Genome-Wide Characterization of Nitrogenase Reductase (nifH) Genes in the Sweet Potato [Ipomoea batatas (L.) Lam] and Its Wild Ancestors

Zengzhi Si 1, Chong Wang 2, Mingming Zhao 1, Zhixin Ji 1, Yake Qiao 1 and Lianjun Wang 2,*

1 Hebei Key Laboratory of Crop Stress Biology, Hebei Normal University of Science and Technology, Qinhuangdao 066000, China
2 Institute of Food Corps, Hubei Academy of Agricultural Sciences, Wuhan 430072, China
* Correspondence: wanglianjun10@163.com

Abstract: The sweet potato (Ipomoea batatas (L.) Lam.) is an important and widely grown crop, and the nitrogenase reductase (nifH) gene is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea. There have been many examples of the isolation of the diazotrophic endophytes in sweet potatoes, and there has been no report on whether sweet potatoes and their wild ancestors harbored nifH genes. In this study, a comprehensive analysis of nifH genes has been conducted on these species by using bioinformatics and molecular biology methods. A total of 20, 19 and 17 nifH genes were identified for the first time in sweet potatoes, I. trifida and I. triloba, respectively. Based on a phylogenetic analysis, all of the nifH genes, except for g10233.t1, itf14g14040.t1 and itb14g15470.t1, were clustered into five independent clades: I, II, III, IV and V. The nifH genes clustered in the same phylogenetic branch showed a more similar distribution of conserved motifs and exons–introns than those of the other ones. All of the identified genes were further mapped on the 15 chromosomes of the sweet potato, I. trifida and I. triloba. No segmental duplication was detected in each genome of three Ipomoea species, and 0, 8 and 7 tandemly duplicated gene pairs were detected in the genome of the sweet potato, I. trifida and I. triloba, respectively. Syntenic analysis between the three Ipomoea species revealed that there were 7, 7 and 8 syntenic gene pairs of nifH genes detected between the sweet potato and I. trifida, between the sweet potato and I. triloba and between I. trifida and I. triloba, respectively. All of the duplicated and syntenic nifH genes were subjected to purifying selection inside duplicated genomic elements during speciation, except for the tandemly duplicated gene pair itf11g07340.t2_itf11g07340.t3, which was subjected to positive selection. Different expression profiles were detected in the sweet potato, I. trifida and I. triloba. According to the above results, four nifH genes of the sweet potato (g950, g16683, g27094 and g33987) were selected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis in two sweet potato cultivars (Eshu 15 and Long 9) under nitrogen deficiency (N0) and normal (N1) conditions. All of them were upregulated in the N1 treatment and were consistent with the analysis of the RNA-seq data. We hope that these results will provide new insights into the nifH genes in the sweet potato and its wild ancestors and will contribute to the molecular breeding of sweet potatoes in the future.

Keywords: sweet potato; wild ancestors; nitrogenase reductase; phylogenetic analysis; chromosome location; expression profile

1. Introduction

The sweet potato, Ipomoea batatas (L.) Lam., is an important food crop widely grown in the world. It is also an alternative source of bioenergy as a raw material for fuel production [1,2]. The sweet potato contains soluble sugar, starch, dietary fiber, protein, fat, calcium and other minerals, as well as antioxidant substances beneficial to human health, such as carotenoids, anthocyanins, vitamins, flavonoids, etc. [2]. Therefore, the sweet potato is rated as the healthiest vegetable by the World Health Organization.
Nitrogen-fixing microorganisms, as the only natural biological source of fixed nitrogen, play important roles in balancing global ecology [3]. Nitrogen fixation is carried out by nitrogenase, and multiple subunits of nitrogenase are encoded by genes nitrogenase reductase (nifH), alpha subunit (nifD) and beta subunit (nifK) [4]. Of them, the nifH gene, which encodes the nitrogenase reductase subunit, is usually used as the marker gene for studying nitrogen-fixing. Thus, a wide range of environments have been sampled for nifH gene diversity [3], such as marine [5], terrestrial [6], extreme [7], anthropogenic [8], host-associated [9] and agricultural [10] environments. Rhizobia is a kind of prokaryotic bacteria with nitrogen fixation abilities [11]. After infecting legumes, it forms many root nodules and converts N$_2$ in the air into NH$_3$ through root nodules for legumes to use. At the same time, rhizobia can obtain the necessary water and nutrients from root nodule cells [12]. Cyanobacteria, which also plays an important role in the balance of nitrogen elements in nature, is also the pioneer plant in barren lands. Cyanobacteria have a great amount of nitrogen fixation, which also promotes the occurrence of photosynthesis [13]. In addition, Azolla imbircata (Roxb.) Nakai, as a floating plant, has the functions of nitrogen fixation, photosynthesis and ammonia release [14–16]. The nitrogen fixation of duckweed mainly depends on its photosynthesis, and the energy and reductant required for nitrogen fixation come from photosynthesis [17].

The nitrogenase reductase (nifH) gene, as the marker gene for studying nitrogen-fixing, has been studied in various fields. Cheng et al. (2018) cloned genes by encoding nifH, nifE, nifN and nifB from Helio bacterium chlororum into the potato virus X (PVX)-basedvector and generated PVX/HisG-nifH, PVX/HisG-nifE, PVX/HisG-nifB and PVX/HisG-nifN, and then they inoculated Nicotiana benthamiana plants with these recombinant viruses and detected the expression on the translation of all the levels of nifH, nifE, nifN and nifB in plants. It was found that nifH can be expressed in abundance in plant cells, and the expression of nifE, nifN and nifB are not detectable on SDS-PAGE [18]. Jiang et al. (2021) built a knowledge-based library containing 32 nitrogenase nifH sequences from prokaryotes of diverse ecological niches and metabolic features and combined rapid screening in tobacco to identify superior nifH variants for plant mitochondria expression. Three nifH variants outperformed in tobacco mitochondria and were further tested in yeast [19]. Klarenberg et al. (2022) evaluated the effect of warming and warming-induced shrub expansion on the moss bacterial community composition and diversity, and nifH gene abundance and the results showed that the abundance of nifH genes was negatively affected by litter abundance [20].

The sweet potato, as a low-nitrogen-tolerant crop, grows well in nitrogen-poor infertile soils. Previous studies have shown that the growth ability of the sweet potato is partially due to the functions of diazotrophic growth-promoting bacteria that contain endo- and epiphytic microorganisms of this plant [21–24]. Various endophytic bacteria were isolated from sweet potatoes [18], such as Azospirillum sp. [25], Gluconacetobacter sp. (formerly Acetobacter sp.) [26], Klebsiella sp. [27], Pantoea sp. and Enterobacter sp. [28], Bradyrhizobium sp., Pseudobacillus sp., Pseudomonas sp. [29], Enterobacter sp., Rahnella sp., Rhodanobacter sp., Pseudomonas sp., Xanthomonas sp. and Phyllobacterium sp. [30], etc.

Although there have been many examples of the isolation of the diazotrophic endophytes in sweet potatoes, there has been no report on whether the sweet potato and its wild ancestors harbor nifH genes. In this study, a total of 20, 19 and 17 nifH genes were first identified in sweet potatoes, I. trifida and I. triloba, respectively. A phylogenetic analysis grouped these genes into five independent clades: I, II, III, IV and V. Conserved motifs and the gene structures of the nifH genes were analyzed. All of the identified genes were further mapped on the 15 chromosomes. Duplication, synteny and Ka/Ks analysis were performed, and the expression profiles of the identified genes were obtained.

2. Materials and Methods

2.1. Identification and Classification of the nifH Genes

The whole genomes of three Ipomoea species, the sweet potato, I. trifida, and I. triloba, were used in this study. The genome sequences of the sweet potato, including the
predicted gene model annotation, were downloaded from the *Ipomoea* Genome Hub (https://ipomoea-genome.org/, accessed on 18 August 2021); the genome sequences of *I. trifida* and *I. triloba*, including the predicted gene model annotation, were downloaded from GenBank BioProject (accessions numbers PRJNA428214 and PRJNA428241). Both a BLAST search and a hidden Markov model search (HMMsearch) were performed as described previously [31]. All protein sequences were first searched for the Fer4_NifH domain (Pfam accession number: PF00142) using hmmsearch with default parameters. Moreover, the extended amino acid sequence of Fer4_NifH domain was used as a query to search for all protein sequences in the sweet potato genome using the BLASTP program. After that, the genes gained by HMMsearch and BLAST methods were merged, and the redundant ones were removed.

2.2. Sequence Alignment and Phylogenetic Analysis

The identified *nifH* genes were aligned using online Clustal Omega (http://www.clustal.org/omega/, accessed on 19 July 2022) [32,33]. According to the previous methods [34], phylogenetic analyses were performed using IQ-TREE (version 1.6.12, http://www.iqtree.org/, accessed on 19 April 2022) with the maximum likelihood algorithm [35]. ModelFinder was used to estimate the best-fit model of nucleotide substitution [36], and branch support values were calculated using SH-aLRT and UFBoot2 [37] with 1000 bootstrap replicates [38]. Thus, the obtained tree was summated to Figtree (version 1.4.3) for visual enhancement (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 6 April 2020).

2.3. Conserved Motif Detection and Gene Structure Analyses of the *nifH* Genes

To investigate the structural motif diversity of the identified *nifH* genes, the protein sequences of them were subjected to motif analysis by online MEME SUITE (https://meme-suite.org/meme/, accessed on 19 July 2022) [39]. The criteria used for MEME analysis were (1) a minimum width of 6; (2) a maximum width of 50; (3) a maximum number of motifs designed to identify 20 motifs; and (4) iterative cycles set by default. The exon–intron structure of the *nifH* genes was acquired from the GFF3 annotation files of the sweet potato, *I. trifida* and *I. triloba*. The distribution of conserved motifs and the exon–intron structures of the *nifH* genes were exhibited using TBtools software (version 1.068) (https://github.com/CJ-Chen/TBtools/releases, accessed on 16 July 2021) [40].

2.4. Chromosome Distribution of the *nifH* Genes

The *nifH* genes with chromosome-located positions were mapped on the chromosomes of the sweet potato, *I. trifida* and *I. triloba* using MapChart (version 2.30) software (https://www.wur.nl/en/show/Mapchart.htm, accessed on 3 July 2020) [41].

2.5. Duplication and Ka/Ks Analysis of the *nifH* Genes

To search for potential duplicated *nifH* genes in the sweet potato, *I. trifida*, and *I. triloba*, the Multiple Collinearity Scan toolkit (MCScanX, version 0.8) (http://chibba.pgml.uga.edu/mcscan2/, accessed on 3 January 2020) was used [42]. All the *nifH* protein sequences of the three species were compared to themselves by using the BLASTP program with an E-value of $1 \times 10^{-10}$. The resulting blast hits were incorporated along with chromosome coordinates of all *nifH* genes as an input for MCScanX analysis. The hits were classified into various types of duplications, including segmental, tandem, proximal and dispersed under a default criterion. The final results were drawn by CIRCOS software for visualization [43]. The aligned protein sequences of the *nifH* genes of the sweet potato, *I. trifida* and *I. triloba* were first converted into the corresponding nucleotide sequences using PAL2NAL software (http://www.bork.embl.de/pal2nal/#RunP2N, accessed on 25 July 2021) [44] and were then submitted to PAML software (version 4.0) (http://abacus.gene.ucl.ac.uk/software/paml.html, accessed on 11 July 2020) [45] for Ka/Ks (nonsynonymous/synonymous) calculation.
2.6. Expression Profiles of nifH Genes of the Sweet Potato, I. trifida and I. triloba

For the expression profile analysis of the nifH Genes in the sweet potato, I. trifida and I. triloba, RNA-Seq datasets were downloaded from the sequence read archive (SRA) of NCBI, which referred to different tissues of sweet potatoes (PRJNA511028), and the expression information (fragments per kilobase of exon model per million mapped fragments, FPKM) of I. trifida and I. triloba was acquired from the sweet potato Genomics Resource (http://sweetpotato.uga.edu/gt4sp_download.shtml, accessed on 13 December 2021). After removing the low-quality reads and adaptor trimming, the clean RNA-Seq reads were aligned to the genome sequences of the sweet potato via Hisat2 [46]. Thereafter, SAMtools software (version 1.11) was used for aligned read counting (https://github.com/samtools/samtools/releases/download/1.11, accessed on 23 December 2020) [47]. Then, the obtained read counts were imported into DEseq2 for the analysis of differentially expressed genes (DEGs) [46]. For each compared course, it was treated as a DEG if |log2FC| > 1 and FDR ≤ 5%, and a mean log2FC value for each gene was calculated. The heat map was produced to distribute the expression levels using the RPKM (i.e., reads per kilobase per million) value in MeV software (version 4.9.0) (https://sourceforge.net/projects/mev/, accessed on 23 August 2020) [48].

2.7. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Two sweet potato cultivars (Eshu 15, medium long vine; Long 9, short vine) were selected for the qRT-PCR analysis of the nifH genes. Freshly cut sweet potato seedlings, with 20- to 30-cm-long stems and five to seven leaves, were dipped into water for transplanting for 3 days. Then, the seedlings were transferred into a Hoagland nutrient solution, and the nutrient solution was set with the following nitrogen levels: N0 (0 mmol·L⁻¹ pure nitrogen) and N1 (14 mmol·L⁻¹ pure nitrogen). Other components remained the same: 1 mmol·L⁻¹ KH₂PO₄, 2 mmol·L⁻¹ MgSO₄, 2.50 mmol·L⁻¹ K₂SO₄, 20 mmol·L⁻¹ FeSO₄·7H₂O, 20 mmol·L⁻¹ EDTA-Na₂, 2H₂O, 5 μmol·L⁻¹ NaI, 0.10 mmol·L⁻¹ H₃BO₃, 0.15 mmol·L⁻¹ MnSO₄, 0.05 mmol·L⁻¹ ZnSO₄, 1 μmol·L⁻¹ (NH₄)₂MoO₄, 0.16 μmol·L⁻¹ CuSO₄ and 0.19 μmol·L⁻¹ CoCl₂. Leaf samples were then collected at three time points: 0 h, 3 h and 72 h after treatment. Thereafter, the total RNA of the samples was isolated using the FastPurE® Universal Plant Total RNA Isolation Kit (TransGen, Wuhan, China), and the first-strand cDNA was prepared using EasyScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) (TransGen, Wuhan, China). The sweet potato β-actin gene (Genbank AY905538) was selected and used to normalize the relative quantities of the target genes. Three replicates were performed, and the expression changes were calculated using the 2^ΔΔCt method for each sample. Then, a quantitative real-time polymerase chain reaction (qRT-PCR) for four sweet potato nifH genes (g950, g16683, g27094 and g33987) was performed. The primers used for PCR were designed using on line Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 21 July 2022) (Table S1).

3. Results

3.1. Identification of the nifH Genes of the Sweet Potato, I. trifida and I. triloba

A total of 20, 19 and 17 nifH genes were identified in the genomes of the sweet potato, I. trifida and I. triloba, respectively (Table 1). Among the 20 nifH genes in the sweet potato, the shortest (g56705.11) was of 67 amino acids, whereas the longest (g4650.11) was of 939 amino acids, and the average length of the genes was 430.55 amino acids (Table 1). Among the 19 nifH genes in I. trifida, the shortest (itf12g22980.11) was of 289 amino acids, whereas the longest (itf12g19260.11) was of 530 amino acids, and the average length of the genes was 372.21 amino acids (Table 1). Among the 17 nifH genes in I. triloba, the shortest (itb14g03920.t3) was of 278 amino acids, whereas the longest (itb14g15470.t1) was of 611 amino acids, and the average length of the genes was 385 amino acids (Table 1).
Table 1. The \textit{nifH} gene information of sweet potato, \textit{I. trifida} and \textit{I. triloba}.

| Species  | Gene_Id | Number of Amino Acids | Exon Number | Chromosome Location | Strand | Gene_Start | Gene_End |
|----------|---------|-----------------------|-------------|---------------------|--------|------------|----------|
| sweet potato | g950.t1 | 315 | 2 | Ibchr1 | + | 5,540,056 | 5,541,373 |
| | g3730.t1 | 315 | 3 | Ibchr1 | + | 26,923,828 | 26,934,096 |
| | g4650.t1 | 939 | 8 | Ibchr2 | + | 3,249,593 | 3,253,657 |
| | g10233.t1 | 426 | 13 | Ibchr3 | + | 5,697,280 | 5,701,545 |
| | g16683.t1 | 488 | 13 | Ibchr5 | − | 550,499 | 558,988 |
| | g17404.t1 | 864 | 8 | Ibchr5 | + | 5,150,173 | 5,157,672 |
| | g19650.t1 | 541 | 16 | Ibchr5 | + | 22,748,809 | 22,759,672 |
| | g22847.t1 | 378 | 8 | Ibchr6 | − | 14,879,621 | 14,883,661 |
| | g25326.t1 | 343 | 4 | Ibchr7 | − | 363,695 | 365,842 |
| | g27090.t1 | 289 | 6 | Ibchr7 | + | 13,784,034 | 13,787,412 |
| | g27094.t1 | 594 | 8 | Ibchr7 | + | 13,802,034 | 13,807,229 |
| | g27100.t1 | 317 | 4 | Ibchr7 | + | 13,837,054 | 13,839,787 |
| | g30380.t1 | 460 | 11 | Ibchr8 | − | 761,393 | 764,513 |
| | g31149.t1 | 440 | 12 | Ibchr8 | − | 4,991,243 | 4,995,748 |
| | g33987.t1 | 237 | 3 | Ibchr9 | + | 217,387 | 219,435 |
| | g38504.t1 | 207 | 3 | Ibchr10 | − | 2,286,298 | 2,293,174 |
| | g50721.t1 | 522 | 7 | Ibchr12 | − | 28,604,621 | 28,610,924 |
| | g56575.t1 | 464 | 10 | Ibchr14 | − | 8,492,679 | 8,500,020 |
| | g56705.t1 | 67 | 2 | Ibchr14 | + | 9,444,490 | 9,445,508 |
| | g61682.t1 | 405 | 9 | Ibchr15 | + | 12,526,772 | 12,530,022 |
| \textit{I. trifida} | ifi05g19180.t1 | 329 | 1 | Chr05 | − | 21,097,894 | 21,099,282 |
| | ifi06g08100.t1 | 358 | 5 | Chr06 | − | 10,472,405 | 10,476,280 |
| | ifi06g08100.t1 | 358 | 5 | Chr06 | − | 10,472,405 | 10,476,280 |
| | ifi06g08100.t1 | 358 | 5 | Chr06 | − | 10,472,558 | 10,476,272 |
| | ifi06g08100.t1 | 358 | 5 | Chr06 | − | 10,472,558 | 10,476,272 |
| | ifi06g08340.t1 | 323 | 4 | Chr06 | + | 10,684,647 | 10,686,646 |
| | ifi09g21630.t1 | 357 | 7 | Chr09 | + | 18,324,833 | 18,330,898 |
| | ifi10g25730.t1 | 377 | 3 | Chr10 | − | 24,717,023 | 24,719,626 |
| | ifi11g00790.t1 | 359 | 7 | Chr10 | − | 370,700 | 373,523 |
| \textit{I. triloba} | ifi11g07340.t1 | 410 | 12 | Chr11 | − | 3,956,871 | 3,961,420 |
| | ifi11g07340.t1 | 410 | 11 | Chr11 | − | 3,957,323 | 3,961,420 |
| | ifi11g07340.t1 | 421 | 10 | Chr11 | − | 3,957,967 | 3,961,420 |
| | ifi11g07340.t1 | 299 | 9 | Chr11 | − | 3,958,281 | 3,961,420 |
| | ifi12g00130.t1 | 409 | 11 | Chr12 | − | 131,709 | 139,045 |
| | ifi12g00130.t1 | 405 | 10 | Chr12 | − | 131,709 | 139,045 |
| | ifi12g19260.t1 | 530 | 16 | Chr12 | + | 18,656,207 | 18,662,081 |
| | ifi12g19260.t1 | 411 | 12 | Chr12 | + | 18,658,050 | 18,662,081 |
| | ifi12g22980.t1 | 289 | 8 | Chr12 | − | 21,330,987 | 21,334,731 |
| | ifi14g14040.t1 | 311 | 8 | Chr14 | − | 14,807,579 | 14,810,974 |
| \textit{I. triloba} | ifi11g07630.t1 | 324 | 9 | Chr11 | − | 4,686,363 | 4,690,421 |
| | ifi12g00110.t1 | 297 | 9 | Chr12 | − | 146,125 | 149,815 |
| | ifi12g00110.t1 | 297 | 8 | Chr12 | − | 146,125 | 149,815 |
| | ifi12g00110.t1 | 297 | 9 | Chr12 | − | 146,125 | 149,815 |
| | ifi12g19660.t1 | 530 | 16 | Chr12 | + | 22,066,146 | 22,072,061 |
| | ifi12g19660.t1 | 513 | 15 | Chr12 | + | 22,066,170 | 22,072,003 |
| | ifi12g19660.t1 | 529 | 16 | Chr12 | + | 22,066,146 | 22,072,061 |
| | ifi12g23320.t1 | 289 | 9 | Chr12 | − | 25,103,363 | 25,107,101 |
| | ifi14g15470.t1 | 611 | 15 | Chr14 | − | 18,766,811 | 18,772,688 |
3.2. Phylogenetic Analysis of the nifH Genes of the Sweet Potato, I. trifida and I. triloba

To analyze the phylogenetic relationship of the nifH genes in the sweet potato, I. trifida and I. triloba, a phylogenetic tree was constructed (Figure 1). All of the nifH genes, except for g10233.t1, itf14g14040.t1 and itb14g15470.t1, were clustered into five independent clades: I, II, III, IV and V, with support values > 93% (Figure 1). Each of the five clades contained the nifH genes from all of the three species, which indicated that the ancestries for each clade were differentiated before the species specification of the nifH genes. Among the genes that were not clustered, the same phenomenon was detected; g10233.t1, itf14g14040.t1 and itb14g15470.t1 were identified from the genome of the sweet potato, I. trifida and I. triloba, respectively (Figure 1). Although the number of the nifH genes in the sweet potato, I. trifida and I. triloba for each clade was different, it meant that the nifH genes of each clade may have experienced different duplication during the species specification of the nifH genes (Figure 1).

3.3. Conserved Motif Detection and Gene Structure Analyses of the nifH Genes

Conserved motifs were detected in all of the identified nifH genes except for g25326.t1 in the three Ipomoea species (Figure 2). Among these conserved motifs, motif 1 was the most conserved one, and 54 of the 56 nifH genes harbored motif 1, followed by motif 2 (39 of 56), motif 3 (27 of 56) and motif 6 (25 of 56). Of the identified nifH genes, itb14g15470.t1 contained the greatest number of conserved motifs (#14), followed by g19690.t1 (#11), itf12g19260.t1 (#11), itb12g19660.t2 (#11), itb12g19660.t3 (#11), itb12g19660.t1 (#11), itf11g07340.t1 (#10), itf11g07340.t2 (#10), itb11g07630.t2 (#10) and itb11g07630.t1 (#10). It was also found that the nifH genes clustered in the same phylogenetic branch showed a more similar distribution of conserved motifs and exons–introns (gene structure) than those of the other nifH genes (Figure 2).
3.4. Chromosome Locations of the nifH Genes of the Sweet Potato, I. trifida and I. triloba

Based on the locations of individual nifH genes, all of the identified genes were mapped on the 15 chromosomes of the sweet potato, I. trifida and I. triloba, respectively (Figure 3). However, the chromosome distribution of the nifH genes was similar between I. trifida and I. triloba, whereas it was different between the sweet potato and I. trifida or I. triloba (Figure 3). The nifH genes in the sweet potato were distributed on all of the chromosomes except for chromosome 4, 11 and 13, whereas the nifH genes in I. trifida and I. triloba were mainly located on chromosome 5, 6, 9, 11, 12 and 14 (Figure 3).

3.5. Duplication and Ka/Ks Analysis of the nifH Genes

The duplication analysis of the nifH genes in the three Ipomoea species showed that no segmental duplication was detected in each genome of them, and 0, 8 and 7 tandemly duplicated gene pairs were detected in the genome of the sweet potato, I. trifida and I. triloba, respectively (Figure 3). The syntenic analysis between the three Ipomoea species revealed that there were 7, 7 and 8 syntenic gene pairs of nifH genes detected between the sweet potato and I. trifida, between the sweet potato and I. triloba and between I. trifida and I. triloba, respectively (Figure 4).
Figure 3. Distribution of nifH genes in chromosomes: (A) Distribution in I. batatas chromosomes. (B) Distribution in I. trifida chromosomes. (C) Distribution in I. triloba chromosomes. Randomly replicated nifH genes are shown with a pink background.

Figure 4. Synteny analysis of the nifH genes. The outer circle represents the haploid chromosomes of the sweet potato, I. trifida and I. triloba (gray); red, green and purple lines show the syntenic gene pairs between sweet potato and I. trifida, between sweet potato and I. triloba and between I. trifida and I. triloba, respectively.
The non-synonymous substitution (Ka) to synonymous substitution (Ks) ratio (Ka/Ks) is an informative value of positive selection. To detect whether some nifH genes are under positive selection, Ka/Ks analysis was performed on duplicated and syntenic nifH genes within or between the studied three Ipomoea species. All of the duplicated and syntenic gene pairs showed a Ka/Ks ratio <1, except for the tandemly duplicated gene pair itf11g07340.t2_itf11g07340.t3, which has a Ka/Ks ratio = 1.34 (Table 2). The results revealed that itf11g07340.t2_itf11g07340.t3 was subjected to positive selection, and all of the other duplicated and syntenic nifH genes were subjected to purifying selection inside duplicated genomic elements during speciation.

Table 2. Ka/Ks analysis of the duplicated and syntenic nifH genes in and between the three Ipomoea species.

| Gene Pairs                | Duplication Type | Ka   | Ks   | Ka/Ks |
|---------------------------|------------------|------|------|-------|
| g10333.11_itb14g15470.11 | Segmental replication | 0.06 | 0.11 | 0.54  |
| g10333.11_itf14g14040.11 | Segmental replication | 0.05 | 0.12 | 0.39  |
| g16683.11_itf12g00110.11 | Segmental replication | 0.00 | 0.00 | 0.00  |
| g16683.11_itf12g00130.11 | Segmental replication | 0.00 | 0.00 | 0.00  |
| g19690.11_itf12g19660.11 | Segmental replication | 0.01 | 0.03 | 0.21  |
| g19690.11_itf12g19260.11 | Segmental replication | 0.00 | 0.03 | 0.00  |
| g30380.11_itf11g07750.11 | Segmental replication | 0.00 | 0.05 | 0.02  |
| g30380.11_itf11g00790.11 | Segmental replication | 0.00 | 0.02 | 0.00  |
| g31149.11_itf11g07630.11 | Segmental replication | 0.01 | 0.05 | 0.16  |
| g33987.11_itf10g25730.11 | Segmental replication | 0.11 | 0.13 | 0.82  |
| g56575.11_itb09g24130.11 | Segmental replication | 0.02 | 0.07 | 0.25  |
| g56575.11_itf10g21630.11 | Segmental replication | 0.02 | 0.08 | 0.26  |
| g950.11_itb05g19830.11  | Segmental replication | 0.00 | 0.05 | 0.06  |
| g950.11_itf05g19180.11  | Segmental replication | 0.00 | 0.07 | 0.04  |
| itb05g19830.11_itf05g19180.11 | Segmental replication | 0.00 | 0.06 | 0.00  |
| itb09g24130.11_itf09g21630.11 | Segmental replication | 0.00 | 0.03 | 0.08  |
| itb11g00750.11_itf11g00790.11 | Segmental replication | 0.00 | 0.06 | 0.02  |
| itb11g07630.11_itf11g07340.11 | Segmental replication | 0.00 | 0.04 | 0.03  |
| itb12g00110.11_itf12g00130.11 | Segmental replication | 0.00 | 0.01 | 0.15  |
| itb12g19660.11_itf12g19260.11 | Segmental replication | 0.01 | 0.04 | 0.14  |
| itb12g23320.11_itf12g22980.11 | Segmental replication | 0.00 | 0.02 | 0.00  |
| itb14g15470.11_itf14g14040.11 | Segmental replication | 0.02 | 0.07 | 0.32  |
| itf11g07340.12_itf11g07340.13 | Tandem | 0.08 | 0.06 | 1.34  |
| itf11g07340.13_itf11g07340.14 | Tandem | 0.03 | 0.04 | 0.71  |
| itf12g19260.11_itf12g19260.12 | Tandem | 0.02 | 0.02 | 0.62  |
| itf11g00750.11_itf11g00750.12 | Tandem | 0.00 | 0.00 | 0.30  |
| itf11g07630.12_itf11g07630.13 | Tandem | 0.01 | 0.01 | 0.59  |

3.6. Expression Patterns of the nifH Genes in the Sweet Potato, I. trifida and I. triloba

In the sweet potato, nearly half of the identified nifH genes (9 genes) were nonregulated in all of detected tissues. The others were mainly upregulated in the fibrous roots (FR) and leaves and were mainly downregulated in the proximal ends (PE) and root stalks (RS) (Figure 5). In the I. trifida, 8, 6, 1, 4 and 2 of the 19 identified nifH genes were upregulated in the callus flowers, callus stems, flowers, flower buds, leaves and stems, respectively, and other genes in the tissue mentioned above or the 19 identified nifH genes in other tissues (i.e., root1 and root2) were mainly downregulated (Figure 5). In the I. triloba, 3, 1, 10, 5 and 4 of the 17 identified nifH genes were upregulated in the flowers, flower buds, leaves, root1, root2 and stems, respectively, whereas other genes in the tissue mentioned above were mainly downregulated (Figure 5).
Figure 5. Heatmaps of expression profiles for nifH genes in multiple tissues: (A) Expression profiles of nifH genes in different sweet potato tissues: fibrous roots (FR), initiative storage roots (ISR), leaves, distal ends (DE), proximal ends (PE), root bodies (RB) and root stalks (RS); (B) Expression profiles of nifH genes in different I. trifida tissues: callus flowers, callus stems, flowers, flower buds, root1, root2, leaves and stems; (C) Expression profiles of nifH genes in different I. triloba tissues: root1, root2, flowers, flower buds, leaves and stems.

3.7. qRT-PCR Analysis of the nifH Genes under Treatments

According to the above results, four nifH genes of the sweet potato (g950, g16683, g27094 and g33987) were selected for qRT-PCR analysis. All of the four genes were up-regulated in N1 treatment and were consistent with the above analysis of RNA-seq data (Figure 6). Compared with the control conditions (0 h), the transcripts of the four nifH genes all peaked after 3 h N1 treatment in the two sweet potato cultivars. Then, the expression level declined, and the expression level of Eshu 15 was higher than that of Long 9 (Figure 6). In Eshu 15, g950, peaked at 3 h with an 8.11 fold higher expression level than that of the control, which was of the highest expression levels of the four nifH genes, followed by g27094 (7.99 fold), g33987 (3.66 fold) and g16683 (3.58 fold). In Long 9, g27094, peaked at 3 h with a 6.60 fold higher expression level than that of the control, which was of the highest expression levels of the four nifH genes, followed by g950 (5.16 fold), g33987 (3.21 fold) and g16683 (2.44 fold) (Figure 6). However, in the N0 treatment, the transcripts of g950, g16683 and g33987 had all almost no change compared with the control conditions (0 h), and the transcripts of g27094 peaked after 3 h N0 treatment, with expression levels of only 1.69 and 1.69 fold higher than that of the control in Eshu 15 and Long 9, respectively (Figure 6).
Figure 6. Expression analysis of g950, g16683, g27094 and g33987 of Eshu 15 and Long 9. The significance of differential gene expression levels compared with control are denoted as *<0.05, **<0.01.

4. Discussion

Nitrogen is an essential element for plants, and it is also one of the most important limiting factors for obtaining a high agricultural yield. Biological nitrogen fixation is an important part of the terrestrial nitrogen cycle, which contributes 90–130 Tg N to the biosphere every year and is mainly completed by bacteria and leguminous plants [49]. nifH is a marker gene, and researchers have been able to characterize aspects of the diversity and ecology of nitrogen-fixing bacteria and archaea. The biological nitrogen fixation of plants, rather than by association with microorganisms, can generate crops that are less dependent on synthetic nitrogen fertilizers and can increase agricultural productivity and sustainability [50]. The sweet potato, the seventh largest food crop in the world [2], grows well in nitrogen-poor infertile soils, and it is believed that sweet potato endo- and epiphytic microorganisms play important roles in acting upon this growth ability in this plant [21–24]. Are these all the reasons? Do the sweet potato and its wild ancestors harbor nifH genes, even with nitrogen fixation potentiality?

Previous studies have demonstrated that, among the 291 tested accessions of cultivated sweet potatoes, all contained one or more transfer DNA (T-DNA) sequences, which is believed to be harbored by Agrobacterium [51]. The study suggested that an agrobacterium infection occurred in evolutionary times, and the T-DNA integration, the interruption of an F-box gene and the subsequent fixation of foreign T-DNA into the sweet potato genome occurred during the evolution and domestication of the sweet potato [51]. Based on the above demonstration, the sweet potato and its wild ancestors harboring nifH genes is possible.

In order to answer the question mentioned above, in this present study, the genome-wide identification of nifH genes was conducted for the first time in this study. A total of 20, 19 and 17 nifH genes were identified in the genome of the sweet potato, I. trifida and I. trifida, respectively. The number of nifH genes in the three investigated species was comparable. The phylogenetic analysis revealed that the identified nifH genes can be clustered into five independent clades: I, II, III, IV and V, with high support values. Moreover, each of the five clades contained the nifH genes from all of the three species, which indicated that the ancestries for each clade were differentiated before the species
specification of the \textit{nifH} genes. All of the \textit{nifH} genes could be located on chromosomes of the three species, and the distribution of them on sweet potatoes were different from \textit{I.trifida} and \textit{I.triloba}. Previous reports have suggested that the whole-genome triplication (WGT) occurred in an ancient ancestor of the \textit{Ipomoea} lineage around 46.1 million years ago (Mya), much earlier than the divergence of \textit{I.nil} from the lineage containing \textit{I.trifida} and \textit{I.triloba} (~3.6 Mya) and the \textit{I.trifida-I.triloba} divergence (~2.2 Mya). The results of the comparison between the genomes of \textit{I.trifida} (or \textit{I.triloba}) and \textit{I.nil} limited large-scale interchromosomal rearrangements over the last 3.6 million years [52], and in the sweet potato, two recent whole-genome duplication (WGD) events occurred about 0.8 and 0.5 million years ago [53]. Therefore, the two recent whole-genome duplications in the sweet potato may be the reason for the discrepancies of chromosome distribution.

Conserved motif detection and gene structure analyses showed that the detected motifs behaved with different degrees of conservation among the \textit{nifH} genes, and the \textit{nifH} genes clustered in the same phylogenetic branch showed a more similar distribution of conserved motifs and exons–introns than that of the other \textit{nifH} genes. Similar results have been found in other gene families in various species, such as the WRKY gene family in pineapples [54], the AP2/ERF gene family in buckwheat [55], the superoxide dismutase (SOD) gene family in rapeseed [56], etc.

Segmental and tandem duplications have significantly contributed to gene family expansion in plants [57,58]. The duplication analysis in the present study shows that no segmental duplication was found in each genome of the three \textit{Ipomoea} species, and 0, 8 and 7 tandemly duplicated gene pairs were detected in the genome of the sweet potato, \textit{I.trifida} and \textit{I.triloba}, respectively. The results suggest that there were no segmental duplications of \textit{nifH} genes through polyploidy followed by chromosome rearrangements, and in several members of them in \textit{I.trifida} and \textit{I.triloba}, there occurred duplications within the same intergenic region or in neighboring intergenic regions of their genomes [59]. A total of 7, 7 and 8 syntenic gene pairs of \textit{nifH} genes were detected between the sweet potato and \textit{I.trifida}, between the sweet potato and \textit{I.triloba} and between \textit{I.trifida} and \textit{I.triloba}, respectively. Similar results have been demonstrated by other closely related species. For example, in the Brassicaceae family, a number of NBS loci were identified: \textit{Arabidopsis lyrata} (#78), \textit{A.thaliana} (#58), \textit{Brassica rapa} (#100), \textit{Capsella rubella} (#52) and \textit{Thellungiella salsuginea} (#59) [57]. These may be generated by different extents of the gene duplication of the ancestor genes [60]. The Ka/Ks analysis of the duplicated and syntenic \textit{nifH} genes in the three \textit{Ipomoea} species revealed that nearly all of the duplicated and syntenic \textit{nifH} genes were subjected to purifying selection inside duplicated genomic elements during speciation, since they had a ratio of Ka/Ks < 1 [61].

The expression profile of the \textit{nifH} genes showed that all but nine of the sweet potato genes detected upregulation or downregulation in various tissues of their corresponding species. Four \textit{nifH} genes of the sweet potato were selected for qRT-PCR analysis, and the expressions were consistent with the transcriptome data analysis. It was also found that the \textit{nifH} genes expressed differently in the three species, and the \textit{nifH} genes expressed differently in the different tissues of the same species as well. The results suggest that the \textit{nifH} genes experienced functional differentiation after the whole genome duplication. The phenomenon of the member of the family gene acting differently in expression profiles has been reported in various studies, for example, in wheat [62], tomatoes [63], tea [64], etc., and even in the duplicated genes [65]. In potatoes, it was found that there was variation within tandemly duplicated genes among cultivated, non-cultivated and wild potato genotypes in terms of bias in functional specificities, the proportion of lineage-specific clusters, diverged expression and promoter similarities [66].

5. Conclusions

In this study, A total of 20, 19 and 17 \textit{nifH} genes were identified for the first time in the sweet potato, \textit{I.trifida} and \textit{I.triloba}, respectively. Following the identification, a phylogenetic tree was formed to cluster the identified \textit{nifH} genes into five independent clades. All of the
nifH genes could be located on chromosomes of the three species, and the distribution of them on sweet potatoes were different from I. trifida and I. triloba. The expression profiles revealed that the nifH genes were expressed differently in various tissues of the three species. A total of 4 nifH genes of the sweet potato were selected for qRT-PCR analysis in two sweet potato cultivars (Eshu 15 and Long 9) under nitrogen deficiency (N0) and normal (N1) conditions. The results acquired in this study may provide new insight into the nifH genes in the sweet potato and its wild ancestors, and they may contribute to the nitrogen-fixing breeding of sweet potatoes in the future.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes13081428/s1, Table S1: Primers used in qRT-PCR.

Author Contributions: Design of the study: Z.S. and L.W; identification of nifH genes in the four Ipomoea species: Z.S. and L.W; motif analysis: Z.S., L.W. and Y.Q.; phylogenetic analysis: Z.S., L.W. and Z.J.; chromosome location: Z.S., M.Z. and C.W.; duplication pattern analysis: Z.S., L.W. and Z.J.; syntenic analysis: Z.S., L.W., Y.Q., M.Z. and Z.J.; Ka/Ks analysis: Z.S., M.Z. and C.W.; expression profile analysis: Z.S., M.Z. and Y.Q.; qRT-PCR analysis: Z.S., C.W. and L.W.; manuscript preparation: Z.S., L.W., Y.Q., M.Z., Z.J. and C.W. All authors have read and agreed to the published version of the manuscript.

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References
1. Liu, Q. Sweet potato omics and biotechnology in China. *Plant Omics* 2011, 4, 295–301.
2. Liu, Q. Improvement for agronomically important traits by gene engineering in sweetpotato. *Breed. Sci.* 2017, 67, 15–26. [CrossRef] [PubMed]
3. Gaby, J.C.; Buckley, D.H. A comprehensive evaluation of pcr primers to amplify the nifH gene of nitrogenase. *PLoS ONE* 2012, 7, e42149. [CrossRef] [PubMed]
4. Rubio, L.M.; Ludden, P.W. The gene products of the nif regulon. In *Nitrogen Fixation at the Millennium*; Elsevier Science: Amsterdam, The Netherlands, 2002; pp. 101–136.
5. Man-Aharonovich, D.; Kress, N.; Zeev, E.B.; Berman-Frank, I.; Béjà, O. Molecular ecology of nifH genes and transcripts in the eastern mediterranean sea. *Environ. Microbiol.* 2007, 9, 2354–2363. [CrossRef]
6. Rösch, C.; Bothe, H. Diversity of total, nitrogen-fixing and denitrifying bacteria in an acid forest soil. *Eur. J. Soil Sci.* 2009, 60, 883–894. [CrossRef]
7. Mehta, M.P.; Butterfield, D.A.; Baross, J.A. Phylogenetic diversity of nitrogenase (nifH) genes in deep-sea and hydrothermal vent environments of the juan de fuca ridge. *Appl. Environ. Microbiol.* 2003, 69, 960–970. [CrossRef]
8. Héry, M.; Philippot, L.; Mériaux, E.; Poly, F.; Le Roux, X.; Navarro, E. Nickel mine spoils revegetation attempts: Effect of pioneer plants on two functional bacterial communities involved in the N-cycle. *Environ. Microbiol.* 2005, 7, 486–498. [CrossRef]
9. Yamada, A.; Inoue, T.; Noda, S.; Hongob, Y.; Ohkuma, M. Evolutionary trend of phylogenetic diversity of nitrogen fixation genes in the gut community of wood-feeding termites. *Mol. Ecol.* 2007, 16, 3768–3777. [CrossRef]
10. Roesch, L.F.W.; Camargo, F.A.; Bento, F.M.; Triplett, E.W. Biodiversity of diazotrophic bacteria within the soil, root and stem of field-grown maize. *Plant Soil* **2008**, *302*, 91–104. [CrossRef]

11. Swain, H.; Abhijitita, S. Nitrogen fixation and its improvement through genetic engineering. *J. Glob. Biosci.* **2013**, *2*, 98–112.

12. Schulte, C.C.; Borah, K.; Wheatley, R.M.; Terpolilli, J.J.; Saalbach, G.; Crang, N.; de Groot, D.H.; Ratcliffe, R.G.; Kruger, N.J.; Papachristodoulou, A.; et al. Metabolic control of nitrogen fixation in rhizobium-legume symbioses. *Sci. Adv.* **2021**, *7*, eabk2433. [CrossRef]

13. Herrero, A.; Muro-Pastor, A.M.; Flores, E. Nitrogen control in cyanobacteria. *J. Bacteriol.* **2001**, *183*, 411–425. [CrossRef]

14. Ekman, M.; Tollbäck, P.; Bergman, B. Proteomic analysis of the cyanobacterium of the azolla symbiosis: Identity, adaptation, and *nfH* modification. *J. Exp. Bot.* **2007**, *59*, 1023–1034. [CrossRef]

15. Bhuvaneshwari, K.; Singh, P.K. Response of nitrogen-fixing water fern Azolla biofertilization to rice crop. *3 Biotech* **2015**, *5*, 523–529. [CrossRef]

16. Eily, A.N.; Pryer, K.M.; Li, F.W. A first glimpse at genes important to the azolla–nostoc symbiosis. *Symbiosis* **2019**, *78*, 149–162. [CrossRef]

17. Chen, W.X.; Tan, Z.Y.; Gao, J.L.; Li, Y.; Wang, E.T. *Rhizobium hainanense* sp. Nov., isolated from tropical legumes. *J. Bacteriol.* **2002**, *48*, 860–862. [CrossRef]

18. Cheng, Q.; Sun, W. Expression of nitrogen structural scaffold genes in higher plant. *BAOJ Biotech* **2018**, *4*, 038. [CrossRef]

19. Jiang, X.; Payá-Tormo, L.; Coroian, D.; García-Rubio, I.; Castellanos-Rueda, R.; Eseverri, A.; Lpez-Torrejón, G.; Burén, S.; Rubio, L.M. Exploiting genetic diversity and gene synthesis to identify superior nitrogenase *nfH* protein variants to engineer N2-fixation in plants. *Commun. Biol.* **2021**, *4*, 4. [CrossRef]

20. Klarenberg, I.J.; Keuschnig, C.; Colmenares, A.; Warshon, D.; Jungblut, A.D.; Jóttir, I.; Vilhelmsson, O. Long-term warming effects on the microbiome and *nfH* gene abundance of a common moss species in sub-arctic tundra. *New Phytol.* **2022**, *204*, 2044–2056. [CrossRef]

21. Itoh, K.; Ohashi, K.; Yakai, N.; Adachi, F.; Hayashi, S. Changes in acetylene reduction activities and *nif H* gene expression associated with field-grown sweet potatoes with different nursery farmers and cultivars. *Horticulture 2019*, *5*, 53. [CrossRef]

22. Hill, W.A.; Hortense, D.; Hahn, S.; Mulongoy, K.; Adeyeye, S. Sweet potato root and biomass production with and without nitrogen fertilization. *Agron. J.* **1990**, *82*, 1120–1122. [CrossRef]

23. Yonebayashi, K.; Katsumi, N.; Nishi, T.; Okayama, M. Activation of nitrogen-fixing endophytes is associated with the tuber growth of sweet potato. *Mass Spectrom.* **2014**, *3*, 10032. [CrossRef]

24. Yoneyama, T.; Terakado, J.; Masuda, T. Natural abundance of 15n in sweet potato, pumpkin, sorghum and castor bean: Possible contribution of n2-derived nitrogen in sweet potato. *Biol. Fertil. Soils* **1997**, *26*, 152–154. [CrossRef]

25. Hill, W.A.; Bacon-Hill, P.; Crossman, S.M.; Stevens, C. Characterization of N2-fixing bacteria associated with sweet potato roots. *Can. J. Microbiol.* **1983**, *29*, 860–862. [CrossRef]

26. Paula, M.d.; Reis, V.; Diöbereiner, J. Interactions of glomus clarum with acetobacter diazotrophicus in infection of sweet potato (*Ipomoea batatas*), sugarcane (*Saccharum spp.*), and sweet sorghum (*Sorghum vulgare*). *Biol. Fertil. Soils* **1991**, *11*, 111–115. [CrossRef]

27. Adachi, K.; Nakatani, M.; Mochida, H. Isolation of an endophytic diazotroph, *klebsiella oxytoca*, from sweet potato stems in Japan. *Soil Sci. Plant Nutr.* **2002**, *48*, 889–895. [CrossRef]

28. Assis Jr, C.; Adachi, K. Isolation of endophytic diazotrophic pantoea agglomerans and nondiazotrophic enterobacter asburiae from sweetpotato stem in Japan. *Lett. Appl. Microbiol.* **2004**, *38*, 19–23. [CrossRef] [PubMed]

29. Terakado-Tonooka, J.; Fujihara, S.; Ohwaki, Y. Possible contribution of bradyrhizobium on nitrogen fixation in sweet potatoes. *Plant Soil* **2013**, *367*, 639–650. [CrossRef]

30. Khan, Z.; Doty, S.L. Characterization of bacterial endophytes of sweet potato plants. *Plant Soil* **2009**, *322*, 197–207. [CrossRef]

31. Shao, Z.Q.; Zhang, Y.M.; Hang, Y.Y.; Xue, J.Y.; Zhou, G.C.; Wu, P.; Wu, X.Y.; Wu, X.Z.; Wang, Q.; Wang, B.; et al. Long-term evolution of nucleotide-binding site-leucine-rich repeat genes: Understanding gained from and beyond the legume family. *Plant Physiol.* **2014**, *166*, 217–234. [CrossRef] [PubMed]

32. Sievers, F.; Higgins, D.G. Clustal omega for making accurate alignments of many protein sequences. *Protein Sci.* **2018**, *27*, 135–145. [CrossRef]

33. Sievers, F.; Wilm, A.; Dineen, D.; Gibson, T.J.; Karplus, K.; Li, W.; Lopez, R.; McWilliam, H.; Remmert, M.; Söding, J.; et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* **2011**, *7*, 539. [CrossRef]

34. Liu, Y.; Zeng, Z.; Zhang, Y-M.; Li, Q.; Jiang, X-M.; Jiang, Z.; Tang, J.-H.; Chen, D.; Wang, Q.; Chen, J.-Q.; et al. An angiosperm *nrl* atlas reveals that *nrl* gene reduction is associated with ecological specialization and signal transduction component deletion. *Mol. Plant* **2021**, *14*, 2015–2031. [CrossRef]

35. Minh, B.Q.; Nguyen, M.A.; von Haeseler, A. Ultrafast approximation for phylogenetic bootstrap. *Mol. Biol. Evol.* **2013**, *30*, 1188–1195. [CrossRef]

36. Kalyaanamoorthy, S.; Minh, B.Q.; Wong, T.K.F.; von Haeseler, A.; Jermiin, L.S. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* **2017**, *14*, 587–589. [CrossRef]
37. Anisimova, M.; Gil, M.; Dufayard, J.F.; Dessizou, C.; Gascuel, O. Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst. Biol.* **2011**, *60*, 685–699. [CrossRef]

38. Meyers, B.C.; Kozik, A.; Griego, A.; Kuala, H.; Michelmore, R.W. Genome-wide analysis of NBS-LRR-encoding genes in arabidopsis. *Plant Cell* **2003**, *15*, 809–834. [CrossRef]

39. Timothy, L.B. MEME SUITE: Tools for motif discovery and searching. *Nucleic Acids Res.* **2009**, *37*, 202–208.

40. Chen, C.; Xia, R.; Chen, H.; He, Y. Ttools, a toolkit for biologists integrating various his-data handling tools with a user-friendly interface. *BioRxiv* **2018**, *2018*, 289660.

41. Voorrips, R.E. Mapchart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* **2002**, *93*, 77–78. [CrossRef]

42. Wang, Y.; Tang, H.; DeBarry, J.D.; Tan, X.; Li, J.; Wang, X.; Lee, T.-H.; Jin, H.; Marler, B.; Guo, H.; et al. MCScanX: A toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res.* **2012**, *40*, e49. [CrossRef]

43. Krzywinski, M.; Schein, J.; Birol, I.; Connors, J.; Gascoyne, R.; Horsman, D.; Jones, S.J.; Marra, M.A. Circos: An information aesthetic for comparative genomics. *Genome Res.* **2009**, *19*, 1639–1645. [CrossRef]

44. Suyama, M.; Torrents, D.; Bork, P. PAL2NAL: Robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Res.* **2006**, *34*, 609–612. [CrossRef]

45. Yang, Z. PAML 4: Phylogenetic Analysis by Maximum Likelihood. *Mol. Biol. Evol.* **2007**, *24*, 1586–1591. [CrossRef]

46. Wen, G. A simple process of RNA-sequence analyses by Hisat2, Htsseq and DSESeq2. In Proceedings of the 2017 International Conference on Biomedical Engineering and Bioinformatics, Bangkok, Thailand, 14–16 September 2017; pp. 11–15.

47. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. The sequence alignment/map format and samtools. *Bioinformatics* **2009**, *25*, 2078–2079. [CrossRef]

48. Howe, E.A.; Sinha, R.; Schlauch, D.; Quackenbush, J. Rna-seq analysis in MeV. *Bioinformatics* **2011**, *27*, 3209–3210. [CrossRef]

49. Galloway, J.N.; Schlesinger, W.H.; Levy, H.; Michaels, A.; Schnoor, J.L. Nitrogen fixation: Anthropogenic enhancement-environmental response. *Glob. Biogeochem. Cycles* **1995**, *9*, 235–252. [CrossRef]

50. Payá-Tormo, L.; Coroian, D.; Martín-Muñoz, S.; Badalyan, A.; Green, R.T.; Veldhuizen, M.; Jiang, X.; López-Torrejón, G.; Balk, J.; Seefeldt, L.C. A colorimetric method to measure in vitro nitrogenase functionality for engineering nitrogen fixation. *Sci. Rep.* **2022**, *12*, 10367. [CrossRef] [PubMed]

51. Kyndt, T.; Quispe, D.; Zhai, H.; Jarrett, R.; Ghislain, M.; Liu, Q.; Ghesyen, G.; Kreuze, J.F. The genome of cultivated sweet potato contains agrobacterium t-dnas with expressed genes: An example of a naturally transgenic food crop. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, 5844–5849. [CrossRef] [PubMed]

52. Wu, S.; Lau, K.H.; Cao, Q.; Hamilton, J.P.; Sun, H.; Zhou, C.; Eserman, L.; Gemenet, D.C.; Olukolu, B.A.; Wang, H.; et al. Genome sequences of two diploid wild relatives of cultivated sweetpotato reveal targets for genetic improvement. *Nat. Commun.* **2018**, *9*, 4580. [CrossRef]

53. Yang, J.; Moeinzadeh, M.-H.; Kuhl, H.; Helmuth, J.; Xiao, P.; Haas, S.; Liu, G.; Zheng, J.; Sun, Z.; Fan, W.; et al. Haplotype-resolved sweet potato genome traces back its hexaploidization history. *Nat. Plants* **2017**, *3*, 696–703. [CrossRef]

54. Xie, T.; Chen, C.; Li, C.; Liu, J.; Liu, C.; He, Y. Genome-wide investigation of WRKY gene family in pineapple: Evolution and expression profiles during development and stress. *BMC Genom.* **2018**, *19*, 490. [CrossRef]

55. Liu, M.; Sun, W.; Ma, Z.; Zheng, T.; Huang, L.; Wu, Q.; Chen, H. Genome-wide investigation of the AP2/ERF gene family in tartary buckwheat (*Fagopyrum tataricum*). *BMC Plant Biol.* **2019**, *19*, 84. [CrossRef]

56. Su, W.; Raza, A.; Gao, A.; Jia, Z.; Zhang, Y.; Hussain, M.A.; Zou, X. Genome-wide analysis and expression profile of superoxide dismutase (SOD) gene family in rapeseed (*Brassica napus* L.) under different hormones and abiotic stress conditions. *Antioxidants* **2021**, *10*, 1182. [CrossRef]

57. Cannon, S.B.; Mitra, A.; Baumgarten, A.; Young, N.D.; May, G. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol.* **2004**, *4*, 10. [CrossRef]

58. Jiang, S.Y.; González, J.M.; Ramachandran, S. Comparative genomic and transcriptomic analysis of tandemly and segmentally duplicated genes in rice. *PLoS ONE* **2013**, *8*, e63551. [CrossRef]

59. 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]

60. Zhang, Y.M.; Shao, Z.Q.; Wang, Q.; Hang, Y.Y.; Xue, J.Y.; Wang, B.; Chen, J.Q. Uncovering the dynamic evolution of nucleotide-binding site-leucine-rich repeat (NBS-LRR) genes in Brassicaceae. *J. Integr. Plant Biol.* **2016**, *58*, 165–177. [CrossRef]

61. Gaut, B.S.; Doebly, J.F. DNA sequence evidence for the segmental allotetraploid origin of maize. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 6809–6814. [CrossRef]

62. Kesawat, M.S.; Kherawat, B.S.; Singh, A.; Dey, P.; Kabir, M.; Debnath, D.; Saha, D.; Khandual, A.; Rout, S.; Manorama; et al. Genome-wide identification and characterization of the *brassinazole-resistant* (*BZR*) gene family and its expression in the various developmental stage and stress conditions in wheat (*Triticum aestivum* L.). *Int. J. Mol. Sci.* **2021**, *22*, 8743. [CrossRef]

63. El-Sappah, A.H.; Elrys, A.S.; Desoky, E.-S.M.; Zhao, X.; Bingwen, W.; El-Sappah, H.H.; Zhu, Y.; Zhou, W.; Zhao, X.; Li, J. Comprehensive genome wide identification and expression analysis of MTP gene family in tomato (*Solanum lycopersicum*) under multiple heavy metal stress. *Saudi J. Biol. Sci.* **2021**, *28*, 6946–6956. [CrossRef] [PubMed]
64. Zhang, S.; Liu, J.; Zhong, G.; Wang, B. Genome-Wide Identification and Expression Patterns of the C2H2-Zinc Finger Gene Family Related to Stress Responses and Catechins Accumulation in <i>Camellia sinensis</i> [L.] O. Kuntze. <i>Int. J. Mol. Sci.</i> <b>2021</b>, <i>22</i>, 4197. [CrossRef]

65. Li, W.H.; Yang, J.; Gu, X. Expression divergence between duplicate genes. <i>Trends Genet.</i> <b>2005</b>, <i>21</i>, 602–607. [CrossRef]

66. Bonthala, V.S.; Stich, B. Genetic divergence of lineage-specific tandemly duplicated gene clusters in four diploid potato genotypes. <i>Front. Plant Sci.</i> <b>2022</b>, <i>13</i>, 875202. [CrossRef] [PubMed]