24 h, and increased by 2 times at 24 h post-stress. In the shoots, the level of monosaccharides was increased at 2 h, then fluctuated in the opposite way to the control at 4–24 h, and returned to the control value 24 h after the post-stress treatment. The amount of sucrose fluctuated from control values (2 h) to decrease (4 h), increase (6–24 h), and another sharp decrease almost to zero (24 h post-stress) (Figure 7, Supplementary Figure S3).

In the ABA-treated roots, the content of all sugars showed similar dynamics; glucose and fructose levels first decreased (2–4 h) and then increased by ~1.2–1.8 times (6–24 h and 24 h post-stress) and sucrose levels also slightly decreased at the early points (2–6 h), later returned to normal (24 h), and then increased by 1.7 times (24 h post-stress). In the shoots, the content of monosaccharides remained practically unchanged during treatment and then increased by ~1.2–1.3 times at 24 h post-stress, whereas that of sucrose fluctuated in the opposite way to the control at 4–24 h, and became equal to the control value 24 h after the post-stress treatment (Figure 7, Supplementary Figure S3).

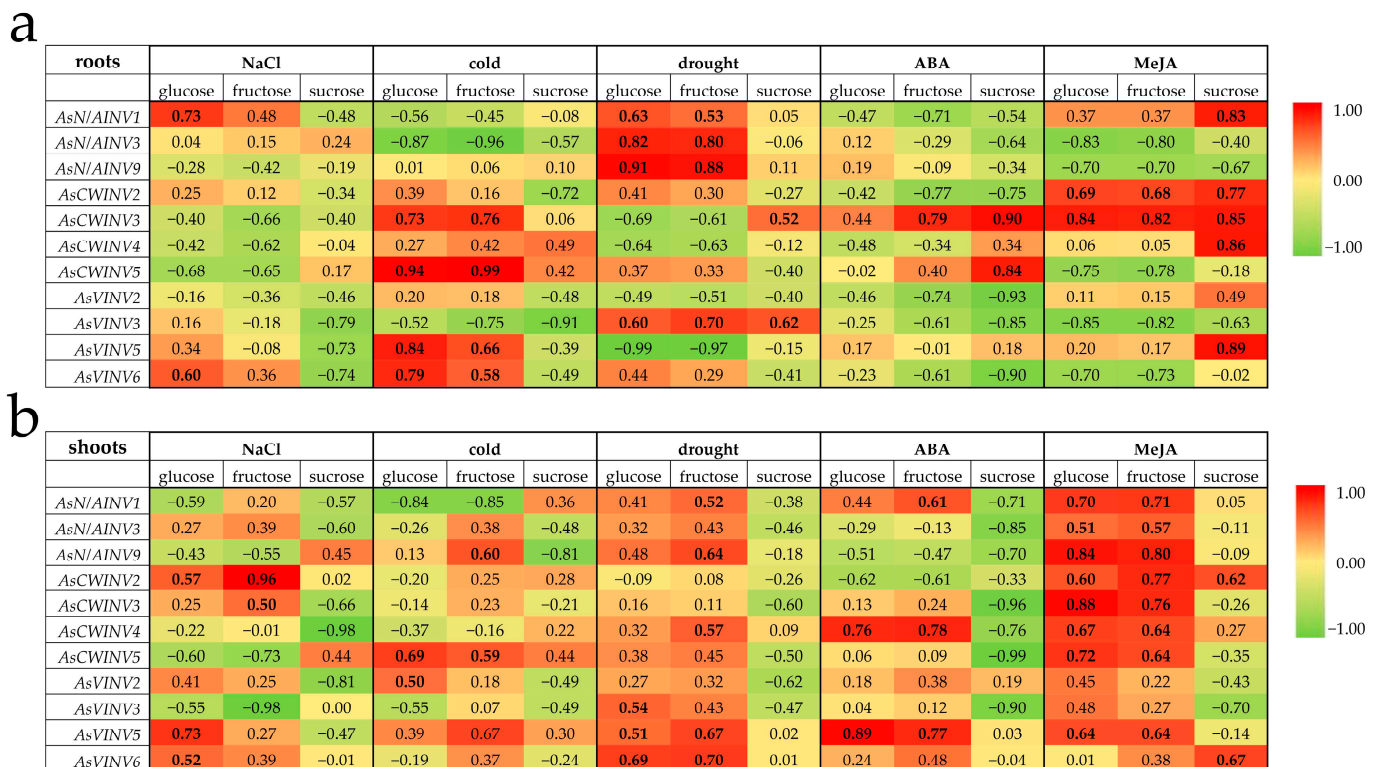


**Figure 7.** Changes in the soluble sugar content in garlic cv. Sarmat roots and shoots in response to stress (salinity, drought, and cold) and phytohormones (ABA and MeJA). The treatment was performed as described in the legend for Figure 6. The number in the box is the ratio of the experimental and control values, obtained by dividing the value for the stressed plant by the value for the unstressed plant. The number shows how many times the sugar concentration in the tissue of a stressed plant is lower (<1) or higher (>1) than in the control (non-stressed plant) at each specific time point. The color gradient from blue to red indicates changes in the sugar content from decrease to increase, respectively, relative to control.

In the MeJA-treated roots, all analyzed sugars were decreased at all time points, except for glucose and fructose, which were increased by ~1.3 times at 6 h of treatment. In the shoots, the contents of glucose and fructose practically coincided with the control, except for increasing by ~1.3 times at 6 h of treatment. Sucrose content increased by ~4–15 times at 2–6 h and became equal to the control values at the end of stress period (24 h) and at 24 h post-stress (Figure 7, Supplementary Figure S3).

Thus, the most dramatic changes in the content of all analyzed sugars were observed under drought conditions; moreover, the roots and shoots reacted in the opposite way. Cold stress practically reversed the daily fluctuations in sugar concentrations; at 24 h after stress, only the values of glucose and fructose returned to normal, while sucrose still showed a significant difference with the control (higher in roots and lower in shoots). Salt stress did not affect the content of monosaccharides, but significantly increased the sucrose level in the roots and reversed the daily fluctuations of sucrose in the shoots. When treated with phytohormones, we note that a day after the plants returned to normal conditions, the sugar content in the roots still remained significantly lower (MeJA) or higher (ABA) than the control, while in the shoots, it returned to normal (except for elevated glucose levels in case of ABA treatment).

These findings show that in garlic, various stress conditions affected soluble sugar content, which may be associated with the expression of invertase genes. Indeed, correlation analysis indicated that in the roots, glucose content was positively correlated with AsN/AINV1 and AsVINV6 expression under salinity stress, AsCWINV3, 5 and AsVINV5, 6 expression under cold stress, AsN/AINV1, 3, 9 and AsVINV3/4 expression under drought stress, and AsCWINV2, 3 expression after MeJA treatment (Figure 8a). At the same time, glucose content had negative correlation with AsCWINV5 expression under salt stress, AsN/AINV1, 3 and AsVINV3/4 expression under cold stress, AsCWINV3, 4 and AsVINV5 expression under drought stress, and AsN/AINV3, 9, AsCWINV5, and AsVINV3/4, 6 expression after MeJA treatment. Positive association was found between fructose content and AsCWINV3, 5 and AsVINV5, 6 expression under cold stress, AsN/AINV1, 3, 9 and AsVINV3/4 expression under drought stress, and AsCWINV3 and AsCWINV2, 3 expression after ABA and MeJA treatment, respectively. Fructose content was negatively correlated with AsCWINV3–5 activity under salt stress, AsN/AINV3 and AsVINV3/4 activity under cold stress, AsCWINV3, 4 and AsVINV2, 5 activity under drought stress, AsN/AINV1, AsCWINV2, and AsVINV2, 3/4, 6 activity after ABA treatment, and AsN/AINV3, 9, AsCWINV5, and AsVINV3/4, 6 activity after MeJA treatment (Figure 8a). Sucrose content showed negative correlation with the activity of AsVINV3/4, 5, 6 under salt stress, that of AsN/AINV3, AsCWINV2, and AsVINV3/4 under cold stress, that of AsN/AINV1, 3, AsCWINV2, and AsVINV2, 3/4, 6 after ABA treatment, and that of AsN/AINV9 and AsVINV3/4 after MeJA treatment. Positive correlation of sucrose content was observed with AsCWINV3 and AsVINV3/4 expression under drought stress, AsCWINV3, 5 expression after ABA treatment, and AsN/AINV1, AsCWINV2–4, and AsVINV 5 expression after MeJA treatment (Figure 8a).



**Figure 8.** Correlation between soluble sugar content and invertase gene expression in the roots (a) and shoots (b) after exposure to abiotic stresses (NaCl, drought, and cold) and phytohormones (ABA, MeJA). The number in the box is the value of the Pearson's coefficient, which represents the linear correlation between two sets of data (sugar content and invertase gene expression level) for a stressed plant. A Pearson's coefficient >0.5 represents a positive correlation, <-0.5 a negative correlation, and a range of -0.5–0.5 indicates no correlation.

In the shoots, glucose content was directly correlated with AsCWINV2 and AsVINV5, 6 activity under salt stress, AsVINV3/4, 5, 6 activity under drought stress, AsCWINV5 and AsVINV2 activity under cold stress, AsCWINV4 and AsVINV5 activity after ABA treatment, and the activity of all genes except AsVINV2, 3/4, 6 after MeJA treatment. Inverse correlation of glucose content was detected with AsN/AINV1, AsCWINV5, and AsVINV3/4 expression under salt stress, AsN/AINV1 and AsVINV3/4 expression under cold stress, and AsN/AINV9 and AsCWINV2 expression after ABA treatment. Fructose levels were positively associated with the activity of AsCWINV2, 3 under salt stress, AsN/AINV1, 9, AsCWINV4, and AsVINV5, 6 expression under drought stress, AsN/AINV9, AsCWINV5, and AsVINV5 expression under cold stress, AsN/AINV1, AsCWINV4, and AsVINV5 expression after ABA treatment, and the activity of all genes except AsVINV2, 3/4, 6 after MeJA treatment. Negative association was found between fructose levels and AsN/AINV9, AsCWINV5, and AsVINV3/4 expression under salt stress, AsN/AINV1 expression under cold stress, and AsCWINV2 expression after ABA treatment (Figure 8b). Sucrose content mostly showed negative correlation with the expression of invertase genes: AsN/AINV1, 3, AsCWINV3, 4, and AsVINV2 under salt stress, AsN/AINV9 under cold stress, AsCWINV3, 5 and AsVINV2 under drought stress, AsN/AINV1, 3, 9, AsCWINV3-5, and AsVINV3/4 after ABA treatment, and AsVINV3/4 after MeJA treatment. Positive correlation of sucrose content was found only with AsCWINV2 and AsVINV6 expression after MeJA treatment (Figure 8b).

Thus, the expression level of 12 analyzed genes was linked to varying degrees with the garlic plant response to all tested treatments. Overall, the expression level of neutral/alkaline invertase genes can be most closely associated with the response to drought (roots) and MeJA (shoots); AsCWINVs—with the response to cold (roots) and phytohormones (roots, shoots); AsVINVs—with the response to cold (roots) and drought (shoots).

#### 4. Discussion

This study provides the first comprehensive characterization of the *A. sativum* invertase genes, which are considered to be critical players in plant growth, development, and stress response [12]. By analyzing the *A. sativum* cv. Ershuizao genome, we found eleven neutral/alkaline (cytosolic) and twelve acid (six cell wall and six vacuolar) invertase-encoding genes (Table 1); the number of genes was comparable to that reported in both dicots and monocots [17]. Although the genes of acid invertases are thought to be much more extensively duplicated and more diverse than those of neutral/alkaline invertases [15], the invertase family in garlic contained almost the same numbers of the two types of invertases.

Using vacuolar invertases as an example, we confirmed the accuracy of the assembly of AsVINV1, 5, 6 CDSs and made corrections to the AsVINV2–4 gene sequences (Figure 1c, Supplementary Table S3), among which the most important was the 9 bp mini-exon found in AsVINV2–4 and also assumed to be present in AsCWINV2. Thus, four of the twelve acid invertase isogenes in garlic contain this mini-exon, which is thought to be common for acid invertases in plants. Analysis of the invertase phylogeny in view of the presence of the mini-exon suggests the emergence of isogenes, in which the 9 bp sequence is a part of other exons, through exon fusion events during evolution [16]. The 9 bp sequence encodes the functionally important DPN consensus [43], and its presence in all identified acid invertases suggests their catalytic activity.

The conserved domains and motifs found in all putative garlic invertases (Figure 3b) point to their functional conservation, including the role in plant growth, development, and stress response [12]. This notion is supported by the detection of numerous *cis*-regulatory elements in invertase gene promoters, which are associated with hormone and stress responses and binding to TFs (Figure 4). Considering the data on the motif profiles and promoter composition as well as tandem location of the genes on chromosomes, similarity of exon structure, and phylogenetic relationships (Figures 1–3), it can be suggested that garlic invertase genes AsN/AINV5-7, AsVINV3, 4, and AsVINV1, 5, 6 should encode three groups of proteins with most similar functional activity and redundant roles within each.

The influence of invertases on plant growth may vary depending on the organ/tissue. Analysis of the expression patterns of the garlic invertase genes based on the transcriptome data [34] (Figure 5) revealed genes with predominant transcription in the roots (*AsN/AINV1*, 3, 8, 9, *AsCWINV2–4*, and *AsVINV3–6*), bulbs (*AsN/AINV3*, 8, 9, *AsCWINV3*, 5, and *AsVINV3–6*), pseudostems (*AsN/AINV3*, 8, 9, *AsCWINV2–5*, and *AsVINV2–4*, 6), leaves (*AsN/AINV3*, 8–10, *AsCWINV2*, and *AsVINV3*, 4, 6), sprouts (*AsN/AINV3*, 9, *AsCWINV5*, and *AsVINV5*), and reproductive organs (*AsN/AINV1*, 3, 9, *AsCWINV3–5*, and *AsVINV3*, 4, 6). These results are consistent with previous studies, indicating the requirement of both neutral/alkaline and acid invertases for vegetative growth and reproductive development [12,16,47]. It should be noted that seven of the twenty-three invertase genes identified in garlic had very weak or no expression in any organs (Figure 5), suggesting their pseudogenic nature or unknown functions, which could be associated with certain processes, time periods, or responses to exotic stress types.

It is known that invertases may play a role in plant adaptation to unfavorable conditions through changes in their activity, including transcription, which is sensitive to the effects of various abiotic and biotic factors [14]. Therefore, we analyzed the expression of 12 genes encoding neutral/alkaline, cell wall, and vacuolar invertases, which were selected based on their high mRNA levels revealed in silico (Figure 5), and tested their expression in the roots and shoots of garlic plants exposed to cold, drought, high salinity, and phytohormones. All 12 genes were found to be differentially expressed under stresses, and the responses differed depending on the organ and stress factor (Figure 6, Supplementary Figure S2), which is in agreement with previous observations that in unfavorable conditions, individual invertase genes are transcribed in an organ- and developmental stage-specific manner [12]. These results indicate that neutral/alkaline, cell wall, and vacuolar isoenzymes could jointly regulate sugar metabolism in garlic, protecting the plant from adverse environmental effects.

Since sugars play a key role in the perception and transmission of stress signals, osmotic adaptation, and neutralization of reactive oxygen species [47–49], the activity of invertases is critical for plant survival under stress. Studies in citrus, kiwi, wheat, and other plants indicate that the concentration of soluble sugars (sucrose, fructose, and glucose) depends on the expression and function of vacuolar invertases [48,50]. Protection from stress-related effects is facilitated by changes in the content of hexoses through hydrolysis of sucrose by all invertases and fructans by acid invertases and FEHs [14,15]. Consistent with these data, transcriptomic analyses indicate that the adaptive response of garlic under salt stress includes regulation of sucrose metabolism [27,28] and that fructans, which along with sucrose are important storage sugars, can stimulate garlic resistance to low temperatures [26] and drought [29].

In our study, we found that the expression of the 12 analyzed garlic invertase genes had statistically significant correlation with the content of glucose, fructose, and/or sucrose (Figure 8), which should affect the synthesis of fructans and, consequently, stress resistance of garlic. In this scenario, sucrose could be hydrolyzed by all invertases and fructans—by acid invertases, since we did not find full-length *FEH* genes or their transcripts in the genome and transcriptome of *A. sativum* [34].

## 5. Conclusions

We identified and characterized twenty-three invertase genes in *A. sativum* cv. Er-shuizao; among them, eleven encoded alkaline/neutral (cytosolic) and twelve—acid (six vacuolar and six cell wall) invertases. The coding sequences of the six vacuolar invertase genes were confirmed or corrected through alignment with the sequences amplified from cDNA of cv. Sarmat. Transcriptomic analysis revealed that 16 invertase genes were expressed at considerable levels in garlic organs. Expression profiling of garlic shoots and roots exposed to abiotic stresses (cold, drought, salinity) and hormones (ABA, MeJA) showed that 12 genes had distinct expression patterns depending on the organ and stress factor, which were significantly correlated with the content of soluble sugars. Our results

indicate that garlic invertases may be involved in plant defense by maintaining the required balance of soluble sugars. The identification and characterization of the invertase isogenes in *A. sativum* provides the basis for their further functional analysis and may contribute to the breeding of stress-tolerant garlic cultivars.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae10060581/s1>, Table S1: List of primers for *Allium sativum* invertase gene amplification, sequencing, and expression analysis; Table S2: Sequences of putative FEH proteins based on *A. sativum* cv. Ershuizao genome data; Table S3: Characteristics of vacuolar invertases in cv. Sarmat; Table S4: Predicted characteristics of *A. sativum* invertases; Table S5: Invertase amino acid sequences used for structural and phylogenetic analysis; Table S6: *Cis*-regulatory elements in the promoters of *A. sativum* cv. Ershuizao invertase genes; Table S7: FPKM values for invertase gene expression in *A. sativum* cv. Ershuizao; Figure S1: Alignment of the AsN/AINV1–11 (a) and AsCWINV1–6/AsVINV1–6 (b) proteins; Figure S2: Time-dependent invertase gene expression in response to abiotic stresses. *A. sativum* cv. Sarmat seedlings were exposed to the indicated factors for 2, 4, 6, and 24 h and then returned to normal conditions for 24 h (24 h R); Figure S3: Changes in the soluble sugar content in garlic cv. Sarmat roots and shoots in response to stress (salinity, drought, and cold) and phytohormones (ABA and MeJA).

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