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Soybean *GmVIT1* Gene Confers Plant Tolerance to Excess Fe/Mn Stress

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Abstract: Iron (Fe) and (Mn) are essential for the plant but are toxic when in excess. Vacuolar iron transporters (VITs) are involved in plant metal storage and detoxication. In this study, we screened two soybean cultivars (HN51 and SN37) with different responses to iron stress. From HN51 and SN37, we identified a new gene *GmVIT1*, for which expression is closely related to iron stress response by transcriptomic and quantitative analysis. We obtained *GmVIT1* and *GmVIT1* promoter from the iron deficiency-tolerant soybean variety Heinong51. Sequence analysis showed that GmVIT1 contained a conserved 170-residue VIT domain and localized at the tonoplast. Moreover, *GmVIT1* is expressed in soybean leaves, stems, and roots. The expression of *GmVIT1* was significantly induced by excessive Fe/Mn in leaves and stems. GUS assay showed that excess Fe/Mn enhanced *GmVIT1* promoter activity. Furthermore, overexpression of *GmVIT1* in Arabidopsis seedlings showed reduced phytotoxic effects induced by excess Fe/Mn stress, including yellowing in leaves, decreased chlorophyll content, and accumulated MDA. *GmVIT1* overexpression in Arabidopsis showed relatively higher soluble sugar content and SOD, POD, and CAT activity. In addition, the ferric reductase activity in *GmVIT1* overexpression in Arabidopsis decreased under excess Fe, while it increased under excess Mn. By integrating all these results, we found that *GmVIT1* plays a vital role in plant response to excess Fe/Mn. The results showed that *GmVIT1* was worthy of metal homeostasis mechanism research in plants and could be applied in the metal toxic-tolerance improvement in crops.

Keywords: soybean; *GmVIT1*; transporter; iron; manganese

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1. Introduction

Iron (Fe) and manganese (Mn) are essential micronutrients for plants, have redox-active, enzyme-activating functions, and fulfill a structural role in stabilizing proteins [1,2]. Either Fe or Mn deficiency in plants disturbs many biological processes and impairs chlorophyll and organic matter biosynthesis [3]. Although Fe is indispensable for plants, excess accumulation of Fe causes the overproduction of reactive oxygen species (ROS) via the Fenton reaction, which irreversibly impairs cellular and chromatin structure, triggering lipid peroxidation of cellular membrane structures and loss of cellular integrity [4–6]. Excess Fe easily appears in waterlogged soils, where soluble Fe concentrations cause a drastic reduction in redox potential. Excessive Fe absorption by plants leads to the appearance of Fe toxicity symptoms called "bronzing", brown spots spread from the leaf tips to the leaf base [7]. Yield reductions of 10–100% occur in the case of the world's leading crop species. Fe toxicity is considered one of the most formidable research challenges in rice and wheat cultivation [8,9]. Similarly, plants need Mn in only small quantities and it is toxic in excess. The quantum yield of PSII, CO_2 assimilation rate, and stomatal conductance are decreased by Mn toxicity. Furthermore, Mn toxicity generates severe oxidative stress in plant species such as barley [10], cucumber [11], rice [12], and ryegrass [13]. Accumulation of Mn oxides leads to a reduction in shoot biomass, while leaves exhibit toxicity symptoms,

including proximal epidermal hypertrophy, and formation of necrotic areas [14]. It has been documented that the uptake of Mn^{2+} and that of other divalent cations (such as Ca^{2+}, Mg^{2+}, or Fe^{2+}) are closely interrelated, attributed to competition between ions for the absorption site [15,16]. Chlorotic leaves and necrotic spots are the most common symptoms of Mn toxicity [17,18]. One-third of soils in the world are acidic. Mn toxicity is common in poorly drained and acidic soils, and reduces food production [15].

Tight control of Fe and Mn acquisition and translocation is crucial for all plants' survival and proliferation. As a pivotal intracellular storage organelle, vacuole is essential for regulating metal homeostasis, especially in excess metal detoxification [19]. In addition to the storage role, vacuoles also facilitate the long-distance transport of metals by regulating vacuolar sequestration, which is mainly determined by the interaction between cytoplasmic metal chelators and tonoplast-localized transporters [20]. Transporters at the tonoplast mitigate the toxic effects of excessive metal concentrations on cells by controlling the storage and transport of toxic ions [19]. Some vacuolar metal transporters have been identified, including natural resistance-associated macrophage proteins (NRAMPs) [21,22], metal-tolerance proteins (MTPs) [23], and iron-regulated transporters (IRTs) [24]. Ordinarily, tonoplast transporters have multiple putative trans-membrane domains (TMDs), which carry highly conserved characteristic sequences related to metal selectivity [25]. Vacuolar iron transporters (VITs) are similar to the above proteins. First, *VIT* genes were identified from *Saccharomyces cerevisiae* [26,27], and then isolated in plants including Arabidopsis [28], rice [29], rape [30], wheat [31], barley [32], and so on. Recently, *VIT* was also found as a detoxifier in *Plasmodium falciparum* [33]. The VIT family proteins contain the DUF125 motif in plants [34]. Phylogenetic analysis revealed that VITs have fewer than four transmembrane domains.

The expression of gene in plant tissues suggests their roles at developmental stages. *AtVIT1* in Arabidopsis and *OsVIT1*, *HvVIT1*, *HvVIT1-2*, *TaVIT1*, *TaVIT2*, and *TaVIT2D* in cereals are determined highly expressed in seed embryo and flag leaves [31,32,35,36]. In addition to seeds, *VIT*s are also expressed in the roots and leaves, and nodules [37], indicating that VITs function in all developmental stages of plants. In previous research, *VIT*s were expressed in reproductive organs, while *VIT*s in vegetative organs have received less attention. It was speculated that VITs transport Fe, Mn, Zn, and other metals [37–39]. *VIT* and its homologs can ultimately rescue the growth of Δ*ccc1* yeast under iron toxicity [35,40]. Previous studies found that the *VIT* family members play a crucial role in excess metal detoxification through the sequestration of Fe from the cytosol to the vacuolar in cells [33,35,40,41]. VIT might participate in Fe accumulation in the endodermal cell layer [42].

*VIT*s are essential genes in plant seeds' Fe accumulation by regulating metal partitioning between source and absorbing organs. The seed Fe concentration in the *nramp3/nramp4* mutant overexpressing *AtVTL1*, *AtVTL2*, or *AtVTL5* was 50-60% higher than that in non-transformed double mutants or wild-type plants [43]. In rice, *OsVIT1* and *OsVIT2* mediated the Fe^{2+} and Mn^{2+} uptake and transpot in flag leaf [37]. OsVIT2 distributes Fe to the grains by sequestering Fe into vacuoles in the mestome sheath, nodes, and aleurone layer [44]. *VIT*s also play an important role in Fe homeostasis and distribution in plants. In the *vit1* mutant of Arabidopsis, Fe diffuses from provascular tissues to the subepidermal cells on the abaxial side of the cotyledon [35]. The constitutive expression of *TaVIT2D* in *vit*1 mutant increased the root, leaf, and seed Fe accumulation [36]. AtVIT1 transports Fe into the vacuole for normal seedling development when exposed to excess iron [35]. Excess Fe significantly induces *Brassica napa BnMEB2* gene expression in old leaves and roots [30]. *BnMEB2* overexpressed plants restored the phenotypes with apparent iron toxicity in roots [30]. In tulip and cornflower petals, Fe ions transported by TgVIT1 and CcVIT combine with anthocyanins in vacuoles to form blue stable chelates [45,46]. In recent years, the application of *VIT* biofortification has increased significantly [31,47]. These results demonstrate enhancing vacuolar Fe transport in the specific organization as a viable transgenic strategy to biofortify. They could contribute to improved Fe nutrition to help

eliminate micronutrient malnutrition in at-risk human populations. However, being a well-known iron storage protein, the detoxification function of excess Fe of VIT has yet to be well-investigated in the plant seedling stage. The underlying mechanism also needs to be elucidated. Further, the detoxification function on excess Mn of VIT is yet to receive notice, nor has it been explicitly studied.

As a major oil crop in the world, soybean is also an iron-sensitive plant. However, soybeans are more sensitive to Fe and Mn stress than other legumes, such as *Centrosema pubescens* and *Phaseolus lathyroide* [48–50]. Although the reports of *GmVTL1* involved nitrogen fixation [40] and GmVTL1a transporting of ferrous Fe from the infected root cell cytosol to the symbiosome [51], research on *VIT* genes in soybean still needs to be conducted. We found an evolutionarily unique *VIT* gene that responds to iron deficiency from transcriptomic analysis. The qRT-PCR results showed that iron deficiency and excess iron significantly up-regulated *GmVIT1* in HN51, while not significantly in SN37. The results indicated that the *GmVIT1* gene is directly related to the different responses to iron stress of HN51 and SN37. Based on the *VIT* family gene function in the model plants, *GmVIT1* might respond to excessive heavy metals such as Fe and Mn and play a role in plant heavy metal detoxification. To prove the function of *GmVIT1*, we cloned its cds and promoter, analyzed its expression pattern response to excess iron and excess manganese, and explored whether it participated in plant resistance to heavy metal stress by the transgenic method. We hope this work will deepen the understanding of the mechanism of *VIT* family members in heavy metal stress and provide a candidate gene resource for soybean breeding improvement.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The *Glycine Max* L. cv. Heinong 51 (HN51) and *Glycine Max* L. cv. Suinong 37 (SN37) were supplied by the Crop Tillage and Cultivation Institute, Heilongjiang Academy of Agricultural Sciences. The soybean seeds were soaked in 5% sodium hypochlorite solution for 5 min, disinfected, and rinsed with sterile water. After germination in the dark, soybean seeds were transferred to 1/2 Hoagland nutrient solution. Wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0) seeds were sterilized with 70% (*v*/*v*) ethanol, followed by 10% NaClO, and rinsed three times with sterile water, and germinated on one-half strength MS medium supplemented with 0.9% agar plates in an incubator with a light/dark cycle of 16 h/8 h at 23 °C, and 60% relative humidity. Subsequently, the Arabidopsis seedlings were cultured in pots.

2.2. Iron Deficiency Tolerance Detection

The HN51 and SN37 soybean seedlings were cultured in 1/2 Hoagland solution to the trefoil stage and treated with 0 mM Fe-EDTA (−Fe) and 0.1 mM Fe-EDTA (the control) for 16 days. The hydroponic solution was replaced every 4 days. The root length, the lateral root number, and the iron content were detected. We performed a transcriptomic analysis on HN51 and SN37 soybean seedlings exposed to iron deficiency. The soybean samples were dried and ground into powder, and 0.05 g samples were put into the digestion tube, and had 4 mL 65% nitric acid added and digested at 120 °C for 90 min. When temperature dropped to room temperature, 1 mL of 30% hydrogen peroxide was added into the digestive tube and heated at 120 °C for 90 min. After dropping to room temperature, 750 µL of 30% hydrogen peroxide was added and kept until transparent. Finally, ddH_2O was used to constant volume to 50 mL. The iron content was determined by an inductively coupled plasma emission spectrometer (PE ICP-OES8000).

2.3. Cloning of GmVIT1 and GmVIT1 Promoter

Total RNA was extracted from HN51 soybean seedlings using a Total RNA Kit II (Omega BioTek, Norcross, GA, USA). The cDNA was synthesized using a TOYOBO kit. Coding sequences of *GmVIT1* were amplified from cDNAs. The total DNA of HN51 was

extracted using the plant genomic DNA extraction kit (Omega). The *GmVIT1* 5′-flanking sequence was amplified. The primers are listed in Supplementary Table S1. After purification, the PCR products were sequenced (Sangon Biotech Co., Ltd., Shanghai, China) and analyzed using BLAST (https://www.ncbi.nlm.nih.gov/orffinder/) (accessed on 18 March 2021); the VIT protein sequences from 12 species were downloaded from NCBI (https://www.ncbi.nlm.nih.gov) (accessed on 17 May 2022) and phytozome (https://phytozome-next.jgi.doe.gov/info/Gmax_Wm82_a2_v1) (accessed on 17 May 2022) and analyzed by the MEGA7.0. The analysis of the *GmVIT1* promoter was performed by Plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (accessed on 20 March 2022).

2.4. Subcellular Localization of GmVIT1

The subcellular localization of GmVIT1 was investigated by 35S::*GmVIT1*-GFP transiently expressed in onion epidermal cells. The pBI121-35S::*GmVIT1*-GFP was constructed and transformed into *Agrobacterium tumefaciens* GV3101 and transformed into the onion epidermal cells. After transformation, cells were incubated in the dark for 2 to 3 days and observed by confocal laser scanning microscopy (TCS SP5 LEICA Microsystems, Germany).

2.5. Gene Expression Analysis

The HN51 and SN37 seedlings were cultured in 1/2 Hoagland solution for 15 days and then treated with 1/2 Hoagland solution with 0.3 mM Fe-EDTA(+Fe), 0 mM of Fe-EDTA(−Fe), and 0.1 mM of Fe-EDTA(CK) for 7 days.To further analyze the relative expression pattern of *GmVIT1* in soybean HN51, HN51 seedlings were cultured in 1/2 Hoagland solution for 15 days and then treated with 0.3 mM Fe-EDTA + 0.1 mM of $MnCl_2$ (+Fe), 0.1 mM of Fe-EDTA 0.3 mM $MnCl_2$ (+Mn), and 0.1 mM of Fe-EDTA + 0.1 mM of $MnCl_2$ (CK) for 7 days. Total RNA was isolated from the cell proliferating leaves, the third to fifth leaves, using the RNeasy mini kit (Qiagen, Hilden, Germany), and cDNA was synthesized using the Reverse TraAceaFirst strand cDNA synthesis kit (TOYOBO, Tokyo, Japan). qRT-PCR analysis was carried out using SYBR® Premix Ex TaqTM II (TAKARA, Otsu, Japan) and CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, United States). The relative gene expression was calculated using the ΔΔCt method and was normalized to *GmActin*. In all experiments, qRT-PCR analyses were performed as triplicates on three different RNA samples isolated independently from each treatment. Primers used in these assays are listed in Supplementary Table S1. The *GmVIT1* promoter was cloned into the pBI121 vector to generate a promoter reporter construct *GmVIT1pro*::β-glucuronidase (GUS). The Agrobacteriummediated transformation of Arabidopsis was performed through the floral dip method. The tissues of the *GmVIT1pro*::GUS transgenic plants were incubated in GUS solution (Coolaber, SL7160), overnight at 37 °C, and then 95% ethanol solution was used to remove chlorophyll. The stained tissues were monitored using a microscope (EZ4-HD LEICA, Germany).

2.6. Generation and Character Analysis of GmVIT1 Transgenic Arabidopsis

GmVIT1 was inserted into the pBI121 vector (35S::*GmVIT1*) and introduced into Arabidopsis by *Agrobacterium tumefaciens* (GV3101)-mediated transformation using the floral dip method. T_1 plants were selected by kanamycin and verified by PCR. Established *GmVIT1* transgenic Arabidopsis were transferred to soil for subsequent generations by selffertilization in a greenhouse. T_3 seedlings were grown in pots and identified by PCR (Figure S1). Three T_3 lines and wild-type plants of 25-day-old were treated with 0.3 mM FeEDTA + 0.1 mM $MnCl_2$(+Fe), 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA(+Mn), and 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ (CK) for 3 days for 7 days. Samples were then observed and photographed. The chlorophyll content was quantified by the acetone method. The soluble sugar and MDA contents were determined according to the experimental method of Dhindsa et al. [52]. The SOD, POD, and CAT activities were measured according to the methods of Shi et al. [53] and Aebi et al. [54]. The ferric iron chelate reductase (FCR) activity was determined using a kit (Geruisi, G0147F).

2.7. Statistical Analysis

Each experiment was repeated in triplicate, and statistical analysis was performed using SPSS statistical software.

3. Results

3.1. The Tolerance of HN51 and SN37 to Fe Deficiency

The HN51 and SN37 are two soybean varieties in Northeast China. Under normal conditions, they had no significant difference in their growth states. After 16 days of iron deficiency treatment, the leaves of HN51 soybean showed mild chlorosis compared with SN37. The iron content in HN51 soybean was signifancatly higher than that of SN37 in iron deficiency and iron supply conditions. Compared with SN37, HN51 showed a significantly higher taproot length. These results indicated that HN51 had stronger adaptability and tolerance to iron deficiency (Figure 1).

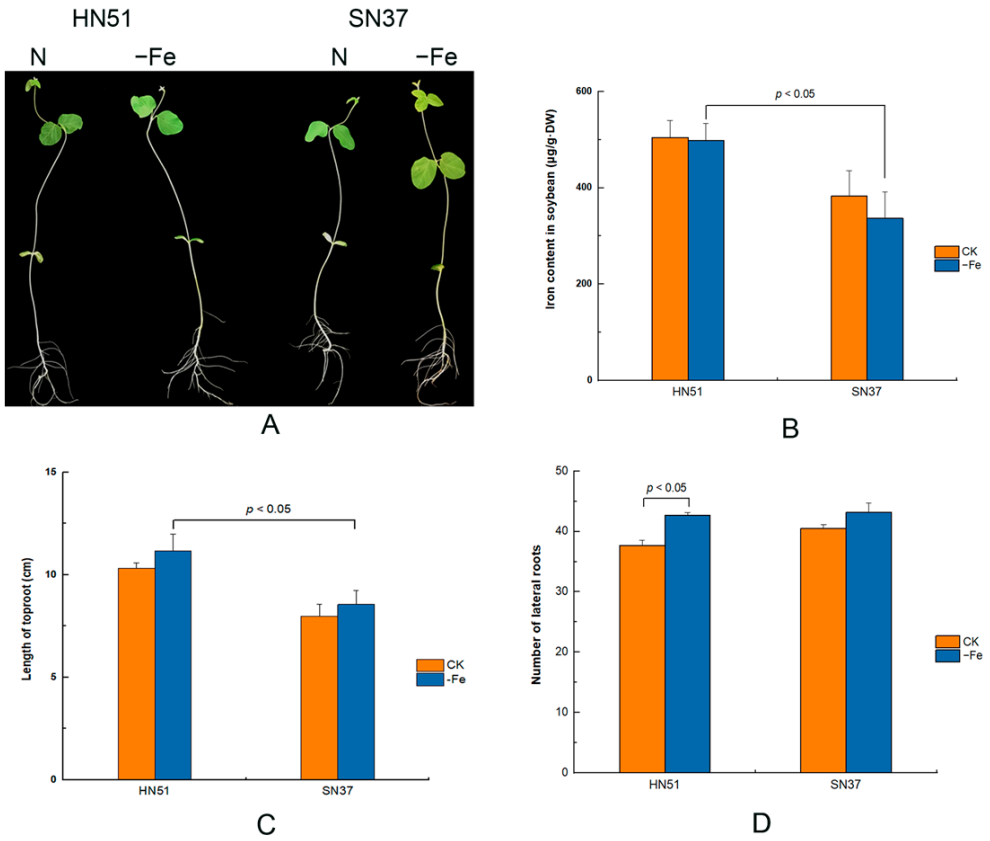


Figure 1. The phenotype and total Fe content of HN51 and SN37 under iron deficiency. (**A**) Phenotypes of HN51 and SN37. (**B**) Total Fe content in soybean. (**C**) The taproot length of HN51 and SN37. (**D**) The lateral root number of HN51 and SN37. The HN51 and SN37 soybean seedlings were treated with 0 mM of Fe-EDTA(−Fe) for 16 days, with 0.1 mM Fe−EDTA as the control. Experiments were repeated three times, and each treatment group contained five samples. The data represent the means of replicates.

3.2. The Expression of GmVITs in Transcriptomic Analysis

Genome-wide analysis revealed that the soybean genome contains 16 *VIT* family members. There are tandem repeats of *VIT* genes on specific chromosomes (Figure 2A). We performed a transcriptomic analysis on HN51 and SN37 soybean seedlings exposed to iron deficiency. The expression of *GmVIT1* was significantly higher in HN51 than in SN37. The Fe-responsive VIT family members in soybean were identified from comparative transcriptome data of HN51 and SN37 under iron deficiency. A total of 16 *VIT* family members were annotated in the transcriptional profile. A total of six

VIT genes (Glyma.05G121200, Glyma.05G121500, Glyma.08G076200, Glyma.05G121600, Glyma.08G076300, and Glyma.18G228200) were not expressed, and Glyma.08G075900 was weakly expressed. The transcription of nine *VIT* genes in soybean (Glyma.05G240600, Glyma.08G047500, Glyma.08G181900, Glyma.15G050400, Glyma.14G187300, Glyma.16G168200, Glyma.02G082500, Glyma.10G225900, and Glyma.20G166100) was detected. Among them, *GmVIT1*(Glyma.20G166100) showed the highest expression, and its homologs Glyma.10G225900 also showed a higher expression (Figure 2B). The gene structure of *GmVIT1* is shown in Figure 2C.

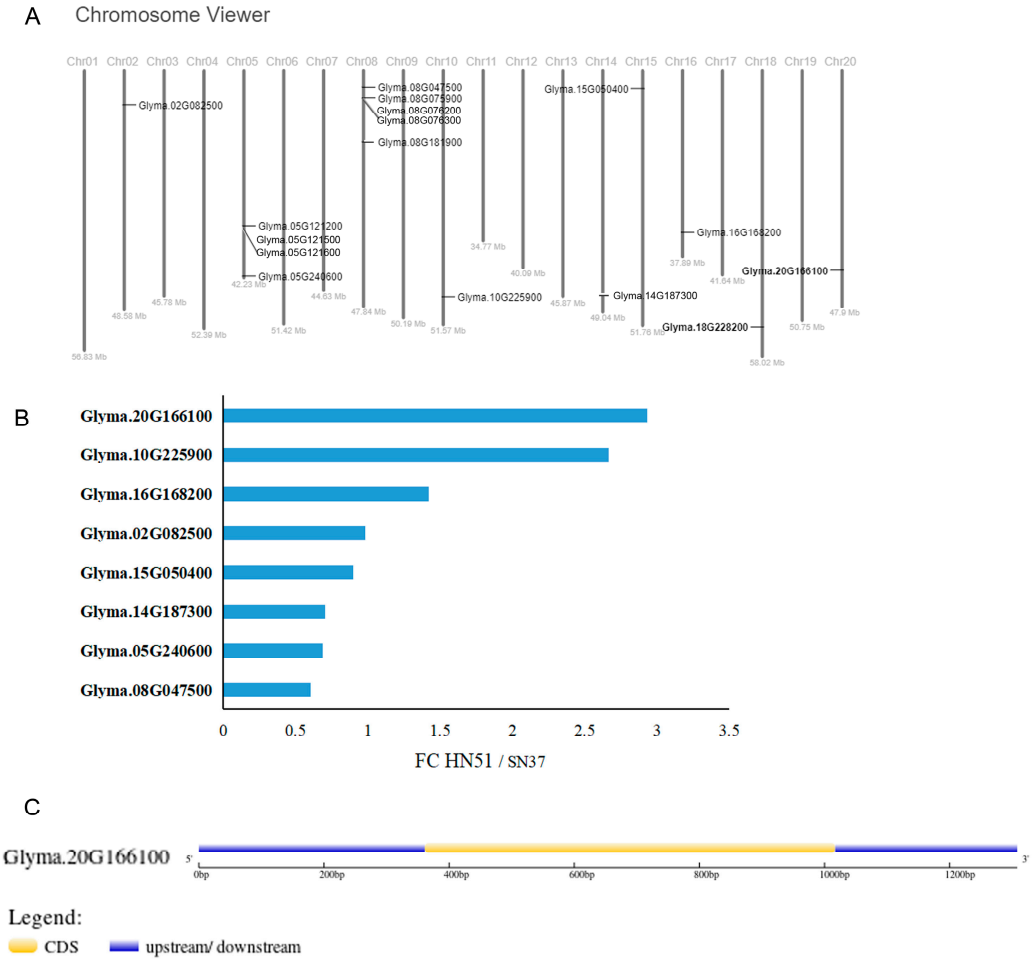


Figure 2. Screening of soybean *GmVIT* genes. (**A**) Chromosome localization of 16 *GmVIT* genes. (**B**) The expression of *GmVIT* genes in HN51 and SN37 under iron deficiency by transcriptomic analysis. (**C**) The gene structure of *GmVIT1*.

3.3. GmVIT1 Response to Iron Deficiency and Excess Iron in HN51 and SN37

In the root, stems, and leaves of SN37, there were no differences in *GmVIT1* expression in −Fe and +Fe treatments, except for the roots under iron deficiency. In HN51 stems and leaves, the expression of *GmVIT1* was significantly higher in −Fe and +Fe treatments than that in the control. The expression of *GmVIT1* was significantly higher in +Fe treatments than that in the control. The expression of *GmVIT1* was not detected in the roots of HN51 under −Fe treatment and showed lower expression than in stems and leaves (Figure 3).

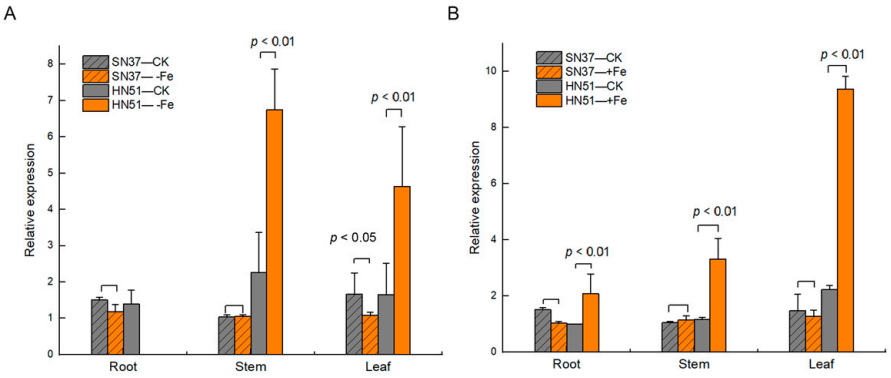


Figure 3. The expression of *GmVIT1* in HN51 and SN37 under iron deficiency (–Fe), excess iron (+Fe), and normal conditions (CK). (**A**) The expression of *GmVIT1* in HN51 and SN37 under iron deficiency (–Fe). (**B**) The expression of *GmVIT1* in HN51 and SN37 under excess iron +Fe: 0.3 mM Fe-EDTA, −Fe: 0 mM of Fe-EDTA, and CK: 0.1 mM of Fe-EDTA. The expression was detected by qRT-PCR. The qRT-PCR experiments were repeated three times, and each treatment group contained five samples. All the data represent the means of three replicates.

3.4. Cloning and Bioinformatics Analysis of GmVIT1

GmVIT1 exists on chromosome 20 and contains an intron (Figure 2A,C). The complete *GmVIT1* CDS spanned 660 bp and encoded a 219 amino acid protein with molecular mass of 23,427.16 KD. GmVIT1 has four transmembrane domains and contains a typical VIT domain at 42-212 amino acids (Figures S2 and 4), indicating that it belongs to the VIT family. An evolutionary tree of 26 VIT proteins from 12 species was constructed using MEGA 7.0. GmVIT1 was assigned to group III, which contained seven VITs from *Glycine max*, *Glycine soja*, *Centaurea cyanus*, *Tulipa gesneriana*, and *Oryza sativa*. GmVIT1 showed a close phylogenetic relationship with Glyma.18G228200v4, GsVIT1, GsVTL4, OsVIT2, CcVIT, and TgVIT1 (Figure 4). GmVIT1 was most close to GsVTL4 from *Glycine soja*, with a 93.24% similarity in the protein sequence. The other two GmVTLs were assigned to the group and were distantly related to GmVIT1. A total of 10 conserved motifs were identified in VIT proteins using MEME. Among them, six motifs (1, 2, 4, 5, 6, and 9) were distributed in GmVIT1 (Figure 4, Table 1).

Table 1. Details of the VIT protein motifs.

Motif	Width	Sites	E-Value	Sequences
1	35	23	1.6×10^{-333}	DSKLVLLAGFAGLVAGAISMGIGEFVSASSZRDVE
2	29	23	2.3×10^{-250}	YSWRGZWVRAAVLGANDGLVSTFSLMMGI
3	41	11	2.0×10^{-162}	MEYNDATPVVNIFRKYPDILVDQRMVADKGLLPADQEVKPW
4	21	26	9.5×10^{-136}	ALASALSFSLGGLVPLLSAIF
5	21	22	2.9×10^{-122}	SAVRTLLGGAJAMAIAFGLTK
6	21	26	3.6×10^{-106}	IVSLLALVLFGVAKARJGGAP
7	29	11	6.3×10^{-102}	RERRVTZWDVINVPDTEQAEJVEIYQQLG
8	15	6	2.9×10^{-33}	IPFTDNDSVKFLGAC
9	18	8	9.3×10^{-26}	REDEDEIEKEKLPNPLQA
10	41	3	1.7×10^{-14}	DDDVENLJDNQENYDLYCPSCGSCITKNVILKKRKRPKHVN

3.5. Subcellular Localization of GmVIT1 Protein

GmVIT1 was fused in frame to a green fluorescent protein (GFP) and driven by a 35S promoter. The 35S::GmVIT1-GFP fluorescence was observed exclusively in the vacuolar membrane, while 35S::GFP fluorescence was found throughout the whole cells (Figure 5). The results showed that the GmVIT1 protein was localized at the vacuolar membrane and may participate in transmembrane transport function as a vacuolar membrane transporter.

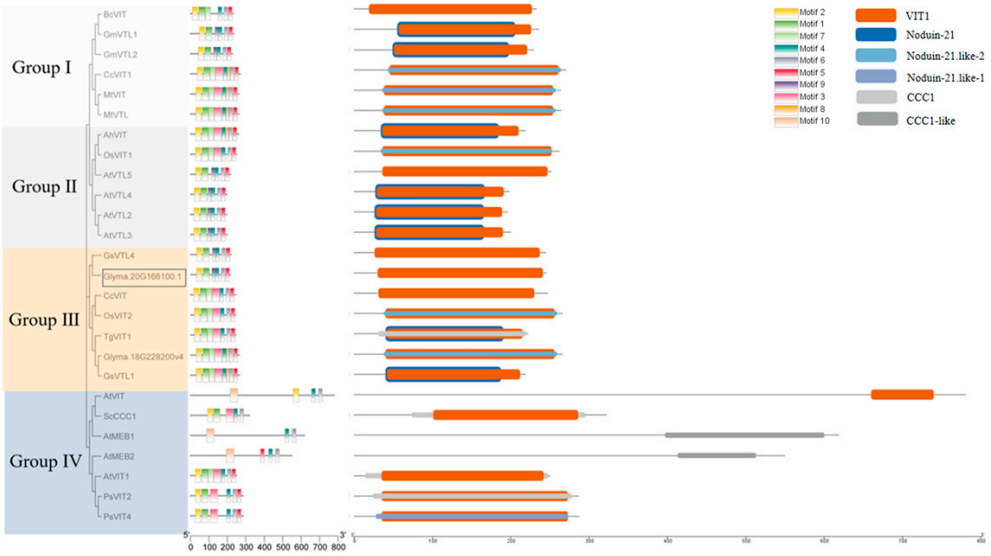


Figure 4. Phylogenetic relationship, motif, and domain composition of the 26 vacuolar iron transporter proteins. Using the Neighbor-Joining method, the phylogenetic tree was constructed with the MEGA 7.0 software. The length of branches corresponds to the degree of divergence. Numbers in the figure represent bootstrap values. Named sequence from *Arabidopsis thaliana* are AtVIT1 (At2g01770), AtVTL1 (At1g21140), AtVTL2 (At1g76800), AtVTL3 (At3g43630), AtVTL4 (At3g43660), AtVTL5 (At3g25190), from *Centaurea cyanus* is CcVIT1 (BAO52026), from *Glycine max* are GmVIT1(Glyma18g228200v4,MN547956.1), GmVTL1 (Glyma.05g121600, NP_001236825), GmVTL2 (Glyma.08g076300, XP_003531056), from *Glycine soja* are GsVIT1 (D0Y65_013220) and GsVTL1(XP_028233991.1), from *Oryza sativa* are OsVIT1 (Os04g0463400, BAS89575) and OsVIT2 (Os09g0396900, Q6ERE5.2), from *Tulipa gesneriana* is TgVIT1 (BAH59029), from *Medicago truncatula* are MtVTL(Medtr7g076320.1) and MtVIT (XP_024625458.2), from *Saccharomyces cerevisiae* is ScCCC1(WN66_04396), from *Bradyrhizobium cosmicum* is BcVIT(FNV92_15665), from *Arachis hypogaea* is AhVIT (DS421_4g129730), from *Cajanus cajan* is CcVIT1(XM_020363549.2), from *Papaver somniferum* are PsVIT2 (XM_026526985.1) and PsVIT4 (XM_026599962.1), and from *Tulipa gesneriana* is TgVIT1(BAH59029). Different motifs of the VIT proteins are displayed in different-colored boxes. The positions of the structural domain are marked with orange, blue, and grey, respectively.

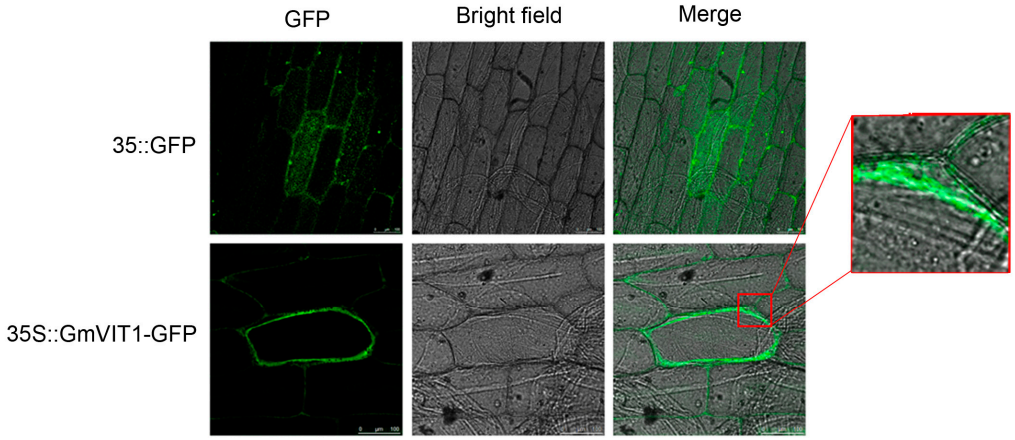


Figure 5. Subcellular localization of 35S::GmVIT1-GFP fusion proteins in onion epidermal cells. GFP: Field of view observed under excitation of green fluorescence; Bright field: Field of view observed without excitation of green fluorescence; Merge: merged green fluorescence and bright-field images. The images were obtained by confocal microscopy. The 35S::GFP was used as a control.

3.6. Expression Patterns of GmVIT1 Gene in Soybean

The tissue-specific expression of GmVIT1 in young soybean seedlings was detected by qRT-PCR. The results showed that GmVIT1 was expressed in three tissues, i.e., leaves, stems, and roots. Specifically, the highest expression level of GmVIT1 was in soybean leaves. In the stems and the leaves, the GmVIT1 transcript level was significantly up-regulated ($p < 0.01$) by +Fe/+Mn stress compared with that in the control. In the root, GmVIT1 was significantly up-regulated ($p < 0.01$) by +Fe stress while down-regulated ($p < 0.01$) by +Mn stress. (Figure 6).

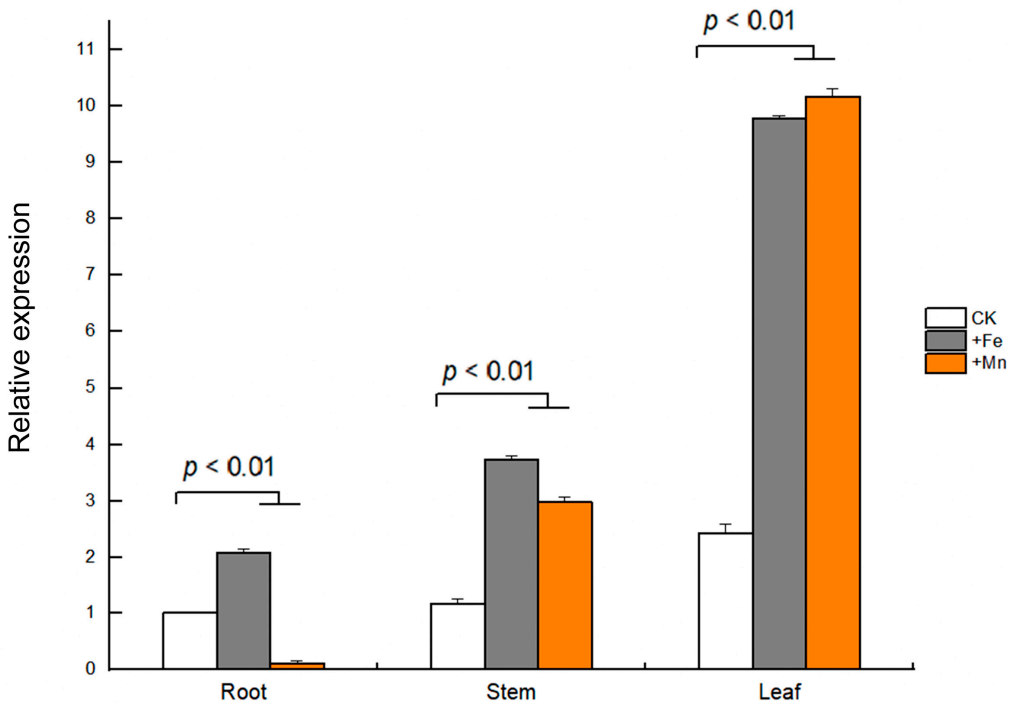


Figure 6. Relative expression of *GmVIT1* in soybean tissues under excessive Fe/Mn treatment. The HN51 seedlings were cultured in 0.3 mM Fe-EDTA + 0.1 mM $MnCl_2$ (+Fe), 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA (+Mn), and 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ as the control (CK) for 3 days. qRT-PCR analysis of the GmVIT1 transcript in the roots, the stems, and the leaves of the soybean plants was conducted. Experiments were repeated three times, and each group contained five samples. All the data represent the means of three replicates.

3.7. GmVIT1 Promoter Activity in Response to +Fe or +Mn Stress in Arabidopsis

The *GmVIT1* promoter contains some cis-elements in response to abscisic acid, gibberellin, salicylic acid, methyl jasmonate, anaerobic, low temperature, and light. MYB recognition sites were also detected in the *GmVIT1* promoter (Figure 7A). The slight GUS staining for *GmVIT1*pro::GUS in roots, stems, and leaves confirmed the widespread and weak expression of *GmVIT1* under normal conditions. The +Fe and +Mn stress significantly enhanced GUS activity in Arabidopsis seedlings, confirming the gene expression analysis results. The +Fe stress enhanced GUS activity in the roots, leaves, and petiole of Arabidopsis, especially in old leaf veins. The +Mn stress enhanced GUS activity in the hypocotyl, leaves, and petiole, especially in young leaves (Figure 7B).

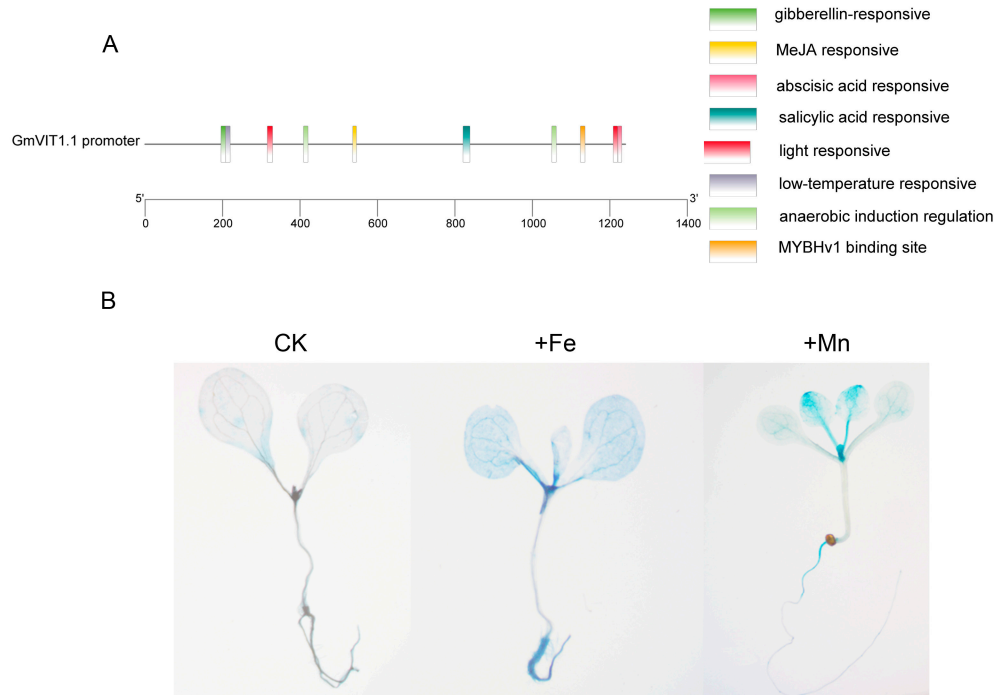


Figure 7. Cis−elements in the *GmVIT1* promoter and histochemical staining of transgenic Arabidopsis harboring the *GmVIT1*pro::GUS constructs. (**A**) Predicted positions and functions of cis-elements in the *GmVIT1* promoter. (**B**) GUS staining results of *GmVIT1*pro::GUS transgenic Arabidopsis under 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ (CK), 0.3 mM Fe-EDTA+0.1 mM $MnCl_2$ (+Fe), and 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA (+Mn) for 7 days. The seedlings were then stained for detection of β-glucuronidase activity.

3.8. GmVIT1 Transgenic Arabidopsis Exhibit Enhancement of Tolerance to +Fe or +Mn Stress

We generated *GmVIT1* transgenic Arabidopsis lines and raised T_3 lines in this study. The transgenic lines, L1, L4, and L10, which have high expression levels of *GmVIT1*, were exposed to +Fe and +Mn stress (Figure 8A). *GmVIT1* transgenic Arabidopsis lines showed better growth status than wild-type plants. Under +Fe and +Mn stress, both transgenic Arabidopsis and wild-type Arabidopsis showed visible leaf chlorosis compared to non-stress conditions (Figure 8B). The MDA content of transgenic lines and WT increased dramatically under +Fe or +Mn stress ($p < 0.01$), indicating that +Fe or +Mn resulted in membrane damage of cells in plants. However, MDA content in L1, L4, and L10 were significantly lower than that in the wild-type plants under +Fe or +Mn stress ($p < 0.05$) (Figure 8C). The MDA results showed that overexpression of *GmVIT1* partially rescued damage to the membrane in plant cells. The chlorophyll content in *GmVIT1* transgenic Arabidopsis was significantly lower than that in the wild-type plants under normal conditions ($p < 0.05$). When exposed to +Fe or +Mn stress, chlorophyll content was significantly reduced in transgenic and wild-type Arabidopsis ($p < 0.01$). The chlorophyll content of L1, L4, and L10 were significantly higher than that in the wild-type plants ($p < 0.05$) (Figure 8D).

3.9. GmVIT1 Transgenic Arabidopsis Showed Higher Soluble Sugar Content and Antioxidant Enzyme Activity under +Fe or +Mn Stress

Soluble sugars are important osmolytes that protect plants against abiotic stress. The soluble sugar content showed significant differences ($p < 0.05$) between *GmVIT1* transgenic Arabidopsis and wild-type plants under normal conditions. The +Fe or +Mn stress significantly increased soluble sugar content in all plants ($p < 0.01$), and the soluble sugar content in *GmVIT1* transgenic Arabidopsis was significantly higher than that in the wild-type plants ($p < 0.05$) (Figure 9A). When grown in normal conditions, the activity of

SOD, POD, and CAT showed significant differences ($p < 0.05$) between *GmVIT1* transgenic and wild-type plants. Excess Fe or Mn stress caused a significant increase in SOD, POD, and CAT activities in *GmVIT1* transgenic Arabidopsis and wild-type plants ($p < 0.01$), and the SOD, POD, and CAT activities of *GmVIT1* transgenic Arabidopsis were significantly higher than that in the wild-type ($p < 0.05$) (Figure 9B–D). The results showed that the protective enzyme activity increased in *GmVIT1* transgenic Arabidopsis, which could protect plants from oxidative damage.

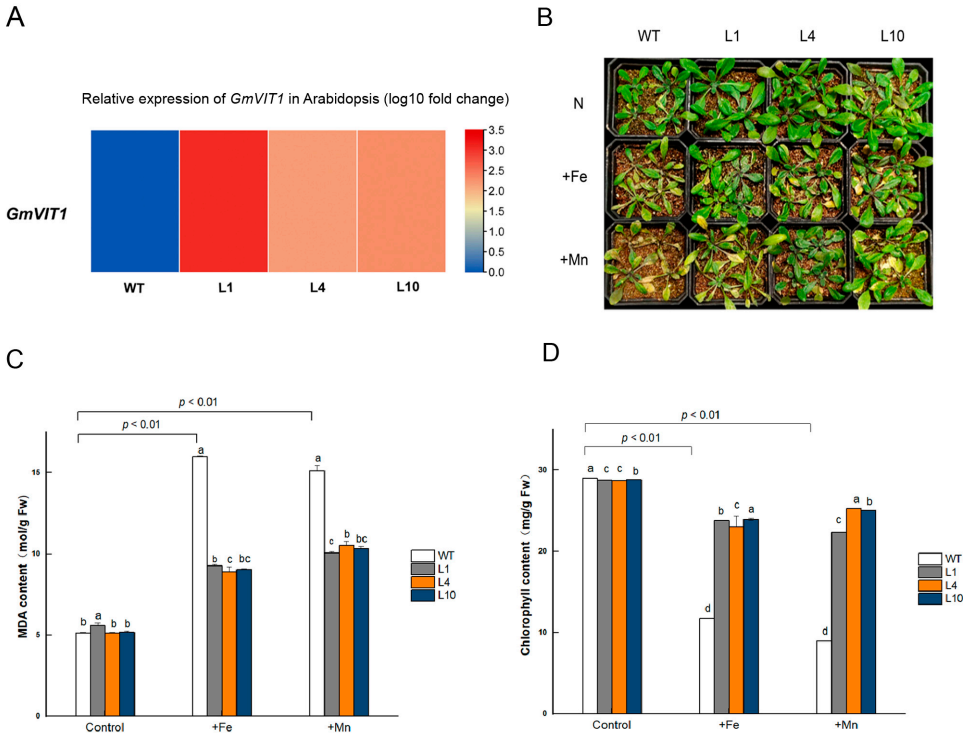


Figure 8. Phenotype and physiological indicators of *GmVIT1* transgenic Arabidopsis and wild-type lines under excess iron or manganese stress. The plants were treated with 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ (CK), 0.3 mM Fe-EDTA + 0.1 mM $MnCl_2$ (+Fe), and 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA (+Mn) (**A**) Relative expression levels of the *GmVIT1* gene in *GmVIT1* transgenic and wild-type Arabidopsis. (**B**) Growth status of *GmVIT1* transgenic lines and wild-type Arabidopsis under +Fe/+Mn stress. (**C**) The MDA content and (**D**) the chlorophyll content of *GmVIT1* transgenic Arabidopsis and wild-type plants. Experiments were repeated three times, and each group contained five samples. All the data represent the means of three replicates. Lowercase letters represent a statistically significant difference between *GmVIT1* transgenic Arabidopsis and wild-type plants under the same condition, $p < 0.05$.

3.10. FCR Activity Changed in GmVIT1 Transgenic Plants under +Fe or +Mn Stress

Grown under the normal condition, the FCR activity in *GmVIT1* transgenic Arabidopsis roots was significantly higher than that in wild-type plants ($p < 0.05$). When exposed to excess Fe or Mn, the FCR activity significantly decreased ($p < 0.01$). The FCR activity in *GmVIT1* transgenic Arabidopsis was significantly lower than that in the wild-type plants under +Fe stress ($p < 0.05$), while significantly higher than that in the wild-type plants under +Mn stress ($p < 0.05$) (Figure 10). Although the FCR activity in *GmVIT1* transgenic plants showed a downward trend, it reflected an opposite response to +Mn and +Fe stress.

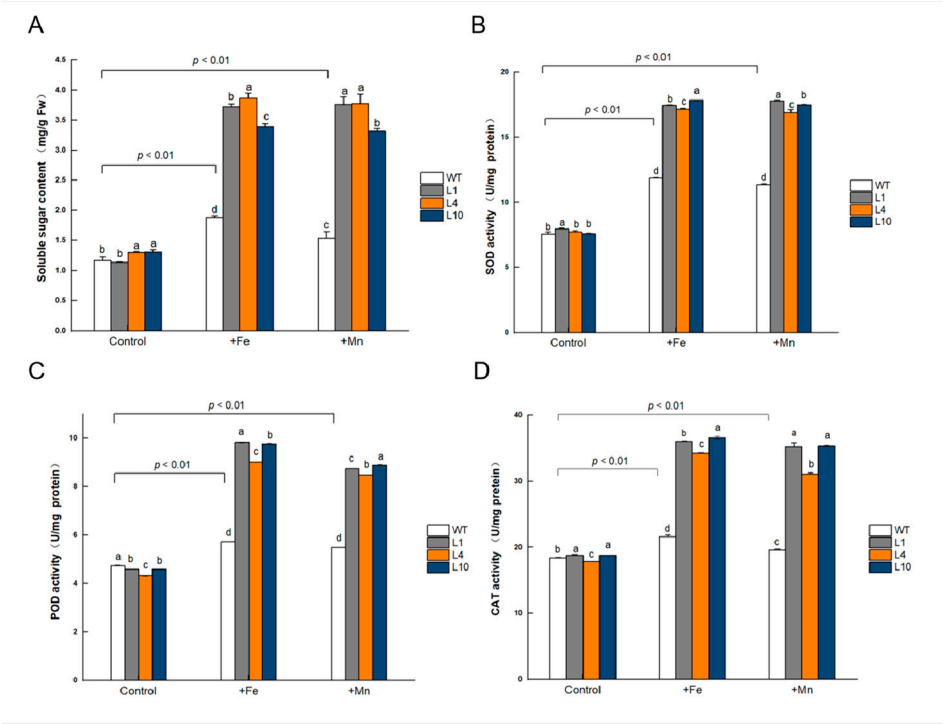


Figure 9. The physiological index of *GmVIT1* transgenic lines and wild-type plants grown under excess iron or manganese. (**A**) Soluble sugar content. (**B**) Superoxide dismutase activity. (**C**) Peroxidase activity. (**D**) Catalase activity. The plants were grown in 1/2 Hoagland nutrient solution for 14 days and treatedwith 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ (CK), 0.3 mM Fe-EDTA + 0.1 mM $MnCl_2$ (+Fe), and 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA (+Mn) for 7 days. Experiments were repeated three times, and each group contained five samples. All the data represent the means of three replicates. Lowercase letters represent a statistically significant difference in GmVIT1 transgenic lines and wild-type plants under the same condition, $p < 0.05$.

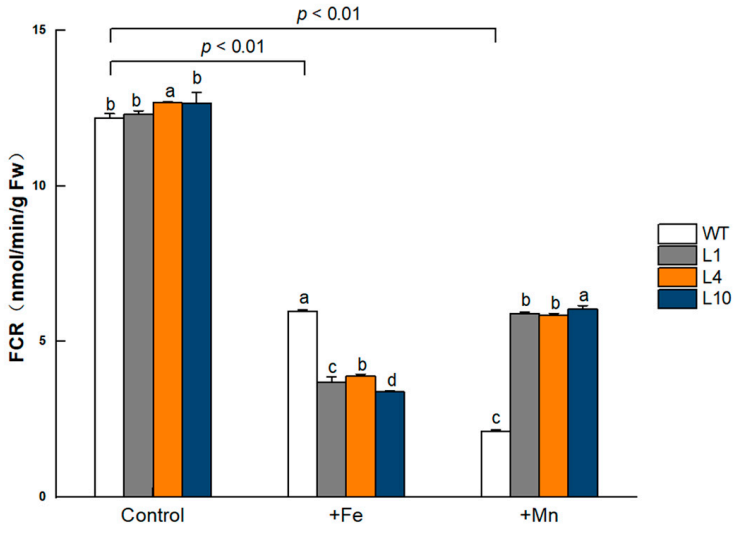


Figure 10. FCR activity of *GmVIT1* transgenic lines and wild-type plants under excess iron or excess manganese stress. Plants were grown in 1/2 Hoagland solution for 14 days and and treatedwith 0.1 mM Fe-EDTA + 0.1 mM $MnCl_2$ (CK), 0.3 mM Fe-EDTA + 0.1 mM $MnCl_2$ (+Fe), and 0.3 mM $MnCl_2$ + 0.1 mM Fe-EDTA (+Mn) for 7 days. Experiments were repeated three times, and each group contained five samples. All the data represent the means of three replicates. Lowercase letters represent a statistically significant difference in *GmVIT1* transgenic plants and the wild-type under the same condition, $p < 0.05$.

4. Discussion

Fe and Mn are redox-active metals and necessary micronutrients for all organisms. When the Fe or Mn concentrations are excessive, free radicals may hinder plant growth [55]. Then, the uptake, compartmentalization, and translocation of Fe and Mn must be strictly regulated in plants. Vacuolar iron transporters have been identified in model plants Arabidopsis, rice, and soybean [56,57]. GmVTL1a is an iron transporter on the symbiosome membrane with an essential function in nitrogen fixation [51], while other soybean VIT functions have not been well-claimed. Here, we cloned *GmVIT1* and its promoter from the HN51 soybean and conducted a functional study.

4.1. GmVIT1 Is a Unique Gene in Soybean VIT Family

Most *GmVIT* genes are present in tandem, and over 61.9 percent of *VIT* genes are cluster distributed in chromosomes 5 and 8 [34], while *GmVIT1* is alone on chromosome 20 in the soybean genome (Figure 2). We found that *GmVIT1* has a low similarity with other GmVITs except for Glyma.10G225900. Notably, GmVIT1 shows over 90% similarity to GsVTL1 and GsVTL4 in *Glycine soja*. Therefore, we believe that *GmVIT1* is a relatively primitive *VIT* gene of soybean from its ancestor *Glycine soja*. The phylogenetic and motif analysis strongly confirm this conclusion (Figure 4). GmVIT1 contains a conserved VIT domain and four transmembrane domains corresponding to the transport function (Figure S2). GmVIT1, GsVTL1, and GsVTL4 both contain motifs 4, 6, and 9, while other VITs do not contain motif 9 (Figure 4). We believed that *GmVIT1* retained in the soybean genome with significance in function during the long-term evolution process. GmVIT1 was localized to the tonoplast, similar to most VIT proteins [40] (Figure 5). Together with structural and phylogenetic results, *GmVIT1* is a unique gene and might perform vacuole metal transport function in the plant cell.

4.2. GmVIT1 Was Highly Expressed in Leaves and Induced by Fe/Mn Stress

Metal transporters vary in tissue expression patterns. *AtVIT1*, *BnMEB2*, *OsVIT1*, *OsVIT2*, *TaVIT1*, *TaVIT2*, and *TgVIT1* were found to be expressed in mature leaves, flowers, and developing seeds and were involved in the development of propagative organs [28,31,35,36,40,45]. Cao et al. found that soybean *VITs* were expressed in vegetative and reproductive organs. Almost no expression of *GmVTL1* and *GmVTL2* was found in the root and leaves of soybean [40]. In this study, we found that *GmVIT1* was expressed in the vegetative organs and showed the highest expression level in leaves (Figure 6), consistent with the results of Cao et al. [34]. The results indicated that *GmVIT1* might play an important role in soybean leaves.

The transcriptomic analysis revealed that the expression of *GmVIT1* was significantly higher in HN51 than that of SN37. The qRT-PCR results showed that iron deficiency and excess iron significantly up-regulated *GmVIT1* in HN51, while not significantly in SN37. The results indicated that the *GmVIT1* gene is directly related to the different responses to iron stress of HN51 and SN37. The expression of several *VITs* induced by Fe stress indicated that *VITs* are involved in the Fe storage of vacuolar to regulate plant Fe homeostasis. The transcript of *AtVTL1*, *AtVTL2*, *AtVTL5*, and *BnMEB2* responded more to exogenous Fe [30,43,58]. Fe stress induced the expression of *Glyma.16G168200*, *Glyma08G076300*, and *Glyma08G075900* in soybean [34]. In this study, *GmVIT1* was induced in roots, stems, and leaves by +Fe treatment. Dramatically, the expression of *GmVIT1* increased nearly to 9.7-fold in the leaves (Figure 6). The GUS staining verified this result. Therefore, we infer that the *GmVIT1* gene may play a more important role in excess Fe response. The unique nature of the leaf makes it prone to the damage of iron overload. Iron is incorporated into iron proteins or transported to cellular organelles, and excess cytosolic iron is sequestered and stored. The up-regulating of *VIT1* might help to detoxifying excess Fe [33]. The up-regulation of *GmVIT1* in leaves suggests that, during iron excess, *GmVIT1* activates vacuolar iron deposition, which appears to be the major mechanism of metal detoxification in soybean. The typical CCC1-like superfamily domains in VIT are also

demonstrated for Mn transport from the cytosol to the vacuole [59]. A study has shown that accumulation of *TaVTL2* and *TaVTL4* in wheat shoots during Mn deprivation [60]. In our results, *GmVIT1* was also induced by excessive Mn, suggesting that *GmVIT1* might also be involved in detoxifying of excess Mn. Kurt et al. found that *VIT*s were highly transcribed under cold, heat, salt, and drought perturbations [28]. We found abundant stress–response cis-elements present in the *GmVIT1* promoter and speculated that *GmVIT1* might be regulated by abiotic stress, which remains to be elucidated.

4.3. Overexpression of GmVIT1 Enhances the Tolerance to +Fe/+Mn Stress in Arabidopsis

Leaf chlorosis is one of the most remarkable phenotypes of plants exposed to Fe and Mn toxicity [56,61]. The wild-type Arabidopsis exhibits characteristic chlorosis in leaves under excess Fe in this study. In contrast, *GmVIT1* transgenic Arabidopsis old leaves showed mild symptoms, and young leaves showed no symptoms, consistent with less reduction in chlorophyll content. Wheat *VIT* gene overexpression dramatically increases Fe content in the transgenic plant, resulting in altered partitioning of Fe between source and sink tissues [31]. Overexpression of *VIT* genes also restored plant growth in iron homeostasis genes mutants such as *nramp3*/*nramp4*, *vit2*, and *meb2* [29,30]. These findings implied that VITs transferred cytoplasmic Fe into vacuoles and contribute to Fe homeostasis regulation in plants [44]. In agreement, we detected that *GmVIT1* is mainly expressed in leaves and increased plant tolerance to excess Fe. *GmVIT1* might involve in iron vacuolar storage and detoxification. In plant tissues, Fe^{2+} participates in Fenton reactions, catalyzing the generation of hydroxyl radicals (OH^-) and other reactive oxygen species (ROS) [62]. The *GmVIT1* transgenic plants showed lower MDA and protective enzyme activity, which indicates the ability of resistance to excess Fe enhanced (Figure 8). Previous studies showed that Mn toxicity functions on young leaves more than on old leaves [57]. In the present study, plants exhibited a mild leaf chlorotic phenotype and a better oxidation index under excess Mn. The results supported that the *GmVIT1* gene may alleviate Mn toxicity in Arabidopsis. The structural similarity suggests a shared function in Mn-binding and transport of MTP, NRAMP, and VITs [15,57]. It has been discussed previously that VIT1 sequestrates Mn to the vacuole. Expression of Arabidopsis *VIT1* also increases the Mn content of yeast cells, but knockout of *AtVIT1* does not alter the Mn distribution [35]. *OsVIT1* and *OsVIT2* may also contribute to sequestrate Mn [40]. *TaVIT2* complemented an Mn transporter mutant *Dpmr1* yeast and increased barley grain Mn content [31]. Our finding in *GmVIT1* indicated that VITs might act as transporters for Mn. Similar to the previous results, *GmVIT1* transgenic Arabidopsis improved tolerance to excess Mn. In contrast, some different conclusions were given. The Mn concentration decreased significantly in *Osvit2* [29], but a significant change in Mn was observed in brown rice of *Osvit1-1* and *Osvit2-1* [40]. In our future work, analysis on Fe and Mn contents in *GmVIT1* transgenic Arabidopsis under control and stress conditions would demonstrate *GmVIT1* gene function. FCR is mainly involved in the catalytic reduction of Fe^{3+} to Fe^{2+}. The FCR activity was relatively low under sufficient or excess Fe conditions [63]. In this study, the FCR activity of transgenic plants was significantly lower than that of wild-type plants under excess Fe stress, demonstrating that *GmVIT1* might reduce the FCR activity (Figure 10). Such a mechanism could be significant and allow plants to adapt to high-iron environments. Metal transporters located in the tonoplast impact on long-distance transport in addition to intracellular metal homeostasis [31]. Wheat *VIT1* response to nicotianamine is important for the distribution of Fe [64]. Studies have shown that *AtVTL2* expression is regulated by *ILR3* [65]. Although *GmVIT1* plays a vital role in excess Fe and excess Mn detoxification, the precise molecular mechanism remains to be resolved.

Taken together, the results demonstrated GmVIT1 localized in tonoplast as a vacuole iron transporter family member. *GmVIT1* is highly expressed in leaves, and also expressed in stems and roots of soybean. *GmVIT1* is induced by iron deficiency and excess Fe and Mn. Overexpression of *GmVIT1* confers Arabidopsis tolerance to excess Fe or excess Mn.

This study provides new insight into *VIT* gene fouction and GmVIT1 could be applied in metal toxic tolerance improvement of crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020384/s1, Figure S1: Seclection of *GmVIT1* trangenic Arabidopsis; Figure S2. Transmembrane domains of GmVIT1; Table S1: The primer sequence.

Author Contributions: Y.-D.B. and C.-H.G. designed the experiments and obtained funding for the research. T.L., L.W., L.S. and J.-L.G. performed the experimental analyses. D.-L.G. and T.L. contributed to compiling and analyzing the data and wrote the manuscript. Y.-J.S. conducted statistical analysis. Y.-C.L. and X.-M.Z. participated in the data analysis and supervised the writing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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