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Genome-Wide Association Analysis for Submergence Tolerance at the Early Vegetative and Germination Stages in Wild Soybean (*Glycine soja*)

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Abstract: Cultivated soybean is an important legume crop that is generally sensitive to flooding stress, including submergence and waterlogging treatments. Wild soybeans, the ancestor of cultivated soybeans, have been potential genetic resources for resistance to abiotic or biotic stresses. The present study aimed to evaluate 163 wild soybean accessions for foliar damages at the early vegetative stage and 105 accessions for germination rates, normal seedling rates, and electrical conductivity at the germination stage under submergence stress. In addition, a genome-wide association study (GWAS) was conducted to identify genomic regions associated with phenotypic measurements at these two growth stages by using MLM and FarmCPU models with publicly available genotypic data. The phenotypic evaluation revealed six and three accessions were tolerant to submergence at the early vegetative and germination stages, respectively. Notably, only one wild soybean accession showed a tolerance reaction to submergence at two stages. Through GWAS analysis, 16 and 20 SNPs across different chromosomes were determined for the submergence-related traits at the early vegetative and germination stages, respectively. Based on the linkage disequilibrium block on the detected genomic regions, ten and four putative genes were identified at the early vegetative and germination stages, respectively. Of these genes, certain genes may be related to submergence stress in wild soybeans. Further studies should be performed to validate the function of these putative genes in the responses of wild soybeans to submergence stress.

Keywords: wild soybean; flooding tolerance; submergence; genome-wide association analysis

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1. Introduction

Flooding is a stressful condition that, due to climate change, poses numerous challenges to plants maintaining homeostasis. Flooding circumstances impact gas exchange, soil microorganisms, and nutrient availability in a plant's surrounding environment, which lead to limited plant growth and development [1–4]. Flooding stress can be categorized depending on the water level relative to plant height as either waterlogging, which covers the part of the plant, or submergence, when the entire plant is covered by water [5].

Soybean (*Glycine max* (L). Merr.) is highly valued for its rich protein and vegetable oil, making it an important resource for humans and animals. Soybeans have typically exhibited vulnerability to flooding stress [6,7]. Yield reduction by flooding stress in soybeans differs by growth stages and several studies reported the response of flooding stress at different soybean growth stages. At the germination stage, the germination rate (GR) of soybeans is influenced by the duration of flooding and temperatures. The GR decreased significantly after 24 to 48 h of flooding stress at 15 and 25 °C [8,9]. Soybean seeds exposed to flooding stress at 15 °C were more susceptible than those at 25 °C. Moreover, the GR of soybean seeds under submergence conditions in the field for two days was less than 10% [10]. Submergence stress has a negative impact on the GR and normal seedling rate (NR) of sensitive soybean seedlings, whereas tolerant genotypes showed a high GR and NR [11,12]. In addition to GR and NR, an increase in electrical conductivity (EC) of leakage solute was associated with the flooding tolerance of seeds [13,14]. Increased EC values were contributed by releasing chemicals from submerged seeds into the water.

Exposing soybean plants to flooding during the vegetative or reproductive stages inhibited nodulation and nitrogenase activities in their roots, which was caused by the lack of oxygen in the saturated soil [15,16]. Flooding stress inhibited root growth and branching in sensitive soybean seedlings at the early vegetative stage [17]. For the aboveground growth, foliar damage score (FDS), which is determined by the presence of a yellowish color, chlorosis, and wilting, was the typical phenotypic scoring of flooding stress and exhibited varying levels of responses during the vegetative and reproductive stages [18–20]. The growth rate under flooding stress was discovered to be 25% less at the V4 stage (four trifoliate leaves) and 35% less at the R2 stage (full blooming) [21]. For the soybean yield under flooding stress, the yield reduction was 20–39% compared to normal conditions [22,23].

Several recent research studies have published findings on identifying genetic loci that influence flooding tolerance in soybeans during different growth stages. Two significant single nucleotide polymorphisms (SNPs) were shown to be linked to the EC value and GR of soybean seeds under flooding conditions in two distinct environments. Furthermore, SNPs located on chromosomes 1 and 5 were significantly associated with root length and shoot length under flooding conditions, respectively, at the germination stage [11]. Yu et al. [13] discovered three significant quantitative trait loci (QTLs) for the GR, NR, and EC values at the germination stage. Dhungana et al. [24] applied QTL mapping to discover 20 QTLs associated with the chlorophyll content of leaves and the dry weight of shoots in soybean seedlings subjected to flooding conditions during the V1–V2 stages. Sayama et al. [25] identified four QTLs on chromosomes 2, 4, 8, and 12 associated with seed flooding tolerance through QTL mapping analysis at the germination stage. Recently, genome-wide association studies (GWAS) have been extensively used as a potent method to investigate the genetic factors influencing flooding conditions in plants with a wide genetic diversity of natural populations [11,26,27]. Fourteen SNPs were identified as being associated with flooding tolerance during the early reproductive stage utilizing a mixture of several models in GWAS analysis [19].

Cultivated soybean was domesticated from its wild ancestor (*Glycine soja* Sieb. and Zucc.) in East Asia thousands of years ago [28,29]. Their geographical spread increases genetic diversity due to their adaptation to different environments [30,31]. Wild soybeans were deemed to possess greater tolerance to flooding than cultivated soybeans [32]. However, little information on flooding stress has been reported with wild soybean accessions. Thus, using wild soybean germplasm for GWAS may be expected to detect the unique genomic regions for flooding stress. This study aimed (1) to assess the tolerance of wild soybeans to submergence stress for FDS at the early vegetative stage and for the GR, the NR, and the EC at the germination stage, and (2) to determine the genetic regions associated with phenotypic measurements in wild soybeans during the early vegetative and germination stages through a GWAS approach using available resequencing data.

2. Materials and Methods

2.1. Plant Materials

A total of 386 cultivated soybean accessions were evaluated for their tolerance levels to submergence at the early vegetative stage (V2 stage, two trifoliate leaves) for FDS trait. In addition, one hundred sixty-three, and one hundred five wild soybean accessions were used to assess tolerance to submergence stress at the early vegetative stage for FDS and germination stage for germination-related traits, respectively. These wild soybean accessions were collected from Korea, China, Japan, and Russia. The cultivated and wild soybean accessions were obtained from the Rural Development Administration, Jeonju, Republic of Korea (https://genebank.rda.go.kr/) (accessed on 1 February 2024) [33,34].

2.2. Phenotypic Evaluation of Wild Soybean Accessions under Submergence at the Early Vegetative and Germination Stages

2.2.1. Evaluation Tolerance of Wild Soybean Accessions under Submergence Conditions at the Early Vegetative Stage

The experiment was carried out using the tray method in the greenhouse at Kyungpook National University (35°53′42″ N, 128°36′45″ E) with three replications from July 2020 to September 2020 for the wild soybean accessions and from July 2018 to September 2018 for cultivated soybean accessions. The seeds of 163 wild soybean accessions were sacrificed before planting by using tweezers to create a small incision on the seed coat region opposite the hilum. The five sacrificed seeds of each wild soybean accession and five seeds of each cultivated soybean accession were then planted in a 50-hole plastic tray filled with horticultural soil to a depth of 2 cm (Hanareum, Shinsung Mineral, Goesan, Republic of Korea). Once the seedlings had fully developed unifoliate leaves, they were trimmed so that only two plants remained in each hole, representing a replication. The plants at the V2 stage, with two completely formed trifoliate leaves, were subjected to submergence conditions. During this period, the water level was maintained at a height of 5–10 cm above all the plants for 5 days, followed by a subsequent 2-day drainage period.

The foliar damage was evaluated following the method of Wu et al. [19] with some modification. The damage to the plant was evaluated based on the yellow color chlorosis and wilting level on the leaves of cultivated and wild soybean accessions. The FDS was rated from 1 to 5 on the 2nd day of drainage: 1: plant without damage, 2: 25% plant damage, 3: 50% plant damage, 4: 75% plant damage, and 5: total plant death. An average FDS was calculated using the final mean FDS of each line after three replications and was used to assign them into five groups of response to submergence stress. Accessions with an FDS value less than 1.5 were categorized as highly tolerant (HT-V2), those with a value between 1.5 and 2.5 were categorized as tolerant (T-V2), those with a value between 2.5 and 3.5 were categorized as moderate (M-V2), those with a value between 3.5 and 4.5 were categorized as sensitive (S-V2), and those with a value greater than 4.5 were categorized as highly sensitive (HS-V2) (Figure S1).

2.2.2. Evaluation for Submergence Tolerance Levels of Wild Soybean Seeds at the Germination Stage

The seeds of 105 wild soybean accessions under submergence conditions were evaluated in the laboratory at 25 °C with three replications, following the previously described method with a minor modification [35,36]. Twenty sterilized seeds were submerged in 50 mL conical tubes as the treatment. Germination was evaluated after five days of submergence, followed by five days of drainage. For the control treatment, twenty seeds were placed in the Petri dish with wet towels under normal conditions. The germination seeds with a radical or hypocotyl length of more than 1 cm were considered germinated. Seedlings with stunted roots or no epicotyl were abnormal seedlings (Figure S2). Moreover, EC per seed weight was recorded on the 5th day of submergence using a handheld conductivity meter (TDS and EC meter). These traits were calculated as follows:

$$\text{Germination rate (GR, \%)} = (\text{number of germinated seeds}/\text{number of sowed seeds}) \times 100$$

$$\text{Normal seedling rate (NR, \%)} = (\text{number of normal seedlings after germination}/\text{number of sowed seeds}) \times 100$$

$$\text{Germination index (GI)} = \text{germination rates after 5 days of submergence treatment}/ \\ \text{germination rates after 5 days of normal conditions}$$

$$\text{Normal seedling index (NI)} = \text{normal seedling rates after 5 days of submergence treatment} / \text{normal seedling rates after 5 days of normal conditions}$$

$$\text{EC } (\mu\text{Scm}^{-1}\text{g}^{-1}) \text{ of the leakage solute} = \text{EC value read on EC meter} / \text{weight of 20 submerged seeds}$$

The tolerance levels of wild soybean seeds at the germination stage were evaluated depending on the NI values, which reflected the relative normal growth of seedlings under submergence stress in comparison with normal condition. Accessions with a NI value higher than 0.9 were categorized as highly tolerant (HT-G), those with a value between 0.9 and 0.7 were categorized as tolerant (T-G), those with a value between 0.7 and 0.4 were classified as moderate (M-G), those with a value between 0.4 and 0.1 were categorized as sensitive (S-G), and those with a value less than 0.1 were categorized as highly sensitive (HS-G).

2.3. Identification of Genomic Regions for the Response to Submergence at the Early Vegetative and Germination Stages

2.3.1. Genotype Data

The SNP data for 165 wild soybean accessions were obtained from (https://figshare.com/projects/Soybean_haplotype_map_project/76110) (accessed on 1 February 2024) [37]. The soybean reference genome assembly (Wm82.a2.v1) was used in mapping the physical position of SNPs on chromosomes [38]. After filtering to remove unusual markers with monomorphic SNPs, <0.05 minor allele frequency, >10% heterozygosity, and >5% missing calls from the data set, 3,655,432 high-quality SNPs were used for the GWAS analysis.

2.3.2. Genome-Wide Association Analysis

Genome-wide association analysis was performed for FDS at the early vegetative stage and GI, NI at the germination stage, utilizing a mixed linear model (MLM) and a fixed and random model Circulating Probability Unification (FarmCPU) according to the Genome Association and Prediction Integrated Tool (GAPIT) version 3.0 package of R software [39]. A threshold level ($\geq$6.56) based on the Bonferroni correction method was applied to determine the SNPs associated with the observed traits.

2.3.3. Haplotype Block Analysis and the Identification of Putative Genes

Using the solid spine method, the Haploview software version 4.0 was utilized to examine the linkage disequilibrium (LD) in the genomic region surrounding significant SNPs. Putative genes were discovered on the LD blocks where significant SNPs were located. These genes' physical positions are based on the reference genome (Wm82.a2.v1.), and annotated functions were acquired from the Soybase database (http://www.soybase.org/).

2.4. Statistical Analysis

Analysis of variance (ANOVA) and Pearson's correlation were carried out using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA: IBM Corp). An ANOVA was conducted to analyze the variance in FDS, NI, GI, and EC values under treatment conditions. Pearson's correlation coefficient was applied to indicate the association of phenotypic traits in each treatment condition or growth stage.

3. Results

3.1. Soybean Responses to Submergence Stress

The responses of wild soybeans to submergence stress were evaluated for phenotypic measurements at the early vegetative and germination stages (Tables S1 and S2). The results of the ANOVA of wild soybean responses to submergence conditions revealed that soybean accession had highly significant effects on all measured traits at the early vegetative and germination stages ($p < 0.0001$) (Table 1).

Table 1. ANOVA results show the responses of wild soybeans to submergence at the early vegetative and germination stages.

Stage	Trait	Source of Variance	Degree of Freedom	Sum of Square	Mean of Square	*F*-Value	*p*-Value
Early vegetative	FDS	Accession	162	327.10	2.02	13.17	<0.0001
		Replication	2	0.57	0.29	1.87	0.16
Germination	GI	Accession	104	15.30	0.15	86.01	<0.0001
		Replication	2	0.19×10^{-2}	0.10×10^{-2}	0.57	0.57
	NI	Accession	104	12.73	0.12	125.99	<0.0001
		Replication	2	0.04×10^{-2}	0.02×10^{-2}	0.21	0.81
	EC	Accession	100	3.25×10^{6}	3.25×10^{4}	16.57	<0.0001
		Replication	2	7.97×10^{3}	3.85×10^{3}	2.03	0.14

FDS, foliar damage score; GI, germination index; NI, normal seedling index; EC, electrical conductivity of leakage solute.

3.1.1. Effects of Submergence Stress on V2 Seedlings of 163 Wild Soybean Collection

The phenotypic distribution of the FDS across 386 cultivated and 163 wild soybean accessions under submergence conditions at the early vegetative stage, respectively, is shown in Figure 1. Most cultivated soybean accessions were S or HS genotypes to stress with more than 4.5 FDS values (Figure 1A, Table S3). The FDS values ranged from 1 to 5 for wild soybean accessions, with an average of 4.2. Only two accessions with an FDS no higher than 1.5 were HT-V2 genotypes, and four were T-V2 genotypes with FDS between 1.5 and 2.5. The ratio of M-V2, S-V2, and HS-V2 groups was 18.4%, 24.5%, and 53.4%, respectively. Of the 163 accessions, YWS1022 and YWS294 exhibited a high tolerance level with an FDS of less than 1.5. The wild soybean accessions CHN23, YWS1034, YWS347, and YWS-17 demonstrated tolerance with the FDS ranging from 1.5 to 2.5 (Figures 1B and S3, Table S1). These findings indicate that many wild soybean accessions exhibited a high susceptibility to submergence stress, as evidenced by a significant proportion of leaf damage and a high average FDS.

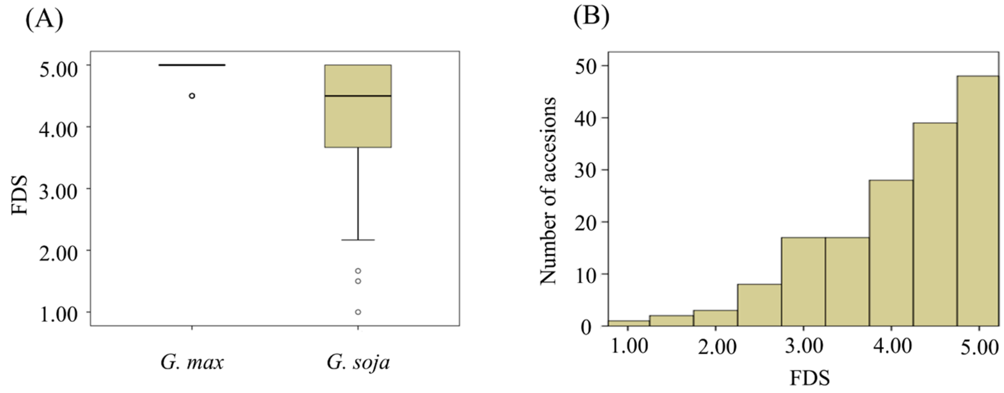

Figure 1. Phenotypic distribution of foliar damage score (FDS) under submergence conditions at the early vegetative stages with cultivated and wild soybeans. (**A**) The box plot shows the phenotypic distribution of FDS of 386 cultivated soybeans (*G. max*) and 163 wild soybeans (*G. soja*) accessions by submergence at the early vegetative stage. (**B**) Frequency distribution of FDS by submergence at the early vegetative stage of 163 wild soybean accessions. FDS, foliar damage score.

3.1.2. Effect of Submergence Stress on Seed Germination of 105 Wild Soybean Collection

The submergence tolerance of wild soybean seeds of 105 accessions was assessed during the germination stage (Figure 2). This assessment included examining directly associated traits such as the GR and the NR, and the relative ratio of each of these traits in treatment and normal conditions was calculated as GI and NI, respectively. Additionally, the EC value was observed on the fifth day after treatment.

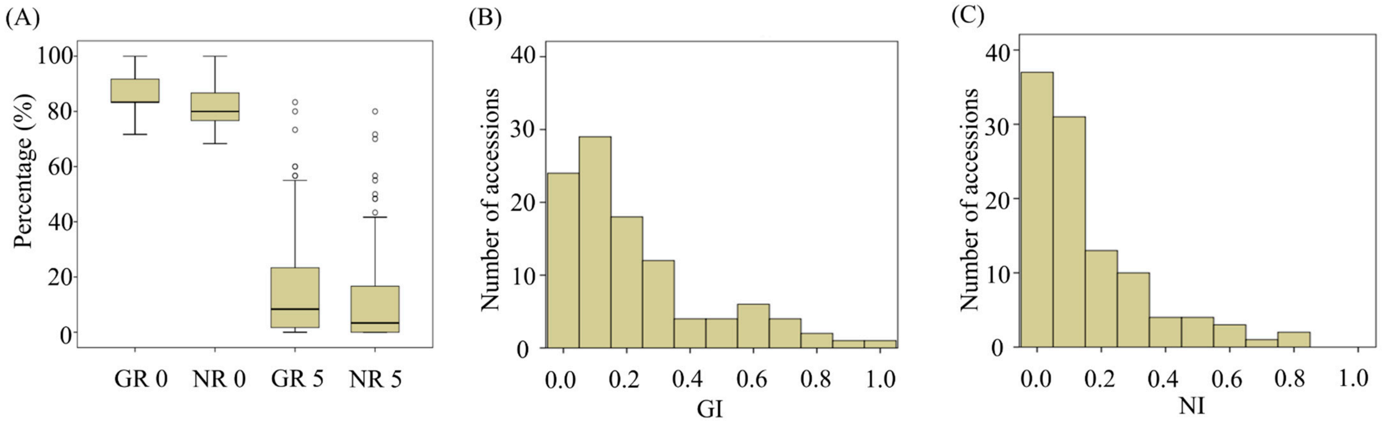

Figure 2. Phenotypic distribution of germination-related traits under submergence conditions at the germination stages with 105 wild soybeans. (**A**) The box plot shows the phenotypic distribution of the germination rate (GR) and normal seedling rate (NR) in control and submergence conditions. (**B**) Frequency distribution of the germination index (GI). (**C**) Frequency distribution of the normal seedling index (NI). GR 0, germination rate under control conditions; NR 0, normal seedling rate under control conditions; GR 5, germination rate under submergence conditions; NR 5, normal seedling rate under submergence conditions.

The GR and the NR distribution are under both control and 5-day submergence conditions (Figure 2A). The average GR and NR values under normal conditions were 85.91% and 81.87%, respectively. Compared with control conditions, however, these values under the 5-day submergence condition decreased to 16.54% and 12.21%, respectively. There were significant variations of GR and NR among accessions and in the interaction of accessions and treatment conditions at p value less than 0.01 (Table S4). Only 11 accessions exhibited more than 50% of the GR during submergence. However, out of them, only six accessions had more than 50% of the NR.

The GI and the NI represented the relative GR and NR comparison between submergence and control conditions (Figure 2B,C). The wide range of the GI and the NI values demonstrated significant variation in wild soybean seed responses to submergence. The GI and the NI values of the wild soybean accessions ranged from 0.00 to 0.91 and 0.00 to 0.86, respectively. In addition, the GI and the NI mean values were 0.18 and 0.14, respectively, suggesting that nearly all soybean lines were sensitive to the 5-day submergence condition. Only three accessions, namely YWS1244, CHN23, and CHN25, had higher GI and NI values than 0.75, and those accessions could tolerate submergence stress at the germination stage, so they were assigned as T-G (Table S2 and Figure S4).

The impact of submergence stress on soybean seeds was assessed by measuring the EC value of the water during germination. The EC value per unit weight of seeds in the 5-day submergence conditions varied from 102.3 to 750.3 $\mu Scm^{-1}g^{-1}$, with an average value of 331.4 $\mu Scm^{-1}g^{-1}$ (Table S2).

Pearson's correlation among germination-related traits of 105 wild soybean accessions in the control and submergence conditions is shown in Table 2. The GR 5 and the NR 5 under submergence were strongly positively correlated with the GI and the NI ($r > 0.967$, $p < 0.01$). Similarly, the GR 0 and the NR 0 under control conditions were highly positively correlated to each other at a significant level ($r = 0.862$, $p < 0.01$). However, the GR 0 and the NR 0 under normal conditions were positively correlated with the GR 5 and the NR 5 under submergence conditions (r-value ranged from 0.449 to 0.529, $p < 0.01$). Furthermore, a negative relationship between EC value and germination-related traits was observed under submergence conditions (r-value range from -0.710 to -0.679, $p < 0.01$) (Table 2).

Table 2. Pearson's correlation coefficients between germination-related traits of 105 wild soybean accessions under control and submergence conditions.

	GR 0	**NR 0**	**GR 5**	**NR 5**	**GI**	**NI**	**EC**
GR 0	1						
NR 0	0.862 **	1					
GR 5	0.483 **	0.529 **	1				
NR 5	0.449 **	0.508 **	0.973 **	1			
GI	0.440 **	0.488 **	0.997 **	0.967 **	1		
NI	0.423 **	0.475 **	0.972 **	0.997 **	0.971 **	1	
EC	−0.479 **	−0.460 **	−0.710 **	−0.679 **	−0.707 **	−0.677 **	1

GR 0, germination rate under control conditions; NR 0, normal seedling rate under control conditions; GR 5, germination rate under submergence conditions; NR 5, normal seedling rate under submergence conditions; GI, germination index by submergence conditions; NI, normal seedling index by submergence conditions; EC, electrical conductivity of the leakage solute; **, statistical significance at the 0.01 level.

Moreover, we investigated the relationship of submergence responses of wild soybean plants to stress at the V2 and germination stages through the correlation coefficients among FDS, GI, and NI. A weak negative correlation between the FDS at the V2 stage and phenotypic measurements at the germination stage was found at a significance level ($r = -0.214$ and $r = -0.207$, respectively, $p < 0.05$) (Table 3).

Table 3. Pearson's correlation coefficients of traits related to submergence responses of 103 wild soybean accessions at the early vegetative and germination stages.

	FDS	**GI**	**NI**
FDS	1		
GI	–0.214 *	1	
NI	–0.207 *	0.951 **	1

FDS, foliar damage score; GI, germination index; NI, normal seedling index; **, *, statistical significance at the 0.01 and 0.05 levels, respectively.

3.2. GWAS for Submergence Tolerance in Wild Soybean

A GWAS was conducted using 3,655,432 SNPs with MLM and FarmCPU models to identify significant SNPs associated with four traits: the FDS, the GI, the NI, and the EC (Figure 3). In the MLM, 55, 19, and 25 SNPs with higher than 5.0 of $-\log_{10}(p)$ were found to be linked with FDS, GI, and NI, respectively. These SNPs are distributed on several chromosomes (Figure 3A,C,E). The FarmCPU analysis revealed 16, 11, and 16 SNPs for FDS, GI, and NI, respectively. The threshold was 6.56 of $-\log_{10}(p)$ based on the Bonferroni correction (Table 3). Sixteen SNPs found on chromosomes 1, 3, 6, 7, 9, 10, 13, 18, and 19 were associated with FDS at the V2 stage with $-\log_{10}(p)$ values ranging from 6.63 to 7.56 (Figure 3). During germination, 11 SNPs were identified on chromosomes 8, 11, and 17, specifically associated with GI traits. Additionally, 17 SNPs related to NI were discovered on chromosomes 4, 6, 8, 11, 13, and 17. Notably, a genomic locus on chromosome 8 contains important SNPs and two other SNPs on chromosomes 11 and 17, which are related to GI and NI (Figure 3B,D,F).

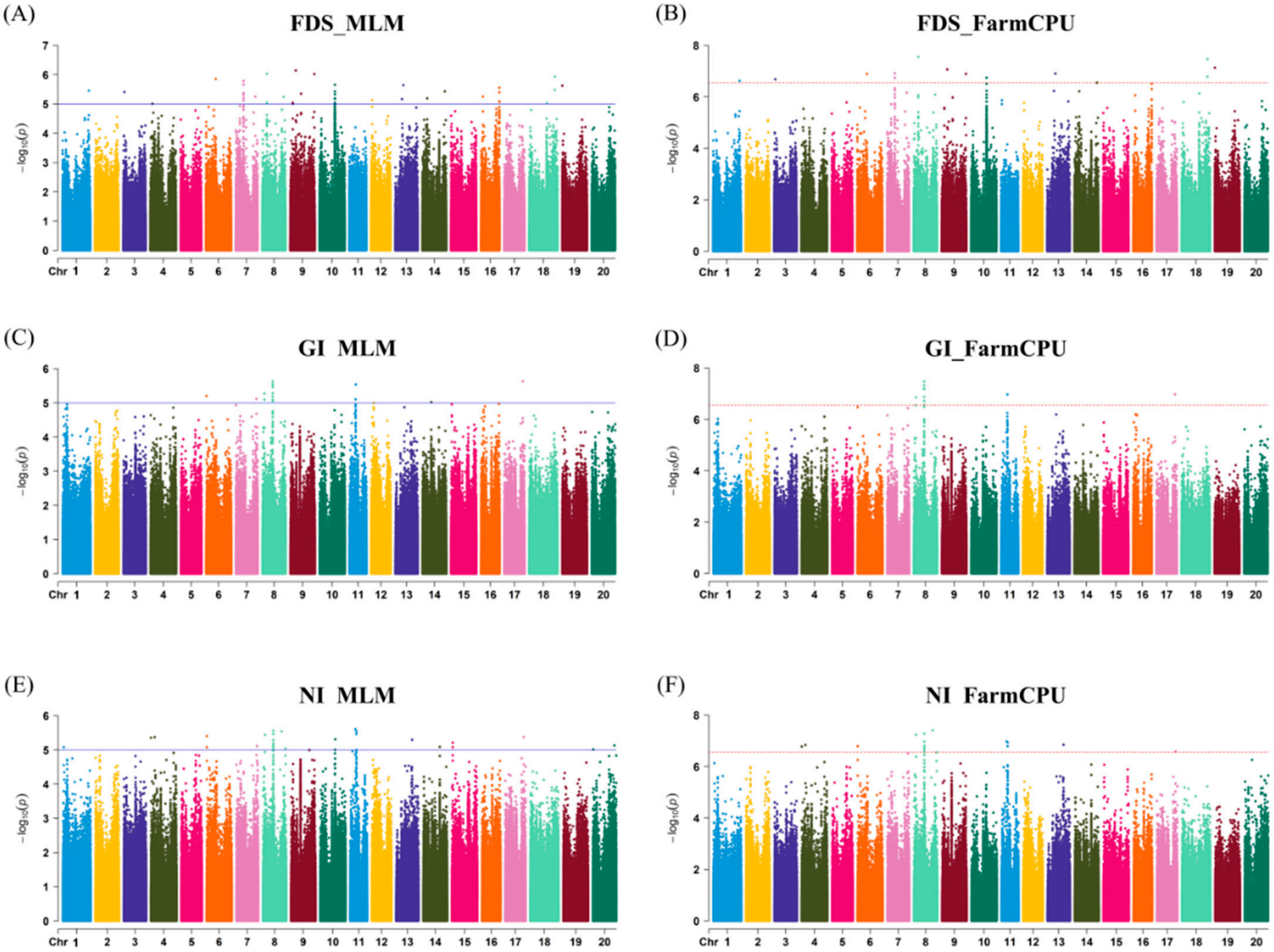

Figure 3. Manhattan plots of genome-wide association study (GWAS) analysis results. (**A**,**B**) Foliar damage score (FDS) at the early vegetative stage. (**C**,**D**) Germination index (GI) at the germination stage, (**E**,**F**) Normal seedling index (NI) at the germination stage. (**A**,**C**,**E**) MLM of GWAS analysis; (**B**,**D**,**F**) FarmCPU model of GWAS analysis. The red line indicates the Bonferroni-corrected threshold $-\log_{10}(p) = 6.56$, and the blue line indicates $-\log_{10}(p) = 5.0$. FDS, foliar damage score; GI, germination index; NI, normal seedling index.

3.3. Predicted Putative Genes Related to Submergence Tolerance in Wild Soybeans

The haplotype blocks were determined on the genomic regions based on significant SNPs in all measured traits from the FarmCPU model (Table 4). The size of the LD blocks in the detected genomic regions in the GWAS varied from 0.1 to 87.0 kbp. Of those LD blocks, 14 contained no annotated genes. For the V2 stage, we determined putative genes in five genomic regions in chromosomes 1, 7, 9, 10, and 18 with LD sizes of 13 kbp to 87 kbp. For the germination stage, two genomic regions in chromosomes 8, 11, and 17 were found to be overlapped and associated with the GI and NI traits, with LD sizes ranging from 1.3 kbp to 56 kbp. However, no gene was found in chromosome 11. The putative genes were associated with submergence tolerance during germination in the overlapping regions on chromosomes 8 and 17 (Figure S5).

Table 4. Significant SNPs associated with foliar damage score (FDS) at the early vegetative stage and germination index (GI) and normal seedling index (NI) by submergence conditions using the FarmCPU model in a genomic-wide association study.

Stage	Trait	Chr	Physical Position	$-\log_{10}(p)$	MAF	Allelic Effect	LD Block	
							Start (bp)	End (bp)
Early vegetative stage	FDS	1	54,141,037	6.63	0.42	0.88	54,130,751	54,144,573
		3	1,998,277	6.68	0.06	−0.70	1,998,277	1,998,458
		6	19,990,938	6.89	0.07	−0.68	19,990,564	19,998,508
		7	15,885,310	6.75	0.11	0.57	15,881,438	15,885,609
			15,885,846	6.92	0.09	−0.58	15,885,656	15,886,110
			15,890,150	6.92	0.09	−0.58	15,886,112	15,893,940
			15,890,983	6.92	0.09	−0.58		
			15,891,177	6.92	0.09	0.58		
		8	9,563,348	7.56	0.07	0.73	9,562,768	9,563,594
		9	11,188,389	7.07	0.07	−1.07	11,184,783	11,192,098
			49,025,346	6.89	0.06	0.95	49,019,854	49,034,099
		10	31,399,639	6.74	0.24	−0.43	31,342,015	31,429,854
		13	16,669,963	6.91	0.06	0.76	16,669,963	16,672,455
		18	52,427,621	6.79	0.07	−0.68	52,426,634	52,427,636
		18	52,439,013	7.46	0.06	−0.72	52,430,505	52,443,633
		19	232,374	7.14	0.07	0.69	231,112	232,480
Germination stage	GI	8	4,273,157	6.86	0.09	0.19	4,267,806	4,279,415
			21,073,167	6.57	0.12	−0.16	21,050,172	21,109,573
			21,083,689	6.88	0.13	−0.15		
			21,086,573	7.21	0.45	−0.30		
			21,087,286	7.18	0.12	0.17		
			21,090,616	7.48	0.45	−0.16		
			21,090,742	7.33	0.11	0.17		
			21,110,694	7.18	0.12	−0.16	21,109,768	21,116,140
			21,111,250	6.72	0.12	−0.15		
		11	12,624,478	6.97	0.05	0.25	12,603,869	12,630,069
		17	37,850,786	6.98	0.05	0.25	37,850,055	37,851,441
	NI	4	292,193	6.78	0.05	0.21	291,401	292,411
			7,867,267	6.84	0.10	0.23	7,866,359	7,867,267
		6	417,071	6.79	0.09	0.18	416,914	417,786
		8	4,268,879	7.24	0.08	−0.16	4,267,806	4,279,415
			21,050,405	6.73	0.09	−0.16	21,050,172	21,109,573
			21,083,689	6.56	0.13	−0.13		
			21,086,573	6.65	0.12	−0.25		
			21,087,286	6.81	0.45	−0.13		
			21,090,616	6.97	0.11	−0.26		
			21,090,742	7.28	0.12	0.14		
			21,110,694	6.81	0.12	−0.13	21,109,768	21,116,140
			37,950,930	7.41	0.05	−0.27	37,850,055	37,952,064
		11	10,489,104	6.98	0.08	0.18	10,486,803	10,492,014
			12,584,954	6.79	0.08	0.17	12,584,597	12,602,962
			12,624,478	6.94	0.05	0.22	12,603,869	12,630,069
		13	32,570,204	6.85	0.08	0.17	32,567,865	32,580,571
		17	37,850,786	6.59	0.05	0.21	37,850,055	37,851,441

Chr, chromosome; MAF, minor allelic frequency.

The putative genes are anticipated to be associated with submergence tolerance-related traits at the V2 and germination stages (Table 5). At the V2 stage, eight putative genes stand out: *Glyma.01g209800*, which has a DnaJ domain, *Glyma.01g209900*, which has a CRAL/TRIO domain, *Glyma.07g134100*, which has a multicopper oxidase domain, *Glyma.09g274300*, which has a protease associated (PA) domain, *Glyma.10g122500*, which has a Clp amino-terminal domain, *Glyma.10g122600*, which has an Expansin C-terminal domain, and *Glyma.18g235900*, which has a RIC1 domain. For the germination stage, four genes were identified as being involved with both the GI and NI: *Glyma.08G055500* (ATP-binding cassette transporter), *Glyma.08G055600* (Thioredoxin), *Glyma.08g244700* (UDP-glucoronosyl and UDP-glucosyl transferase), and *Glyma.17g225400* (protein kinase domain).

Table 5. Putative genes associated with foliar damage score (FDS) at the early vegetative stage, and the germination index (GI) and normal seedling index (NI) at the germination stage under submergence conditions within haplotype blocks.

Stage	Trait	Chr	Near SNP	Gene Name	Start Position (bp)	End Position (bp)	Predicted Function (Pfam)
Early vegetative stage	FDS	1	Chr01_54141037	*Glyma.01g209800*	54,132,733	54,141,714	DnaJ domain
				Glyma.01g209900	54,143,653	54,147,593	CRAL/TRIO domain
		7	Chr07_15885846	*Glyma.07g134100*	15,880,168	15,883,379	Multicopper oxidase
		9	Chr09_49025346	*Glyma.09g274300*	49,026,939	49,033,104	Protease associated (PA) domain
		10	Chr10_31399639	*Glyma.10g122300*	31342355	31,344,766	Expansin C-terminal domain
				Glyma.10g122400	31,351,688	31352397	Damage-control phosphatase ARMT1-like domain
				Glyma.10g122500	31,397,284	31,398,432	Clp amino-terminal domain
				Glyma.10g122600	31,424,536	31,427,271	Expansin C-terminal domain
		18	Chr18_52439013	*Glyma.18g235800*	52,428,946	52,442,014	RIC1
				Glyma.18g235900	52,443,073	52,448,797	Ferric reductase NAD binding domain
Germination stage	GI, NI	8	Chr08_4268879 – Chr08_4273157	*Glyma.08G055500*	4,269,457	4,272,267	ATP-binding cassette (ABC) transporter
				Glyma.08G055600	4,273,355	4,280,318	Thioredoxin
			Chr08_21090742	*Glyma.08g244700*	21,100,094	21,102,535	UDP-glucoronosyl and UDP-glucosyl transferase
		17	Chr17_37850786	*Glyma.17g225400*	37,849,485	37,855,509	Protein kinase domain

Chr, chromosome.

4. Discussion

Flooding treatments, including submergence and waterlogging treatments, are significant abiotic stresses that hamper germination and restrict the growth and development of various plant species. Tolerance to submergence stress was demonstrated by the plants' or seeds' capacities to remain alive and grow during the stress or recovery phase [40,41]. Due to planting during the rainy season or relatively short plant height at the vegetative stages, submergence and waterlogging stresses could occur in wild and cultivated soybeans at the early growth stages [42]. Hence, in this investigation, we examined the reactions of wild soybean seeds and seedlings after 5-day submergence stress. Leaf wilting and chlorosis are the primary visual symptoms detected in plants under flooding stress [43]. Earlier studies used this visual scoring method to screen flooding-tolerant soybean lines [22,44].

For the FDS, the ANOVA result indicates that the genotypes had an important impact on the responses of wild soybeans to submergence stress ($p < 0.0001$), suggesting that these responses were controlled by genetic factors (Table 1). The number of stress-sensitive and extremely stress-sensitive accessions accounted for over 80% in both the early vegetative and germination stages. However, for submergence responses of 386 cultivated soybean germplasm, all accessions were S-V2 or HS-V2 genotypes to submergence stress with an average FDS of 4.9. These findings indicated that some wild soybeans were relatively more tolerant to submergence at the early vegetative stages than cultivated soybeans. The previous study reported that wild soybeans showed higher tolerance to flooding than cultivated soybeans [32]. At the V2 stage, only two wild soybean accessions exhibited HT-V2 genotypes, and four accessions were T-V2 genotypes to submergence stress.

During the germination stage, submergence stress significantly decreased the GR and NR of wild soybean seeds, resulting in an average drop of more than 67%. The comparison of these two traits between stress and normal conditions revealed GI and NI traits with an average value of less than 0.2. The NR and NI values were lower than the GR and GI values, respectively, suggesting that submergence stress disrupts the normal establishment of soybean plants (Figure 2). Submergence has many effects on reduced germination, seed degradation, and the occurrence of abnormal seedlings in soybeans and other plant species [14,18]. Previous investigations have shown that the EC value can be measured when seed components dissolve into water during germination under submergence stress. The elevated EC value observed is likely attributed primarily to the breakdown of the cell membrane [18,45]. The submergence-tolerant lines exhibited higher GR and NR and lower EC values than the submergence-sensitive lines. Previous studies reported that the EC values were negatively correlated with the GR, the NR, the GI, and the NI by submergence conditions in soybeans [9,17,45–48], pea [19], and sudangrass [49]. Upon thorough analysis of the evaluation data, we identified three soybean lines that exhibit GI and NI values over 0.75. These lines are deemed capable of withstanding submergence during the germination stage. Notably, a wild soybean accession, CHN23 (T-V2 and T-G), showed a tolerant response to being submerged throughout the germination and early vegetative stages. Throughout the domestication progress of soybeans to meet the demand of high productivity, taste, and nutrient value, selective breeding resulted in a narrow genetic background and a reduction in the environmental adaptation of modern crops [50]. To overcome these drawbacks, wild soybeans have been chosen as the genetic material because of their wide genetic diversity and their unique resistance genes to abiotic and biotic stresses [51]. Thus, the discovery of tolerant wild soybean accessions in this study could be a valuable genetic resource for further investigation into flooding tolerance in soybeans.

Flooding tolerance in soybeans has been considered a complex quantitative trait controlled by multiple genes. Currently, GWAS and QTL mapping have been used to understand the genetic basis of flooding tolerance or submergence tolerance in soybeans [9,15,17,52–54]. The identified SNPs are found in different genomic regions and chromosomes, indicating the complex genetic basis of flooding-related traits in soybeans [15]. This study conducted a GWAS on 163 wild soybean accessions to investigate FDS at the V2 stage and the GI and NI of 105 accessions at the germination stage. MLM has been the most popular model applied in association mapping in plants including soybeans [11,13,19]. However, MLM leads to the missing of some potential SNPs or genomic regions. Therefore, for quantitative traits, using a FarmCPU model can control the false positives and reduce compute times for GWAS analysis. The application of these two models enables efficient analysis and improves the accuracy when conducting association mapping for quantitative traits [55]. Therefore, MLM and FarmCPU association models, which have been employed in prior research, were utilized in this analysis to identify important SNPs or QTLs associated with soybean agronomic variables [56–59]. Identifying significant SNPs in distinct genomic loci at two growth stages implies that distinct genetic mechanisms govern the submergence tolerance of wild soybeans at different growth stages.

Our investigation has identified significant SNPs and genomic loci harboring probable putative genes. For FDS at the V2 stage, we detected 16 significant SNPs according to the $-\log_{10}(p)$ value. Among them, five SNPs on chromosome 7 and one SNP on chromosome 10 were found in genomic regions with approximate LD sizes of 12.5 kbp and 87.8 kbp, respectively. Furthermore, several genes were detected in the LD block regions linked to significant SNPs on chromosomes 1, 9, and 18. Previous studies reported QTLs related to dry weight index, survival rate, and flooding tolerant index by flooding at vegetative stages in chromosomes 7, 10, and 18; however, their physical positions were around 630 kbp to 10 Mbp away from the genomic regions detected in our study (Table 5) [53,54,60]. During the germination stage, it is worth considering that the overlapped genomic regions for GI and NI on chromosomes 8 and 17 are important regions to link submergence tolerance in wild soybean seeds. Recent studies identified different QTLs and SNPs in chromosome 8 associated with a tolerance of soybean seeds under submergence stress, which was 3.8 Mbp to 9.4 Mbp, distinct from the detection of genomic regions in our study [9,29]. Additionally, three other genomic regions on chromosomes 4, 11, and 13 may be associated with the formation of normal seedlings in soybean seeds under stressful conditions. In this study, the detection of several genomic loci by GWAS indicates the presence of multiple genetic variables controlling submergence tolerance in wild soybeans at different growth stages. In addition, our study showed a weak correlation between submergence responses at the two stages. This conclusion is consistent with the results of cold tolerance between the germination and V2 stage in tomatoes [61] and alkaline–saline tolerance between the seedling and mature stages in wheat [62].

Although the biochemical mechanisms behind plant tolerance to flooding are not currently fully understood, flooding is recognized as a complex abiotic stress. During the period of stress or the recovery stage after stress, plants must interact with other stresses [63]. Oxidative stress, senescence, dehydration, and ABA-related stresses during the recovery stage were identified as factors that induce post-submergence injury, ultimately determining the survival and development of plants following the stress [64–66].

One of the main ways that plants cope with submergence and other abiotic stresses is by recovering their root growth and preserving the function of their roots, which allows them to continue absorbing nutrients and survive after stress [66–68]. *GmEXP1* was observed in the growth of root hairs in wild soybeans [69]. This work identified two paralogous genes, *Glyma.10g122300* (*GmEXP2*) and *Glyma.10g122600* (*GmEXP5*), on chromosome 10 at the V2 stage. These genes belong to the beta-Expansin subfamily of the Expansin family and are commonly involved in modifying cell walls in response to various abiotic stimuli reported in previous studies [70–72]. In addition, Expansin genes are important for elongating or enlarging plant cells [72,73] and are involved in the plant response to phytohormones and abiotic stress in soybeans [71], Populus [74], potato [75], and maize [76]. During waterlogging conditions, the different expression of Expansin genes in the root tissue of tolerant and sensitive maize genotypes was reported [76].

Altering the composition or structure of plant cell walls is seen as a viable approach to cope with abiotic stresses [77]. Flooding stress results in an increase in lignin content in the cell wall, which enhances the ability of teosinte (*Zea nicaraguensis*) to prevent radial oxygen loss during stress [78]. For the FDS trait, we determined a putative gene, *Glyma.07g134100*, in a laccase family containing multicopper oxidase. Plant laccases, which are enzymes that contain several copper oxidases, have a crucial function in the process of lignin production and in responding to abiotic stress [79,80]. In rice, the high content of lignin contributed to lodging resistance to deep flooding or submergence stress [81]. In Arabidopsis, the knockout of *lac17* showed a significant reduction in lignin in the stem, while the complementation of *lac17* with *LAC17* restored the loss of lignin content [82,83]. The laccases play a role in the reactions of maize and its wild relative to the stress caused by submergence during the vegetative stage [84].

This investigation identified a potential gene, *Glyma.08g244700*, for the GI and NI values, which encodes for UDP-glucosyl transferase (UGT). It has been claimed that this gene is involved in the modification of cell wall polysaccharides. The overexpression of the *GmUGT83R1* mutant gene in soybean not only showed a higher content of pectin and cellulose, but also exhibited greater root length compared to the wild type [85]. A rapid reduction in pectin and cellulose content was shown in the root of the sensitive genotype, while it remained relatively high in the tolerant genotype of *Brassica napus* L. under flooding conditions [86]. The UGT gene, *OsUGT75A*, has been identified as a potential gene responsible for the ability of rice seeds to tolerate submergence by controlling the coleoptile length [87]. Therefore, *Glyma.08g244700* may play a role in the response to submergence stress in wild soybeans during the germination stage. Furthermore, in response to flooding stress, plants can modify not just lignin but also cutin and suberin, thereby enhancing the barrier against radial oxygen loss [67,88]. In this study, we identified a putative gene that may be associated with GI and NI at the germination stage, *Glyma.08G055500*, which encodes for ATP-binding cassette (ABC) transporter. Several ABC transporters in subfamily G (ABCG) proteins are associated with suberin and cuticle in plants, which is considered to improve the tolerance of plants under submergence stress. In Arabidopsis, knockout mutants (*abcg5*) showed a lower GR than wildtype; *ABCG5* also contributed to a high cuticular wax content of seedlings, which reduced the survival of submerged seedlings [89].

5. Conclusions

In this study, wild soybean accessions had diverse reactions to being submerged during the early vegetative stages, while cultivated soybeans showed more sensitivity to submergence stress than wild soybeans. The phenotypic evaluation identified two wild soybean accessions that showed high tolerance, four wild soybeans that showed tolerance to submergence stress in the early vegetative stage, and three accessions that showed tolerance at the germination stage. CHN23 exhibited notable tolerance to submergence stress during both growth stages, making it a valuable genetic resource for further investigation into flooding tolerance in soybeans. Specifically, GWAS analysis revealed that 16 and 20 SNPs were linked with the submergence tolerant-related traits at the early vegetative and germination stages, respectively. Furthermore, based on LD analysis, ten putative genes were identified for submergence tolerance at the early vegetative stage, and four putative genes were identified for submergence tolerance at the germination stage. Further studies examining the expressions of putative genes should be conducted on the responses of wild soybeans to submergence and flooding stresses. The results of this study provide information for future programs to determine genetic factors controlling the flooding tolerance of wild soybeans and develop superior soybean cultivars with high productivity under flooding stress by the introgression of tolerance genes from wild soybeans.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture14091627/s1: Figure S1: Phenotypic evaluation for foliar damage score of wild soybeans after submergence. Foliar damage score (FSD) was rated from 1 to 5. Plants were assigned into five tolerance level groups: highly tolerant (HT-V2), tolerant (T-V2), moderate (M-V2), sensitive (S-V2), and highly sensitive (HS-V2). Figure S2: Different phenotypes of soybean seeds germinated under a 5-day submergence condition. (A) Normal seedlings, (B) abnormal seedlings, and (C) decay seeds. White bars indicate 1 cm. Figure S3: Phenotype of selected wild soybean plants under 5-day submergence following 2 days of drainage at the early vegetative stage. YWS294 and YWS1022 were highly tolerant (HT-V2), CHN23 was tolerant (T-V2), CHN25 was moderate (M-V2), and YWS525 and IT182976 were highly sensitive (HS-V2). Figure S4: Phenotype of accessions with high and low germination and normal seedling index after submergence. CHN23 was tolerant (T-G), and IT182976 was highly sensitive (HS-G). Figure S5: Manhattan plot and linkage disequilibrium (LD) heat map surrounding the significant SNPs. (A) Manhattan plot for foliar damage score (FDS) on chromosome 1. (B) Manhattan plot for FDS on chromosome 7. (C) Manhattan plot for FDS on chromosome 9. (D) Manhattan plot for FDS on chromosome 10. (E) Manhattan plot for FDS on chromosome 18. (F,G) Manhattan plot for germination index (GI) and normal seedling index (NI) on chromosome 8. (H) Manhattan plot for GI and NI on chromosome 17. (I) Manhattan plot for NI on chromosome 4. (K) Manhattan plot for NI on chromosome 11. (L) Manhattan plot for NI on chromosome 13. For the Manhattan plot, the red line indicates the Bonferroni significant threshold. For the LD heat map, the blue arrows represent the position of significant SNP in the LD block. The regions indicated by solid lines were used to determine putative candidate genes. Table S1: Foliar damage score of wild soybean plants by 5-day submergence followed by 2 days of drainage at the early vegetative stage. Table S2: The phenotype of 105 wild soybean accessions under 5-day submergence at the germination stage. Table S3: Foliar damage score of cultivated soybean plants after submergence at the early vegetative stage. Table S4: Analysis of variance of germination rate and normal seedling rate of 105 wild soybean accessions in control and submergence conditions.

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