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Genome-Wide Characterization of Glutamine Synthetase Family Genes in *Cucurbitaceae* and Their Potential Roles in Cold Response and Rootstock-Scion Signaling Communication

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Abstract: Glutamine synthetase (GS; EC 6.3.1.2, L-glutamate: ammonia ligase ADP-forming) is the key enzyme responsible for the primary assimilation and reassimilation of nitrogen (N) in higher plants. There are two main isoforms of GS in higher plants, classified as cytosolic GS (GS1) and chloroplastic GS (GS2) by their size and subcellular localization. In order to improve the stress tolerance, quality, and yield of cucurbit crops such as cucumbers (Csa, *Cucumis sativus* L.), pumpkins (Cmo, *Cucurbita moschata* var. Rifu) are often used as rootstocks. Here, the GS family of the two species were comprehensively analyzed using bioinformatics in terms of aspects of the phylogenic tree, gene structure, chromosome location, subcellular localization, and evolutionary and expression patterns. Seven and four *GS* gene family members were screened in pumpkin and cucumber, respectively. *GS* family genes were divided into three groups (one for *GS2* and two for *GS1*) according to their homology and phylogenetic relationships with other species. The analysis of gene ontology annotation of *GS* family genes, promoter regulatory elements, and tissue-specific expression patterns indicates the potential different biological roles of GS isoforms in *Cucurbitaceae*. In particular, we have identified a potentially available gene (*GS*1: CmoCh08G004920) from pumpkin that is relatively highly expressed and tissue-specifically expressed. RT-PCR analysis showed that most CmoGSs are induced by low temperature, and long-term (day 2 to day 9) cold stress has a more obvious effect on the RNA abundance of CmoGS. Our work presents the structure and expression patterns of all candidate members of the pumpkin and cucumber *GS* gene family, and to the best of our knowledge, this is the first time such work has been presented. It is worth focusing on the candidate genes with strong capacity for improving pumpkin rootstock breeding in order to increase nitrogen-use efficiency in cold conditions, as well as rootstock-scion communication.

Keywords: genome-wide; glutamine synthetase; *Cucurbita moschata*; expression profile; phylogeny; rootstock breeding

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1. Introduction

Glutamine synthetase (GS; EC 6.3.1.2, L-glutamate: ammonia ligase ADP-forming) is the key enzyme responsible for primary nitrogen (N) assimilation in higher plants [1,2]. Glutamine synthetase catalyzes the ATP-dependent addition of ammonium (NH_4^+) to the γ-carboxyl group of glutamate to produce glutamine and takes part in the GS–GOGAT cycle, which serves as the cornerstone of N metabolism [3]. The *GS* gene family has been studied in certain plants, including *Arabidopsis* [4], maize (*Zea mays*) [5,6], and Populus

(*Populus trichocarpa*) [7]. However, the entire *GS* gene family has not been identified in any of the species of *Cucurbitaceae*. The sources of ammonium assimilated by GS include the fixation of atmospheric N, direct nitrate or ammonia uptake from the soil, photorespiration, phenylalanine-ammonia lyase-catalyzed phenylalanine deamination, and the release of ammonium during storage via protein mobilization and plant senescence. Hence, in the context of nitrogen assimilation, *GS* is considered a candidate gene for transgenic approaches to increasing nitrogen-use efficiency (NUE). GS also responds to various abiotic stresses, including salt, cold, and drought, which have adverse effects on crop production [3].

Oligomorphic isozymes composed of *GS* polypeptides encoded by multiple nuclear genes are located in the cytoplasm or chloroplasts and are expressed in the nonphotosynthetic and photosynthetic tissues of higher plants [8]. Researchers have reported that the decametric structure of the plant GS holoenzyme consists of two face-to-face cyclic pentamer subunits [9,10]. In vascular plants, there are two main isoforms of GS, classified as cytosolic GS (GS*1*) and chloroplastic GS (GS*2*) according to their size and subcellular localization [11]. The genomic analysis of multiple angiosperm species showed that *GS1* genes belonged to a small, multigene family [1], whereas *GS2* was encoded by one to two genes. The cytosolic GS1 isoform assimilates ammonium from the soil, and the remobilization of ammonia is released via protein degradation in senescing leaves, whereas the larger chloroplast-localized GS2 isoform is responsible for the reassimilation of ammonium released during photorespiration and nitrate reduction in plastids [12,13]. The different expression patterns of these genes regulate glutamine production both spatially and temporally. For example, in rice (*Oryza sativa*), there are three genes coding for cytosolic *GS1* (*OsGS1.1*, *OsGS1.2,* and *OsGS1.3*) and one gene coding for the plastidic *GS2* (*OsGS2*). *OsGS1.1* exists globally but is expressed more in the shoots, while *OsGS1.2* is expressed mostly in the root. *OsGS1.3* is almost undetectable except for the spikelets, and *OsGS2* is abundant in the leaves [14].

The differences between GS isoforms are increasingly being studied. The main cause of ammonium toxicity in *Arabidopsis* was found to be ammonium assimilation of GS2 rather than ammonium accumulation [15]. Isotopic-tracing experiments and genetic evidence indicated that three of the five GS1s work together to remobilize nitrogen and fill seeds in *Arabidopsis* [16]. The different location, gene expression and function of each isoform of GS is well proven in wheat grain (*Triticum aestivum* L.) [17]. In *Cucurbitaceae* crops, it has been reported that GS responded to root-zone low temperature [18], low nitrogen stress [19], and intercropping allelopathy [20]. However, the detailed type, number and mechanism of functional global GS isoforms of *Cucurbitaceae* are still unclear.

There are several economically important species in the *Cucurbitaceae* family [21], such as cucumber, melon, and watermelon. Pumpkins are often used as rootstocks or as scions to afford these *Cucurbitaceae* plants higher stress tolerance, better quality, and higher yield [22–24]. Except for nitrogen sources, NH^{4+} is cytotoxic [25]. Cucumber, as a species of the *Cucurbitaceae* family, is highly influenced by NH^{4+} compared with other plants belonging to the family. Previous studies showed that grafting cucumber (*Cucumis sativus* L./*Cucurbita moschata*), compared with the control (*Cucumis sativus* L./*Cucumis sativus* L.), was less toxic alone, with a decrease in GS. In this study, we identified seven *CmoGS* and four *CsaGS* (Cucumber Glutamine Synthetase) genes through a genome-wide analysis of pumpkin and cucumber with reference to studies of other five species. We predicted the structure, subcellular localization, phylogeny, and function of *GS* family genes using bioinformatics methods to provide new insights into glutamine metabolism in pumpkin.

2. Materials and Methods

2.1. Screening of GS Family Genes in Pumpkin and Cucumber

By setting up a local database, the genome of *Cucurbita moschata* var. Rifu and *Cucumis sativus* L. including all information (cds, pep, DNA, and gff3) was obtained from CuGenDB

(http://cucurbitgenomics.org/) (accessed on 20 May 2021). Gln-synt_N.hmm (PF03951) and Gln-synt_C.hmm (PF00120) were downloaded from Pfam (http://pfam.xfam.org/) (accessed on 20 May 2021) [26]. The pep file was screened for GS proteins on the basis of Gln-synt_N.hmm and Gln-synt_C.hmm using HMMER3.1 software (National Institutes of Health, Bethesda, Maryland, USA, grant number R01HG009116) (E-value $< 1 \times 10^{-5}$). Proteins lacking a complete GLn-synt_N domain or Gln-synt_C domain can be identified by manually checking all GS protein sequences with annotations from the SMART (http://smart.embl.de/) (accessed on 5 May 2021) and Pfam databases [27]. We identified the GS proteins with the program of ExPASy (http://web.expasy.org/protparam/) (accessed on 5 May 2021).

2.2. Phylogenetic Analysis of GS Proteins in Pumpkin and Cucumber

First, to perform multiple-sequence alignment analysis, the full-length GS protein sequences from some representative species, including *Populus trichocarpa*, *Oryza sativa*, *Vitis vinifera*, *Arabidopsis thaliana*, and *Zea mays* were analyzed using MEGA-X software (Center of Evolutionary Functional Genomics Biodesign Institute, Arizona State University, Tempe, AZ, USA) [28]. The sequences of *GS* family genes of *Arabidopsis* and poplar were obtained through TAIR (http://www.arabidopsis.org/) (accessed on 5 May 2021) and based on previous reports [29], respectively. The sequences of *GS* family genes from other species were achieved from NCBI (https://www.ncbi.nlm.nih.gov/) (accessed on 5 May 2021). Then, the reconstructed phylogenetic evolutionary tree was divided into three groups according to the phylogenetic relationships.

2.3. Structure and 5′-Upstream Regions Regulatory-Elements Analysis of GS Family Genes in Pumpkin and Cucumber

The distributions of different regions of *GS* family genes on pumpkin and cucumber chromosomes were drawn from the gff3 file. The potential regulatory elements in the 2000 bp 5′-upstream regions of *GS* genes were identified by TBtools [30] with the default parameters. The information extraction and preliminary drawing of the regulatory-element analysis were also completed using TBtools.

2.4. Evolution Analysis of GS Family Genes in Pumpkin and Cucumber

MCScanX software (Plant Genome Mapping Laboratory, University of Georgia, Athens, GA, USA) [28] was used to analyze the segmental duplications so as to replenish BLAST results. The collinearity of *CmoGS* and *CsaGS* family genes were also visualized using TBtools.

2.5. Gene Ontology Enrichment

GO enrichment analysis was performed using OmicShare tools (Gene Denovo, Guangzhou, China).

2.6. Relative Gene Expression with mRNA Abundance of GS Family Genes

To identify the relative gene expression levels of *GS* family genes, the published transcriptomic data (PRJNA385310 of pumpkin, PRJNA312872 of cucumber, and RNA-seq data in CuGenDB) [31] was downloaded. The data of four tissues, including roots, stems, leaves, and fruits harvested 46 days after pollination in *Cucurbita moschata* var. Rifu, and four tissues, including roots, stems, young leaves, and flesh-3weak-fruits in 12-week-old cucumber (*Cucumis sativus*), were utilized in our study. The parameter of $\log_2{}^{(RPKM)}$ was used to represent the expression level of each gene.

2.7. Semi-Quantitative RT-PCR Assays with Cold-Treated Pumpkin Seedlings

Pumpkin (*Cucurbita moschata* var. Rifu *'Qianglishi'*) was used in this study. About 100 seeds were put into sterile water at 55 °C for 10 min and 25 °C for 4 h, then germinated in a dark room at 28 °C for 24 h. The germinated seeds were sown in 50-hole seedling

trays with a substrate (peat: vermiculite: perlite, volume ratio 2:1:1) in a light chamber (relative humidity: 70%, 28 °C/18 °C day/night; 16 h/8 h light/dark; light intensity 190–600 μmol m^{-2} s^{-1}). Pumpkin seedlings with second true leaf were placed at 4 °C in the previous chamber (relative humidity: 70%; 4° C/4 °C day/night; 16 h/8 h light/dark; light intensity: 190–600 μmol m^{-2} s^{-1}). Samples were taken from roots and leaves at 0 h, 6 h, 12 h, 24 h, 2 d, 3 d, 6 d, and 9 d after the cold treatment. Each replicate includes 3–4 independent plants, and three independent biological replicates were performed.

Total RNA was isolated from different pumpkin tissues using RNA plant Plus Reagent (Huayueyang Biotech, Co., Beijing, China) following manufacturer's instructions. A total of 2 μL of each RNA sample was quantified using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized by using PrimeScriptTM RT reagent kit (Perfect Real Time, Takara Biomedical Technology Co., Ltd., Beijing, China). The internal control PCR was performed with 30 cycles by amplifying ACT7. Semi RT-PCR was conducted with the reaction system (2 × T5 Super PCR Mix (TSE005, TSING KE Co., Beijing, China), 1.5 μM Primer and 2 ng cDNA) in 35 cycles using the primers listed in Supplementary Table S1.

3. Results

3.1. Genome-Wide Characterization of GS Family Genes in Pumpkins and Cucumbers

Seven and four genes encoding GS protein domains were identified by screening the pumpkin and cucumber genome databases, respectively. We analyzed amino-acid length, protein molecular weight, theoretical isoelectric point (PI), grand average of hydropathicity (GRAVY), instability index, predicted subcellular localization, and amino-acid composition of each gene. The predicted *GS* family genes of pumpkin and cucumber were distributed on different chromosomes. Additionally, the GRAVY values of the GS proteins were uniformly negative, indicating that these proteins may be hydrophilic. The difference between pumpkin and cucumber was that there was only one chloroplast-localized *GS2* in cucumber but two in pumpkin. Furthermore, there were three and five cytosol-localized *GS1s* in cucumber and pumpkin, respectively. The longest (*CmoCh06G014450,* 479aa) and shortest (*CmoCh01G003900,* 340aa) of *GS* can be found in pumpkin, while the estimated length of all three *GS1s* of cucumber was 356 amino acids (Supplementary Table S2).

3.2. Evolutionary Relationships of GS Family Genes

To find the phylogenetics and taxonomy of *CmoGS* and *CsaGS* members, a phylogenetic tree was reconstructed based on the alignments of 39 amino-acid sequences, including seven from pumpkins (*Cucurbita moschata* var. Rifu), four from cucumbers (*Cucumis sativus* L.), five from grapes (*Vitis vinifera*), four from rice (*Oryza sativa*), seven from poplar (*Populus trichocarpa*), six from maize (*Zea mays*), and six from *Arabidopsis thaliana* (Supplementary Table S3). According to the phylogenetic relationships, Cucurbitaceae *GS*s were distributed in three groups, called Groups 1–3. *Cucurbitaceae GS1s* were distributed in Group 1 and Group 3, while Group 2 contained only *GS2s.* According to the homology of pumpkin and cucumber *GS*, the *Cucurbitaceae GSs* can be divided into four subfamilies (Groups 1–4). Each subfamily contains one *CsaGS* and one or two *CmoGSs* (Figure 1). The six *CmoGSs*, in addition to *CmoCh15G007570*, occurred in pairs, which may be due to chromosome-doubling events in pumpkin's evolutionary history and a subsequent period of allotetraploidy [15]. It can be understood that *Cucurbitaceae GS1s* have a closer phylogenetic relationship with the *GS1s* of *Arabidopsis*, poplar, and grape than the other two monocotyledonous plants (Figure 1). Interestingly, Group 1 and Group 3 contained only *GSs* of dicotyledonous plants, and *GSs* of dicotyledonous plants were more closely related in other clades (e.g., yellow boxes).

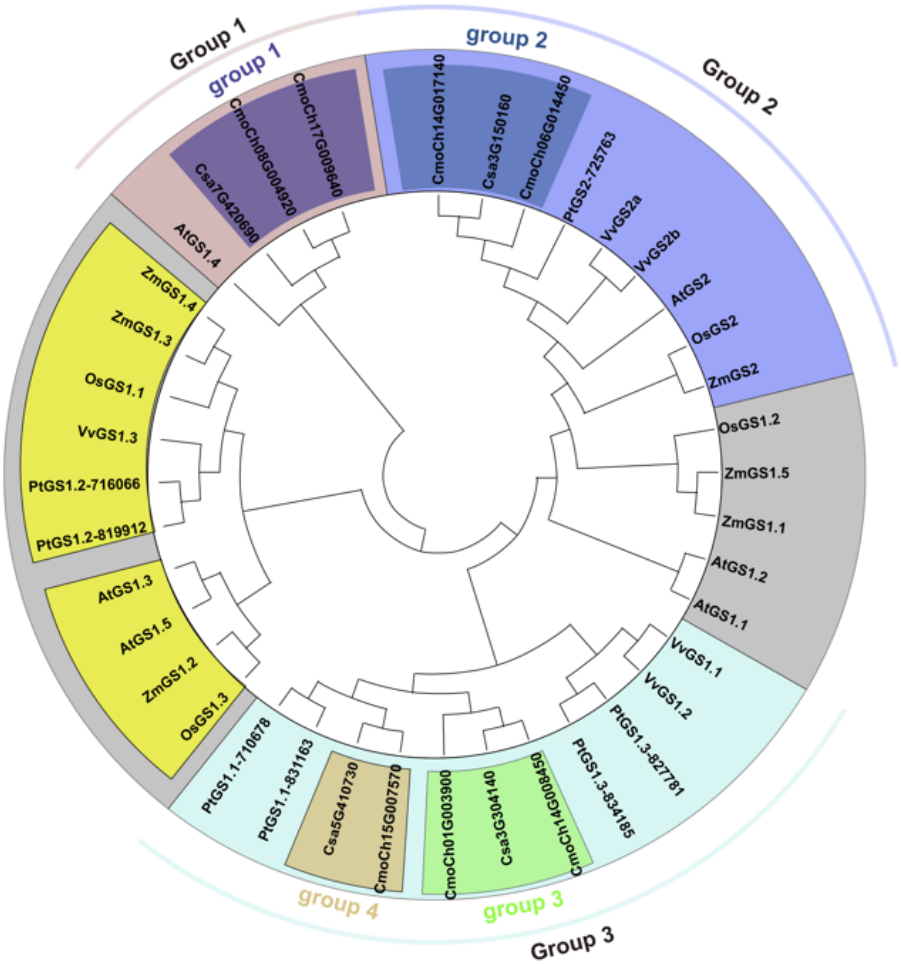

Figure 1. Phylogenetic tree of glutamine synthetase (*GS*) family genes in pumpkin, cucumber, grape, rice, poplar, maize, and *Arabidopsis*. The phylogenetic tree was constructed by the neighbor-joining method, with 1000 bootstrap repeats. The three classifications and four subfamilies are represented by different colors. Pt: *Populus trichocarpa*. Os: *Oryza sativa*. Vv: *Vitis vinifera*. At: *Arabidopsis thaliana*. Zm: *Zea mays*.

3.3. Structural Analysis of Cucurbitacea GS Family Genes

Based on structural analysis, we found that there are 11–13 introns in these pumpkin and cucumber *GS* family genes. Considering their short protein length (340–479 amino acids), the exons of these genes are fully divided by introns (Figure 2). For the *GS* subfamily with two *CmoGSs*, the structure of *CsaGS* in this subfamily is more similar to that of the *CmoGS*, which may imply the evolution sequence of *CmoGS* replications in one subfamily. In particular, the intron length of *CmoCh08G004920* is the longest. Compared to genes on other branches, *CmoCh14G008450* and *CmoCh15G007570* have a similar intron–exon structure, indicating that these sequences may have experienced a replication event.

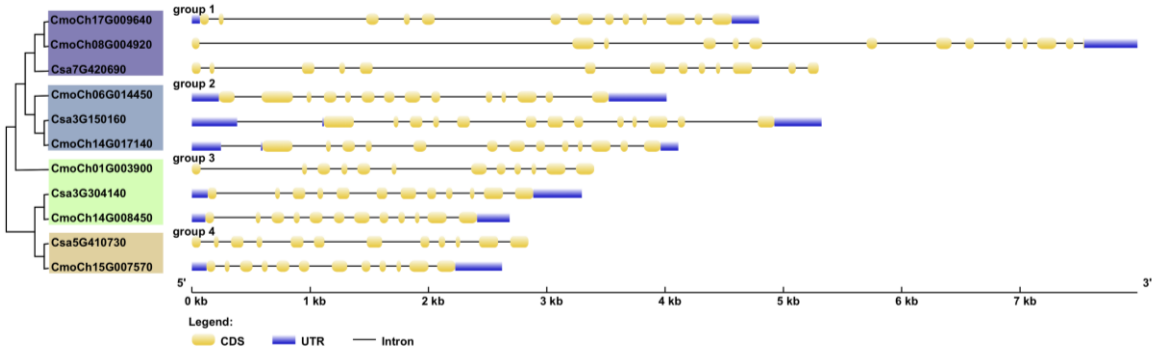

Figure 2. Structural analysis of *CmoGS* and *CsaGS* members. *GS*s in different subfamilies are indicated by different colors. The lower number axis shows the number of deoxynucleotides.

3.4. Regulatory Elements in Cucurbitacea GS Family Genes

We investigated the regulatory elements in the 5′-upstream regions for the purpose of gaining insight into the function of *GS* family genes in pumpkins and cucumbers. In the 5′-upstream regions of *GS* family genes, the putative regulatory elements are abundant and not conserved among these genes. Even if the genes are of the same branch, their putative regulatory elements differ markedly in number and type (Figure 3, Supplementary Table S4). For example, 15, 33, and 13 regulatory elements were identified in the two putative *CmoGS2*s and one *CsaGS2*, of which the auxin-responsiveness element is only present in *CmoCh06G014450*. Phytohormone regulatory elements (abscisicacid responsiveness, auxin responsiveness, gibberellin responsiveness, MeJA responsiveness, and salicylicacid responsiveness) are located unevenly in the upstream regions of all of *Cucurbitacea GS* family genes. There seem to be fewer phytohormone regulatory elements in the 5′-upstream regions of cucumber (20, with an average of 5.00 for every *CsaGS*) than in the 5′-upstream regions of pumpkin (55, with an average of 7.86 for every *CmoGS*). Certain regulatory elements (e.g., low-temperature responsiveness) only exist in the regulatory regions of one to two *GS* genes, indicating that some genes are stimulated by specific signals but not others. Putative regulatory elements involved in light responsiveness and abscisic acid responsiveness were identified in all *GS* family genes. None of the regulatory elements in our study are unique to a subfamily.

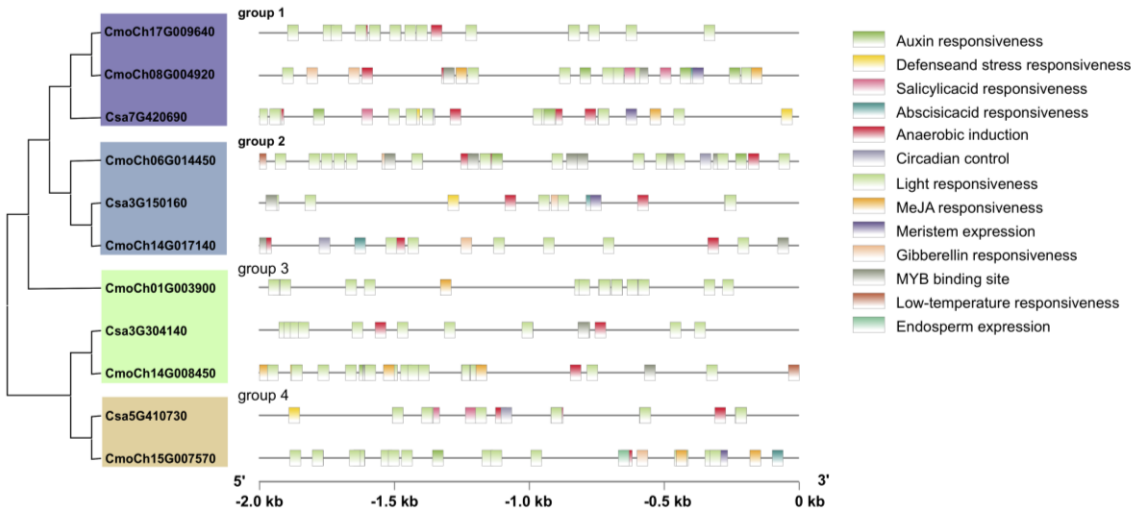

Figure 3. The regulatory regions of *CmoGS* and *CsaGS* gene members. This shows an area of 2000 bp upstream of ATG. ATG is at the position of "0" in the figure. GS gene members in different subfamilies are indicated by different colors on the left. The different color blocks on the right represent different regulatory elements and correspond to the color blocks on the line.

3.5. Distribution and Duplication of GS Family Genes in Pumpkin and Cucumber

Cucurbitaceae GS genes are not located on scaffolds or unanchored contigs and exhibit uneven distribution on the chromosomes (Figure 4). Two *CmoGS* genes are located on Cmo_Chr14, while the others are located on Cmo_Chr1, Cmo_Chr6, Cmo_Chr8, Cmo_Chr15, and Cmo_Chr17. Furthermore, two *CsaGS* genes are located on Csa_Chr3, while the others are located on Csa_Chr5 and Csa_Chr7. Each *CmoGS* contains one collinear gene in cucumber, with the same as *CsaGS* in pumpkin. All *CsaGS* genes, except for Csa5G410730, contain a pair of collinear *CmoGS* genes, which are located in two chromosomes with high homology.

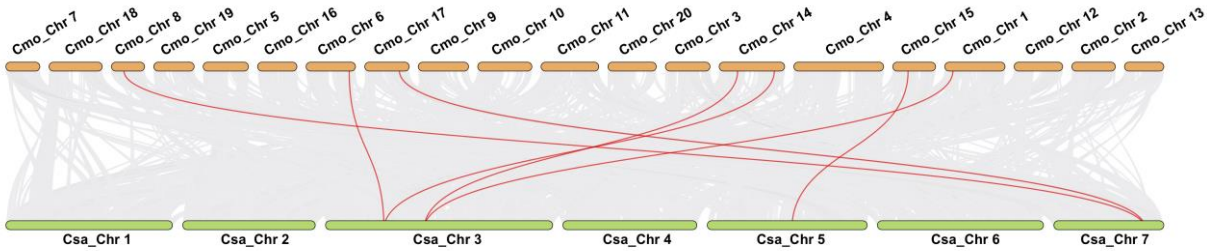

Figure 4. Chromosome localization of duplicated *CmoGS* and *CsaGS* gene members in pumpkin and cucumber. The red lines represent the colinear genes.

3.6. Functional Annotations of GS Gene Members in Pumpkin and Cucumber

To explore the relevant functions of *GS*, the enrichment of the GO (gene ontology) terms was analyzed. According to the GO enrichment analysis, the seven *CmoGS*s and four *CsaGSs* were classified into three ontological categories: biological process, cellular component, and molecular function, with 14 functional terms (Figure 5, Supplementary Table S5). We predicted that the *GS* family genes of pumpkin and cucumber were involved in many plant, biological, and physiological processes, particularly that all genes were involved in the process of glutamine biosynthesis, cell-wall macromolecule biosynthesis, organonitrogen-compound biosynthesis, glutamate–ammonia ligase activity, and ATP binding.

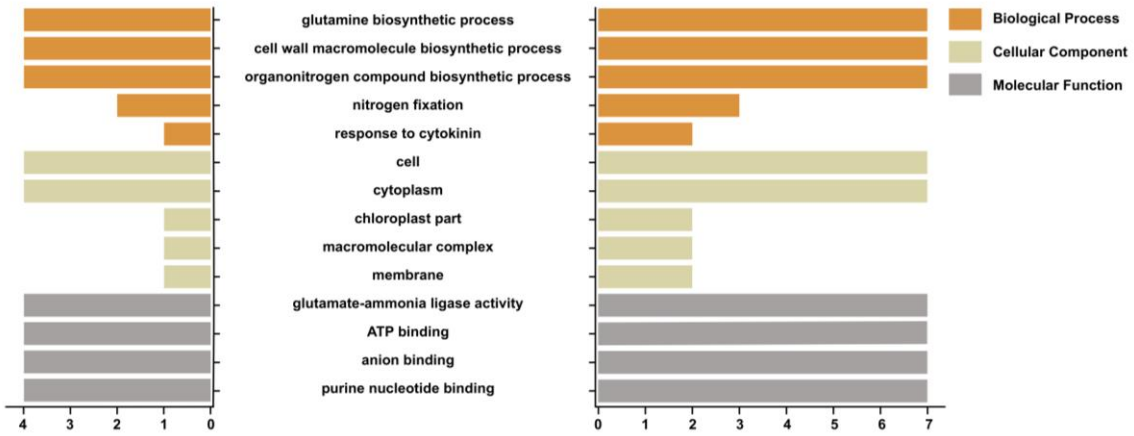

Figure 5. Gene ontology (GO) enrichment of *CmoGS* and *CsaGS* family genes.

3.7. Analysis of the Expression Patterns of CmoGS Family Genes

To further explore the function of the *GS* family genes, a heat map (Figure 6, Supplementary Table S6) was constructed based on the published BioProject RNA-seq data (accession: CmoGSs: PRJNA385310 and CsaGSs: PRJNA312872) in the cucurbit genome

database: CuGenDB. As expected, the three members of the *GS2* subfamily (Group 2) are highly expressed in green tissues and barely expressed in the roots. Compared with *GS2s*, *GS1s* have more diverse expression patterns in a variety of tissues. Unlike other *CmoGS1* replications, *CmoCh08G004920* is highly expressed in all tissues, especially in the stem, suggesting its crucial role in intercellular nitrogen transport. The expression of all *CsaGSs* can be detected in the fruit, and the highest expression of *Csa7G420690* indicates that it may play an important role in the development of the fruit. *CmoCh08G004920*, which belongs to Group 1 with *Csa7G420690*, also shows higher expression in the fruit. In conclusion, the expression pattern of the *GS2* subfamily (Group 2) (*CmoCh06G014450*, *CmoCh14G017140*, and *Csa3G150160*) was similar, but that of the *GS1* subfamily was different. In addition, different from all *GS1* genes with high expression levels in cucumber, only one of the two *GS1* genes in Group 1 and Group 3 was more active in pumpkin.

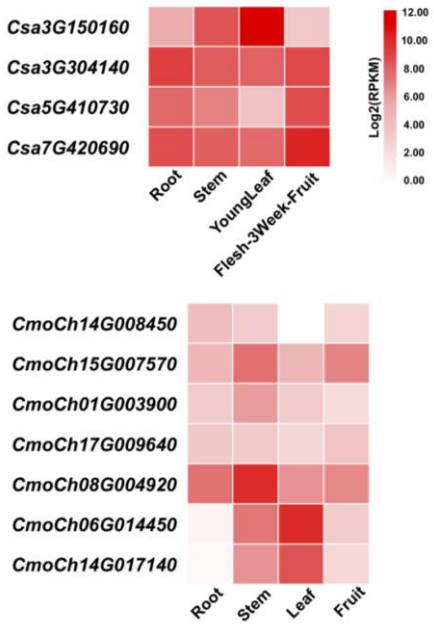

Figure 6. The expression profile of *CmoGS* and *CsaGS* family genes. The RNA-seq data are from BioProject (pumpkin: accession: PRJNA385310 and cucumber: accession: PRJNA312872).

It was previously reported that the expression of *GS* was induced by cold in tea plants [32]. In order to test the potential function of *CmoGS* in pumpkin rootstock in glutamine regulation under cold stress, we conducted RT-PCR experiments on *CmoGS* genes. Semi RT-PCR assays of the first true leaves and roots of pumpkin treated at 4 °C were used to detect the expression level of *CmoGSs* under cold stress. The first true leaves and roots of pumpkin seedlings at 6 h, 12 h, 24 h, 2 days, 3 days, 6 days, and 9 days after cold treatment and before treatment were selected as samples. The result indicate that except for *CmoCh08G004920*, the expression trend of *CmoGSs* was changed to varying degrees by cold induction, although some changes only occurred in the root or leaf, such as those in *CmoCh01G003900*, *CmoCh06G014450*, *CmoCh14G008450*, and *CmoCh14G017140* (Figure 7). The RNA abundance of *CmoGSs* remained unchanged or increased slightly under short-term cold stress within 12 h. Under the long-term cold stress of 2–9 days, the RNA abundance of only *CmoCh01G003900* and *CmoCh14G008450* in the first true leaf increased. Two genes with low-temperature-response regulatory elements, *CmoCh06G014450* and *CmoCh14G008450*, were assumed to respond to cold stress. In general, most *CmoGSs* showed changes in the RNA content at low temperatures, and the trend of changes was different for different sites and genes. Compared with short-term cold stress, *CmoGSs* showed more significant changes in RNA abundance under long-term cold stress.

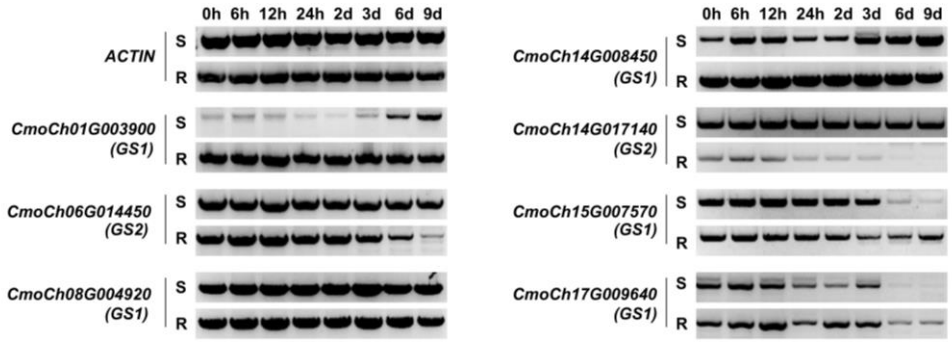

Figure 7. Semi RT-PCR identification of *CmoGS* expression in responding to cold stress. The time for cold treatment is shown at the top of the figure (h: hour, d: day). Actin: *CmoACT7*. S: first true leaf of pumpkin. R: root of pumpkin.

4. Discussion

GS isoforms have been analyzed in many plants, such as soybeans (*Glycine max*) [33], potatoes (*Solanum tuberosum* L) [34], and tomatoes (*Solanum lycopersicum* cv. Micro-Tom) [35], laying an important foundation for analyzing functional GS isoforms. Nevertheless, *GS* family members have not been identified in *Cucurbitaceae*, and few functional studies have been conducted. Thus, the structure and function of *GS* family members were analyzed herein through bioinformatics. According to homology comparison with the *GS* family of *Arabidopsis thaliana* and a domain search, seven and four members of the pumpkin and cucumber *GS* family genes were identified, respectively. Phylogenetic trees were then constructed with GS amino-acid sequences from seven plants, including pumpkins and cucumbers, with the aim of exploring the evolutionary relationships of *Cucurbitaceae GSs*. Promoter region analysis showed that the number and arrangement of regulatory elements in *GSs* differed between family members. The comparison of the number and types in the regulatory elements of *GS* family genes suggests that each *GS* gene may be subject to complex regulation, indicating that the functions of these genes are nonredundant. Chromosome 14 of pumpkins has high homology with Chromosome 1 and Chromosome 6, and *CmoGSs* are distributed pairwise on these chromosomes. Similar situations also occur on Chromosome 8 and Chromosome 17 [15]. This may be related to a doubling of the whole genome of pumpkin, and the duplication was considered to be the main cause of gene-family expansion [36,37]. Therefore, to a certain extent, the uneven distribution of family genes on chromosomes is caused by duplication of fragments. [38].

GS was considered one of the oldest functional genes [39,40]. There are two GS isoforms (GS1 and GS2) in plants, which are considered to have diverged by duplication from a common ancestor [41]. This separation may have occurred later than the appearance of vascular plants but earlier than the divergence of gymnosperms/angiosperms [9]. The majority of plants reported to date have only one *GS2*. By comparing the number of amino acids in the candidate GS proteins of pumpkins and the phylogenetic analysis, we proved that there are two *GS2* duplicates in pumpkins, as in grapes and *Medicago truncatula* [9,42]. In this study, GS proteins from seven plants, including pumpkins and cucumbers, were used to reconstruct a phylogenetic tree (Figure 1). These proteins were divided into three groups based on their homology. Interestingly, GS1 proteins have a tendency to be separated according to whether they belonged to monocotyledons or dicotyledons. This suggests that the evolution of *GS* did not stop after the appearance of angiosperms. Thus, our findings facilitate the study of differentiation between monocotyledons and dicotyledons.

In other plants, members of *GS* family show spatially and temporally specific and nonoverlapping functions [5,43]. A heat map constructed based on the reported RNA-seq data also confirmed the tissue-specific expression patterns of *GS* genes in pumpkin and cucumber (Figure 6). Combined with the similar tissue-specific expression pattern and

collinearity, the close homology between the two replicates of *CmoGS2* was demonstrated. Differential expression between two *CmoGS2* genes may depend on developmental processes or the presence of specific signals, based on their differential promoters. This also explains the differences in *CmoGS1* gene expression; even members of a sister group with close homology have significantly different transcript abundance in tissues. The heat map shows the differential contribution of the *CmoGS1* gene members. The high expression of *CmoCh08G004920* in the roots and stem suggests that it may be endowed with powerful primary nitrogen assimilation and nitrogen-transport capacity, suggesting pumpkin has evolved a spare glutamine-synthesis-regulation mechanism. In other plants, these functions involve several major *GS1*s [32,44], so we should focus on *CmoCh08G004920* in future research. Transcripts of the *CmoGS2*s are clustered in the leaves, where ammonia is produced by photorespiration release and nitrate reduction. While we are making unremitting efforts to improve NUE, we also facing new challenges. Studies have shown that the increase in CO_2 reduces the nitrate reduction of C3 plants, but the ammonium utilization rate does not decrease [2,8,14]. Therefore, the means of cooperation between GS isoforms may change.

5. Conclusions

Our present work, to the best of our knowledge, is the first time the structure and expression patterns of all candidate members of the pumpkin and cucumber *GS* family genes has been studied. The Cucurbitaceae *GS* family genes exhibit complex regulatory and functional possibilities. We found that seven duplicates of *CmoGSs* and four duplicates of *CsaGSs* can be organized into three groups based on their homology and multispecies phylogenetic relationships, with two groups for cytosolic *GS1* and one for chloroplast *GS2*. Based on the published RNA-seq data, we performed an expression analysis of the *GS* family, indicating that the gene expression is spatially specific. In particular, we found tissue-specific expression of pumpkin *GS1* (*CmoCh08G004920*) with relatively high expression of other *GS1*s, indicating a candidate gene to improve nitrogen-utilization efficiency (NUE) in pumpkins. In general, this study paves the way to deepen our understanding of the *GS* family genes for future research on the regulation and function of *GS* family genes in cucurbits crops. It provides confidence in dealing with food safety issues of cucurbits vegetables that may be caused by climate change.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11111156/s1, Table S1: The primers used for RT-PCR in this study, Table S2: Amino acid composition of CmoGS and CsaGS proteins, Table S3: Amino acid sequences of CmoGS and CsaGS proteins used for phylogenetic tree construction, Table S4: Regulatory elements in the 5′-upstream regions of *GS* genes promoter identified by Tbtools, Table S5: Biological process, cellular component and molecular function categories in GO enrichment of *GmoGS* and *CsaGS* genes, Table S6: RPKM value of RNA—seq data in different tissues of pumpkin and cucumber.

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References

1. Bernard, S.M.; Habash, D. The importance of cytosolic glutamine synthetase in nitrogen assimilation and recycling. *New Phytol.* **2009**, *182*, 608–620. [CrossRef] [PubMed]
2. Miflin, B.J.; Habash, D.Z. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J. Exp. Bot.* **2002**, *53*, 979–987. [CrossRef] [PubMed]
3. James, D.; Borphukan, B.; Fartyal, D.; Achary, V.M.M.; Reddy, M.K. Transgenic manipulation of Glutamine Synthetase: A Target with Untapped Potential in Various Aspects of Crop Improvement. In *Biotechnologies of Crop Improvement*; Springer: Berlin/Heidelberg, Germany, 2018; Volume 2, pp. 367–416.
4. Peterman, T.K.; Goodman, H.M. The glutamine synthetase gene family of Arabidopsis thaliana light-regulation and differential expression in leaves, roots and seeds. *Mol. Genet. Genom.* **1991**, *230*, 145–154. [CrossRef] [PubMed]
5. Li, M.G.; Villemur, R.; Hussey, P.J.; Silflow, C.D.; Gantt, J.S.; Snustad, D.P. Differential expression of six glutamine synthetase genes in Zea mays. *Plant Mol. Biol.* **1993**, *23*, 401–407. [CrossRef] [PubMed]
6. Sakakibara, H.; Kawabata, S.; Takahashi, H.; Hase, T.; Sugiyama, T. Molecular Cloning of the Family of Glutamine Synthetase Genes from Maize: Expression of Genes for Glutamine Synthetase and Ferredoxin-Dependent Glutamate Synthase in Photosynthetic and Non-Photosynthetic Tissues. *Plant Cell Physiol.* **1992**, *33*, 49–58. [CrossRef]
7. Castro-Rodríguez, V.; García-Gutiérrez, A.; Canales, J.; Avila, C.; Kirby, E.G.; Cánovas, F.M. The glutamine synthetase gene family in Populus. *BMC Plant Biol.* **2011**, *11*, 119. [CrossRef]
8. Lam, H.-M.; Coschigano, K.T.; Oliveira, I.C.; Melo-Oliveira, R.; Coruzzi, G.M. The Molecular-Genetics of Nitrogen Assimilation into Amino Acids in Higher Plants. *Annu. Rev. Plant Biol.* **1996**, *47*, 569–593. [CrossRef] [PubMed]
9. Seabra, A.R.; Carvalho, H.; Pereira, P.J.B. Crystallization and preliminary crystallographic characterization of glutamine synthetase fromMedicago truncatula. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2009**, *65*, 1309–1312. [CrossRef]
10. Unno, H.; Uchida, T.; Sugawara, H.; Kurisu, G.; Sugiyama, T.; Yamaya, T.; Sakakibara, H.; Hase, T.; Kusunoki, M. Atomic Structure of Plant Glutamine Synthetase-A key enzyme for plant productivity. *J. Biol. Chem.* **2006**, *281*, 29287–29296. [CrossRef]
11. McNally, S.; Hirel, B.; Gadal, P.; Mann, A.; Stewart, G. Evidence for a specific isoform content related to their possible physiological role and their compartmentation within the leaf. *Plant Physiol.* **1983**, *72*, 22–25. [CrossRef]
12. Andrews, M. The partitioning of nitrate assimilation between root and shoot of higher plants. *Plant Cell Environ.* **1986**, *9*, 511–519. [CrossRef]
13. Hirel, B.; Lea, P.J.; Lea, P.J.; Morot-Gaudry, J.-F. Ammonia Assimilation. In *Plant Nitrogen*; Springer: Berlin/Heidelberg, Germany, 2001; pp. 79–99.
14. Sun, H.; Huang, Q.-M.; Su, J. Highly effective expression of glutamine synthetase genes GS1 and GS2 in transgenic rice plants increases nitrogen-deficiency tolerance. *J. Plant Physiol. Mol. Boil.* **2005**, *31*, 492–498.
15. Hachiya, T.; Inaba, J.; Wakazaki, M.; Sato, M.; Toyooka, K.; Miyagi, A.; Kawai-Yamada, M.; Sugiura, D.; Nakagawa, T.; Kiba, T.; et al. Excessive ammonium assimilation by plastidic glutamine synthetase causes ammonium toxicity in Arabidopsis thaliana. *Nat. Commun.* **2021**, *12*, 1–10. [CrossRef]
16. Moison, M.; Marmagne, A.; Dinant, S.; Soulay, F.; Azzopardi, M.; Lothier, J.; Citerne, S.; Morin, H.; Legay, N.; Chardon, F.; et al. Three cytosolic glutamine synthetase isoforms localized in different-order veins act together for N remobilization and seed filling in Arabidopsis. *J. Exp. Bot.* **2018**, *69*, 4379–4393. [CrossRef]
17. Wei, Y.; Xiong, S.; Zhang, Z.; Meng, X.; Wang, L.; Zhang, X.; Yu, M.; Yu, H.; Wang, X.; Ma, X. Localization, Gene Expression, and Functions of Glutamine Synthetase Isozymes in Wheat Grain (*Triticum aestivum* L.). *Front. Plant Sci.* **2021**, *12*. [CrossRef]
18. Anwar, A.; Li, Y.; He, C.; Yu, X. 24-Epibrassinolide promotes NO3- and NH4+ ion flux rate and NRT1 gene expression in cucumber under suboptimal root zone temperature. *BMC Plant Biol.* **2019**, *19*, 1–5.
19. Xin, M.; Wang, L.; Liu, Y.; Feng, Z.; Zhou, X.; Qin, Z. Transcriptome profiling of cucumber genome expression in response to long-term low nitrogen stress. *Acta Physiol. Plant.* **2017**, *39*, 130. [CrossRef]
20. Liu, H.; Gao, Y.; Gao, C.; Liu, S.; Zhang, J.; Chen, G.; Zhang, S.; Wu, F. Study of the physiological mechanism of delaying cucumber senescence by wheat intercropping pattern. *J. Plant Physiol.* **2019**, *234–235*, 154–166. [CrossRef]
21. Sun, H.; Wu, S.; Zhang, G.; Jiao, C.; Guo, S.; Ren, Y.; Zhang, J.; Zhang, H.; Gong, G.; Jia, Z.; et al. Karyotype Stability and Unbiased Fractionation in the Paleo-Allotetraploid Cucurbita Genomes. *Mol. Plant* **2017**, *10*, 1293–1306. [CrossRef]
22. Condurso, C.; Verzera, A.; Dima, G.; Tripodi, G.; Crinò, P.; Paratore, A.; Romano, D. Effects of different rootstocks on aroma volatile compounds and carotenoid content of melon fruits. *Sci. Hortic.* **2012**, *148*, 9–16. [CrossRef]
23. Pulgar, G.; Villora, G.; Moreno, D.; Romero, L. Improving the Mineral Nutrition in Grafted Watermelon Plants: Nitrogen Metabolism. *Biol. Plant.* **2000**, *43*, 607–609. [CrossRef]
24. Xing, W.-W.; Li, L.; Gao, P.; Li, H.; Shao, Q.-S.; Shu, S.; Sun, J.; Guo, S.-R. Effects of grafting with pumpkin rootstock on carbohydrate metabolism in cucumber seedlings under $Ca(NO_3)_2$ stress. *Plant Physiol. Biochem.* **2015**, *87*, 124–132. [CrossRef] [PubMed]
25. Britto, D.T.; Kronzucker, H. NH4+ toxicity in higher plants: A critical review. *J. Plant Physiol.* **2002**, *159*, 567–584. [CrossRef]
26. Bateman, A.; Birney, E.; Cerruti, L.; Durbin, R.; Etwille, L.; Eddy, S.R.; Griffiths-Jones, S.; Howe, K.L.; Marshall, M.; Sonnhammer, E.L. The Pfam protein families database. *Nucleic Acids Res.* **2002**, *30*, 276–280. [CrossRef]
27. Gasteiger, E.; Gattiker, A.; Hoogland, C.; Ivanyi, I.; Appel, R.D.; Bairoch, A. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* **2003**, *31*, 3784–3788. [CrossRef]

28. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
29. Tang, H.; Bowers, J.E.; Wang, X.; Ming, R.; Alam, M.; Paterson, A.H. Synteny and Collinearity in Plant Genomes. *Science* **2008**, *320*, 486–488. [CrossRef]
30. Chen, C.J.; Chen, H.; Zhang, Y.; Thomas, H.R.; Frank, M.H.; He, Y.H.; Xia, R. TBtools: An Integrative Toolkit Developed for Interactive Analyses of Big Biological Data. *Mol. Plant.* **2020**, *13*, 1194–1202. [CrossRef]
31. Wei, G.; Tian, P.; Zhang, F.; Qin, H.; Miao, H.; Chen, Q.; Hu, Z.; Cao, L.; Wang, M.; Gu, X.; et al. Integrative Analyses of Nontargeted Volatile Profiling and Transcriptome Data Provide Molecular Insight into VOC Diversity in Cucumber Plants (*Cucumis sativus*). *Plant Physiol.* **2016**, *172*, 603–618. [CrossRef]
32. Cheng, G.; You, X.; Wu, Y.; Zhang, J. Analysis on gene differential expression of cold-resistance cultivar 'Ziyangyuanye'of Camellia sinensis after low temperature stress. *J. Plant Res. Environ.* **2013**, *22*, 38–43.
33. Miao, G.H.; Hirel, B.; Marsolier, M.C.; Ridge, R.W.; Verma, D.P.S. Ammonia-regulated expression of a soybean gene encoding cytosolic Glutamine-Synthetase in transgenic lotus-corniculatus. *Plant Cell* **1991**, *3*, 11–22. [PubMed]
34. Teixeira, J.; Pereira, S. High salinity and drought act on an organ-dependent manner on potato glutamine synthetase expression and accumulation. *Environ. Exp. Bot.* **2007**, *60*, 121–126. [CrossRef]
35. Scarpeci, T.E.; Marro, M.L.; Bortolotti, S.; Boggio, S.B.; Valle, E.M. Plant nutritional status modulates glutamine synthetase levels in ripe tomatoes (*Solanum lycopersicum cv. Micro-Tom*). *J. Plant Physiol.* **2007**, *164*, 137–145. [CrossRef] [PubMed]
36. Kong, H.; Landherr, L.L.; Frohlich, M.W.; Leebens-Mack, J.; Ma, H.; De Pamphilis, C.W. Patterns of gene duplication in the plant SKP1 gene family in angiosperms: Evidence for multiple mechanisms of rapid gene birth. *Plant J.* **2007**, *50*, 873–885. [CrossRef]
37. Nakano, T.; Suzuki, K.; Fujimura, T.; Shinshi, H. Genome-Wide Analysis of the ERF Gene Family in Arabidopsis and Rice. *Plant Physiol.* **2006**, *140*, 411–432. [CrossRef]
38. Zhu, Y.; Wu, N.; Song, W.; Yin, G.; Qin, Y.; Yan, Y.; Hu, Y. Soybean (*Glycine max*) expansin gene superfamily origins: Segmental and tandem duplication events followed by divergent selection among subfamilies. *BMC Plant Biol.* **2014**, *14*, 93. [CrossRef]
39. Kumada, Y.; Benson, D.; Hillemann, D.; Hosted, T.; Rochefort, D.; Thompson, C.; Wohlleben, W.; Tateno, Y. Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 3009–3013. [CrossRef]
40. Pesole, G.; Bozzetti, M.P.; Lanave, C.; Preparata, G.; Saccone, C. Glutamine synthetase gene evolution: A good molecular clock. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 522–526. [CrossRef]
41. Tingey, S.V.; Tsai, F.Y.; Edwards, J.W.; Walker, E.; Coruzzi, G.M. Chloroplast and cytosolic glutamine synthetase are encoded by homologous nuclear genes which are differentially expressed in vivo. *J. Biol. Chem.* **1988**, *263*, 9651–9657. [CrossRef]
42. Seabra, A.R.; Vieira, C.P.; Cullimore, J.V.; Carvalho, H.G. Medicago truncatula contains a second gene encoding a plastid located glutamine synthetase exclusively expressed in developing seeds. *BMC Plant Biol.* **2010**, *10*, 1–16. [CrossRef]
43. Wang, X.; Wei, Y.; Shi, L.; Ma, X.; Theg, S.M. New isoforms and assembly of glutamine synthetase in the leaf of wheat (*Triticum aestivum* L.). *J. Exp. Bot.* **2015**, *66*, 6827–6834. [CrossRef] [PubMed]
44. Thomsen, H.C.; Eriksson, D.; Møller, I.S.; Schjoerring, J.K. Cytosolic glutamine synthetase: A target for improvement of crop nitrogen use efficiency? *Trends Plant Sci.* **2014**, *19*, 656–663. [CrossRef] [PubMed]