RNA 시퀀싱과 QTL 분석을 통한 콩 내습성 관련 후보유전자 탐색

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Integrating RNA Sequencing and Quantitative Trait Locus Mapping to Identify Potential Candidate Genes for Flooding Tolerance in Soybean

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Abstract Flooding stress causes a significant reduction in soybean yield. The development of flood-tolerant cultivars is an effective way to minimize yield loss due to flooding. Information on candidate genes for flooding tolerance is useful for developing tolerant lines. The objective of this study was to identify potential candidate genes for flooding tolerance in soybean by integrating the results of a quantitative trait locus analysis and RNA sequencing. A total of 19 genes showed good amplification in capillary electrophoresis and were further analyzed through a reverse transcription quantitative polymerase chain reaction (qRT-PCR); two of these genes showed differential expression among tolerant and susceptible lines. The expression of *Glyma.12g030900* and *Glyma.10g050300* in leaf and root tissues, respectively, was higher in several tolerant lines than in the susceptible lines under flooding stress. The chlorophyll index of the tolerant lines was also consistently higher than that of the susceptible lines over two years, supporting the qRT-PCR results. This study provides useful information on flooding tolerance in soybeans.

Keywords flooding stress, gene expression analysis, genetic mapping, marker-assisted selection, transcriptome analysis

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Introduction

Flooding stress is one of the major abiotic stresses that causes substantial yield reduction in soybean (*Glycine max* L. Merrill), one of the most economically important legumes worldwide. Flooding stress in soybean causes different morphological and physiological impairments, such as reduction in leaf chlorophyll content (Cho et al. 2006) and shoot dry weight (VanToai et al. 2001, Shimono et al. 2012), resulting in yield reduction. The increment of soybean cultivation in the converted paddy fields (Singh 2010, Nishida et al. 2013) and possible increase in floods due to climate change (Bailey-Serres et al. 2012) may further reduce the soybean productivity due to flooding

stress. Identification of candidate genes associated with flooding tolerance in soybean, therefore, could be of great significance in developing flooding tolerant cultivars and thereby reducing the negative impacts of flooding stress.

Genetic mapping techniques, such as quantitative trait locus (QTL) analysis and genome-wide association study (GWAS) are helpful for locating the genetic regions associated with quantitative trait variations (Miles 2008, Visscher et al. 2008). Several QTL studies on flooding stress tolerance at different stages of soybean growth have been carried out and documented in Soybase (<http://www.soybase.org>). At the seed germination stage, two studies have identified 25 (Yu et al. 2019) and four (Sayama et al. 2009) QTLs for seed-flooding tolerance. Recently,

20 QTLs for flooding tolerance at the V1-V2 stages of soybean have been identified on nine chromosomes (Dhungana et al. 2020a). In another QTL study conducted at early growth stage has reported seven QTLs (Githiri et al. 2006). Similarly, one (VanToai et al. 2001) and six (Nguyen et al. 2012) QTL associated with flooding tolerance have been identified at the R1 stage and two QTLs are detected at the R2 stage (Comelious et al. 2005).

Molecular techniques, such as microarray profiling and RNA sequencing (RNA-seq) have been used to generate gene expression data. RNA-seq, a more advanced technique than microarray, has become popular (Trapnell et al. 2013, da Maia et al. 2017, Zhang et al. 2017b) to investigate gene expression in many species. A number of studies have been conducted to examine the gene expressions in soybean tissues under different biotic and abiotic stresses, such as drought (Vidal et al. 2012), flooding (Dhungana et al. 2020b, Sharmin et al. 2020) drought and flooding (Chen et al. 2016), common cutworm attack (Du et al. 2019), and salt (Zeng et al. 2019).

Integration of the results obtained from QTL and/or GWAS as well as RNA-seq analyses could more precisely predict candidate genes of a trait as the former is useful in locating the genetic regions associated with the trait and the latter provides a global transcriptome at the whole-genome level. This strategy of combining results to screen candidate genes associated with various target traits is widely applied in many crops, such as cold stress response in rice seedling (Kong et al. 2020), cadmium tolerance in barley (Derakhshani et al. 2020), fiber quality traits in cotton (Liu et al. 2016), flowering time genes in oilseed rape (Jian et al. 2019), and salt tolerance in rice (Wang et al. 2017). By adopting the same approach, a few studies have also been conducted in soybeans to predict candidate genes for traits like pod dehiscence (Hu et al. 2019) and low-phosphorus stress (Zhang et al. 2017a). Despite several separate reports on screening candidate genes for flooding tolerance in soybean through genetic mapping or RNA-seq, no such study has been performed integrating the results of QTL mapping and transcriptome sequencing. The objective of this study was to predict potential candidate genes based on the results of QTL and RNA-seq and to validate them using

real-time quantitative polymerase chain reaction (qRT-PCR). The results could be useful in soybean breeding program for developing flood-tolerant soybeans.

Materials and Methods

Plant materials and growing conditions

Flood-tolerant ‘Paldalkong’, a common cultivar in Korea (Chun et al. 2019), and flood-susceptible ‘NTS1116’ soybean cultivars, which were used as parents to develop the QTL mapping population (Dhungana et al. 2020a) and RNA-seq (Dhungana et al. 2020b) analyses, were considered in this study. For the measurement of leaf chlorophyll content index (CCI), 10 flood-tolerant and 10 flood-susceptible recombinant inbred lines (RILs) from the QTL mapping population were included. The relative expression of the potential candidate genes was analyzed in 2 susceptible (18R47-154 and 18R47-143) and 3 tolerant (18R47-64, 18R47-30, and 18R47-47) RILs, along with the parental cultivars (Paldalkong and NTS1116).

Plants were grown in round-bottomed plastic pots of 16 × 20 cm dimension (top and bottom diameters × height), kept in a plastic house (Department of Southern Area Crop Science, National Institute of Crop Science, Miryang, Republic of Korea). The plants were raised under ambient environment with an average temperature of 33.6 and 33.2°C in 2019 and 2020, respectively. The pots were filled with the soils prepared by mixing upland soil, compost, and nursery soil at 1:1:0.75 ratio (Dhungana et al. 2020b). Five seeds were sown on July 15 in three replicates for each genotype and treatment condition. Seedlings were thinned by the first trifoliate (V1) stage to keep three plants in each pot. All plants were grown in a well-watered condition up to the V1–V2 stage and then the stress-designated plants were flood-stressed by holding ~10 cm water for 14 days, whereas the control-designated plants were grown in well-watered condition during the period.

Measurement of leaf chlorophyll content

The leaf chlorophyll content (CC) was measured on the first trifoliate leaves at 2-5-day intervals using a chlorophyll meter (SPAD-502Plus, Minolta Camera Co., Osaka, Japan).

Leaf chlorophyll index (CCI) was calculated as the ratio of CC under flooded to control conditions and was considered as an indicator of flood tolerance level.

Sample collection, RNA isolation, and cDNA synthesis

Leaf and root samples were collected at 14 days after flooding (DAF) in microtubes, immediately kept into liquid nitrogen, and stored at -80°C until RNA extraction (Dhungana et al. 2020b). Total RNA from the leaf and root samples were isolated using an RNA extraction kit (RNeasy PowerPlant Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions. The cDNA was synthesized through a reverse transcription reaction using the EcoDry cDNA synthesis premix (Takara Bio Inc., Shiga, Japan), following the manufacturer's instructions. The concentrations of RNA and cDNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Screening of potential candidate genes

A few differentially expressed genes in RNA-seq (Dhungana et al. 2020b) within the QTL regions (Dhungana et al. 2020a) were selected on the basis of the FPKM (fragments per kilobase of exon per million reads mapped) values of the tolerant and susceptible parental cultivars (Paldalkong and NTS1116) in RNA-seq and/or functional annotation (gene product) for investigating as potential candidate genes for flooding tolerance.

Primer design and amplification analysis

All the primers for qPCR were designed using Primer3 software (<http://bioinfo.ut.ee/primer3/>). The amplified PCR products of the primers were examined with a QIAxcel DNA High Resolution (Qiagen, Hilden, Germany). The 10 μl PCR reaction mixture consisted of 5 μl master mix (PreMIX-nTaq, Enzynomics, Seoul, Korea), 3 μl distilled water, 0.5 μl forward and reverse primers, and 1 μl cDNA template. The conditions for PCR were: holding at 95°C (5 min), melting at 95°C (20 s), and annealing at 60°C (20 s)/ 72°C (1 min) for 35 cycles.

qRT-PCR and gene expression analysis

Gene expression was determined by qRT-PCR using an ABI

7300 system (Applied Biosystems, Foster City, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Woolston Warrington, UK). The reaction mixture (20 μl) consisted of 10 μl SYBR Green, 8 μl sterilized distilled water, 0.5 μl forward and reverse primers each, and 1 μl cDNA. The conditions for qRT-PCR were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s. The experiments were performed with three biological replicates, and the relative expression of the genes was determined using the $\Delta\Delta\text{Ct}$ (delta delta cycle threshold) values. Actin was used as reference (housekeeping) gene and NTS1116 control as reference sample for the relative expression analysis.

Statistical analysis

Analysis of variance was conducted using SAS 9.4 (SAS Institute, Cary, NC, USA) to compare the means between the control and flooding of different samples, and the significant differences were determined using the least significant difference test at $p < 0.5$.

Results

Variation in chlorophyll index

The average CCI values of the susceptible RILs were lower than that of the tolerant RILs in both years (Fig. 1). The difference in the average CCI between the tolerant and susceptible lines was higher in 2020 (Fig. 1B) than in 2019 (Fig. 1A).

Potential candidate genes and their amplification

A total of 31 genes were selected as potential candidate genes for flooding tolerance based on the QTL and RNA-seq results. Before qRT-PCR, amplifications of the genes were analyzed through capillary electrophoresis and only the primers with good amplification of target-size amplicons were considered for further analysis. Out of the 31 genes, 19 were found to have good amplification (Table 1). So, only the primers of these genes were analyzed through qRT-PCR. Some of the genes such as *Glyma.10g050300*, *Glyma.12g040600*, *Glyma.12g041200*, and *Glyma.13g285500* that had good amplification in leaf tissues showed satisfactory amplification in root tissues as well.

Fig. 1. Chlorophyll index values of the tolerant and susceptible recombinant inbred lines (RILs) in 2019 (A) and 2020 (B).

Table 1. Selected potential candidate genes within the QTL regions for flooding tolerance in soybean.

SN	Name of gene	QTL reported	FPKM (Paldalkong)		FPKM (NTS1116)		Product	Forward primer (5'→3')	Reverse primer (5'→3')
			Control	Flooding	Control	Flooding			
1	<i>Glyma.10g040600</i>	<i>qSFT_10-35</i>	42.034	16.632	37.228	19.162	Photosystem II reaction center PSB28 protein, chloroplastic	ACCCTCCACTCTCTTGCAATT	GGCATGCGTAAAGTTTGACC
2	<i>Glyma.10g041500</i>	<i>qSFT_10-35</i>	0.412	5.922	7.365	5.820	Ketohexokinase, transcript variant X1	CCACCATCAGAGCTTGTTGA	GCTCCTAGATCCCTGCACTT
3	<i>Glyma.10g050100</i>	<i>qSFT_10-43</i>	0.155	1.393	0.341	0.376	Probable beta-1,4-xylosyltransferase IRX9	GGTCGCACTTCCCTCTTCA	GATTTGAAGTCGTGCGAGCA
4	<i>Glyma.10g050300</i>	<i>qSFT_10-43</i>	1.151	3.476	2.425	2.497	Lipid phosphate phosphatase gamma, transcript variant X2	CTCCAGTCACTGCCAGTACA	AGCCCAAGTAAACCCGAGAA
5	<i>Glyma.10g051500</i>	<i>qSFT_10-43</i>	0.136	3.675	1.493	0.016	Zinc finger protein JACKDAW, transcript variant X1	TCACCTTCCTCCCAAATACC	CAACTGCCCCATTGACTTGCA
6	<i>Glyma.10g053500</i>	<i>qSFT_10-46</i>	1.056	8.408	3.067	9.292	Auxin response factor 18, transcript variant X2	GGCAGACATGTTTGGCATAG	CGTTGTACTGTCAGAAATCC
7	<i>Glyma.10g053900</i>	<i>qSFT_10-46</i>	0.010	5.677	0	0.878	Protein CNGC15a, transcript variant X2	TTCCTGGAACAACCTGGCTCT	GTTCAAGGGGCTTCCTCAAT
8	<i>Glyma.10g063900</i>	<i>qSFT_10-52</i>	0.544	1.510	0.325	0.520	RecF/RecN/SMC N terminal domain-containing protein	AGGAGGGTCAATGCTTGCTT	CACCATGAGCAGAAGAACCA
9	<i>Glyma.11g183700</i>	<i>qSFT_11-55</i>	0.073	0.071	0.269	0.047	bZIP transcription factor	TCTTGTTGACACCATTCGGC	CTTGCTGCTTGACGAACCTGT
10	<i>Glyma.11g214500</i>	<i>qSFT_11-65</i>	2.566	1.425	0.045	0.213	U-box domain-containing protein	CAAGAGGGAGGAAGGGGTTT	GTTTCAACAACCGGGTAGCA
11	<i>Glyma.12g018100</i>	<i>qSFT_12-8</i>	3.343	11.455	3.563	5.386	Scarecrow-like protein 4	TCAGGACTGCGTTCAAGTACT	CACTGCTCCTTGTCCTCCAT
12	<i>Glyma.12g019200</i>	<i>qSFT_12-8</i>	6.252	12.799	4.744	5.774	Uncharacterized LOC100787046	ACCAAACCAAGTGCACATATCC	TTTCTGCAGCAACACACCC
13	<i>Glyma.12g022300</i>	<i>qSFT_12-8</i>	0.021	2.449	1.862	0	Gamma aminobutyrate transaminase 3, chloroplastic, transcript variant X2	GCTTGATTCTGGGCACTGAG	GCTACACGAACCAACATCCC
14	<i>Glyma.12g022700</i>	<i>qSFT_12-8</i>	57.231	9.909	6.360	10.522	NAC domain protein NAC6	GCACATCACTACAAATGCCCT	TGGACTATTCAACCTGAGCCCA
15	<i>Glyma.12g023300</i>	<i>qSFT_12-8</i>	0.047	0.061	0.250	2.196	L-ascorbate oxidase homolog	GCTGATTTTCGAGCCTTTGTC	TCCACACACCACCATCCATT
16	<i>Glyma.12g023600</i>	<i>qSFT_12-8</i>	3.407	6.658	1.895	3.515	Aquaporin TIP1-2	GGGCTTGCCAATGGTTTGTT	CCCAATGCCCATTCATAGTG
17	<i>Glyma.12g029200</i>	<i>qSFT_12-8</i>	0.005	0.546	0.614	0.005	DNA repair protein XRCC2 homolog, transcript variant X2	CCTTCTGTCTGGCAGTCCTT	TCGACAAAGACACGAGCATC

Table 1. Continued

SN	Name of gene	QTL reported	FPKM (Paldalkong)		FPKM (NTS1116)		Product	Forward primer (5'→3')	Reverse primer (5'→3')
			Control	Flooding	Control	Flooding			
18	<i>Glyma.12g029300</i>	<i>qSFT_12-8</i>	0.135	0.508	0.271	0.322	BTB/POZ domain-containing protein At1g50280	TGAGAGTCCAAG GATGAAGCA	GTGGCATCTGAA CGTTCGTT
19	<i>Glyma.12g029900</i>	<i>qSFT_12-8</i>	0.039	0.347	0.221	0.161	Uncharacterized LOC100807371, transcript variant X2	AGCCCTTTGAAG CAACTCATG	GCCAGGCATCTC ACTCATAC
20	<i>Glyma.12g030000</i>	<i>qSFT_12-8</i>	1.092	1.759	2.885	4.174	Inactive leucine-rich repeat receptor-like serine/threonine-protein kinase At1g60630	ATGCCGCAACTT TCAAAGGT	CACTGACCGAAC CCACCTAG
21	<i>Glyma.12g030900</i>	<i>qSFT_12-8</i>	0.034	0.264	0	0.279	Auxin influx carrier LAX12	TGTGGGTCTTAG TCGTAGGG	CCATGATGAGGG TGGTGAA
22	<i>Glyma.12g039400</i>	<i>qSFT_12-12</i>	5.077	13.169	3.988	5.736	Cysteine protease RD19A	GTGGCAAACCTC AGTGTCGT	AGGCACCAGAA CCATATCCC
23	<i>Glyma.12g040600</i>	<i>qSFT_12-12</i>	13.963	86.446	9.925	21.666	bZIP transcription factor bZIP124	TCAACATCACCA CGCAACAG	GTGGCATTCAAC ACGTCAAC
24	<i>Glyma.12g041200</i>	<i>qSFT_12-12</i>	0.589	3.460	1.346	1.098	Uncharacterized LOC100780999	CATAGTCGTGTG TTGCGCAG	AGCAATGAAAG CCTCCACAG
25	<i>Loc112998587</i>	<i>qSFT_12-8</i>	0.087	0.905	0.146	0.267	Lysine histidine transporter-like 8	CCTCAAGAAACC TCGTCCAAGA	CCATTGAGAGCT AGAGTCCGAA
26	<i>Glyma.13g276700</i>	<i>qSFT_13-35</i>	1.928	27.212	13.933	10.153	Protein SPA1-RELATED 3, transcript variant X1	ATTACGCGGTCA TGCGAATG	GCGTCGTCCACT TCATTACC
27	<i>Glyma.13g279500</i>	<i>qSFT_13-35</i>	15.67	46.846	46.849	46.129	Uncharacterized LOC100305647	TCACACCAAGTA CAGGACCA	TCCCTTGCCCTT GAGTAGTT
28	<i>Glyma.13g279900</i>	<i>qSFT_13-35</i>	0.714	6.628	0.413	3.059	NAC transcription factor NAC27	TCTTCTTCTTCGT CCCAGCT	CTCGTGGTGCTG TTGTTGTT
29	<i>Glyma.13g282900</i>	<i>qSFT_13-30</i>	0.035	0.360	0.607	1.723	Calcium-binding protein KRP1	TGCATGCTCATA GAGGGTGA	CCCTGAGGAGTG CACATTTT
30	<i>Glyma.13g285500</i>	<i>qSFT_13-30</i>	0.128	0.576	0	0	Calcium/calmodulin-regulated receptor-like kinase 1, transcript variant X2	GGCAGCACTTGC CTACAAAT	GCTGATCAACAT CAATGGAGAC
31	<i>Glyma.17g173000</i>	<i>qSFT_17-115</i>	6.562	15.338	2.187	2.714	Mitogen-activated protein kinase kinase kinase 2-like	CGCGGATATATG GGCTTTGG	TGCGCTCCACCT CTTCTTAG

The candidate genes resided within the QTL regions (Dhungana et al. 2020a) were identified on the basis of the FPKM (fragments per kilobase of exon per million reads mapped) values of the tolerant and susceptible genotypes and/or related gene product (Dhungana et al. 2020b). The genes in bold letters showed good amplification in the capillary electrophoresis assay.

The other genes had a considerable amplification only in the leaf tissue.

qRT-PCR and gene expression

Only two genes *Glyma.10g050300* and *Glyma.12g030900* showed differential expression in root and leaf tissues, respectively, under the control and flooded conditions among tolerant and susceptible genotypes (Fig. 2). The expression of *Glyma.10g050300* in the root tissue of three tolerant genotypes Paldalkong, 18R47-30, and 18R47-47 under flooded condition was higher than in the control condition and that in two susceptible genotypes NTS1116 and 18R47-154 under flooded condition was lower than that in the control condition. However, the expression in a tolerant line 18R47-64 under flooding was lower than that in the control condition and that in a susceptible

line 18R47-143 under flooding was slightly higher than that in the control condition (Fig. 2A).

The relative expression of *Glyma.12g030900* in the leaf tissue of three tolerant genotypes Paldalkong, 18R47-64, and 18R47-30 under flooded condition was higher than in the control condition. Among the three genotypes, Paldalkong had significantly higher expression of *Glyma.12g030900* under the flooded condition. On the other hand, the expression in two susceptible lines 18R47-154 and 18R47-143 under flooded condition was lower than that in the control condition. However, the expression in a tolerant line 18R47-47 under flooding was significantly lower than that in the control condition and in a susceptible genotype NTS1116 under flooding was slightly higher than that in the control condition (Fig. 2B).

Fig. 2. Relative expression of (A) *Glyma.10g050300* (in root tissue) and (B) *Glyma.12g030900* (in leaf tissue) under the control and flooded conditions among tolerant (left-side 4 genotypes in each gene) and susceptible (right-side 3 genotypes in each gene) genotypes. The vertical lines within bars indicate standard error. CL: control leaf, FL: flooded leaf, CR: control root, FR: flooded root.

Discussion

Flooding stress remarkably reduces CC in soybean (VanToai et al. 2001, Cho et al. 2006, Shimono et al. 2012). Higher CCI value denotes low difference between CC under flooding and control conditions, implying minimal effect of flooding stress. So, the genotypes with higher CCI indicates their greater tolerance level to flooding stress. In the present study, the tolerant RILs have higher CCI than the susceptible ones. Similar result of large reduction of CC in the susceptible cultivar Hannamkong than in the tolerant cultivar Sowonkong was found under excess water stress in soybean (Cho et al. 2006).

In this study, potential candidate genes for flooding tolerance were screened by integrating the results of RNA-seq (Dhungana et al. 2020b) and QTL mapping (Dhungana et al. 2020a). RNA-seq was conducted using the tolerant (Paldalkong) and susceptible (NTS1116) parental genotypes that were used in the QTL mapping. From RNA-seq analysis, a total of 31 genes residing in the QTL regions and having contrasting FPKM (fragments per kilobase of exon per million reads mapped) values between the parental genotypes (Paldalkong and NTS1116) under the control and flooded conditions, were sorted as potential candidate genes. However, in qRT-PCR analysis, both parental and RIL genotypes were considered to select

the candidate genes, in which only two genes had contrasting expression in the parental as well as RILs as shown in Fig. 2. Although the other 17 genes had satisfactory amplification in capillary electrophoresis, they did not show contrasting gene expression among tolerant and susceptible RILs in qRT-PCR. Capillary electrophoresis was carried out before qRT-PCR. Analysis of PCR products using capillary electrophoresis helps detect primer dimers and other non-target amplicons that could mislead the qRT-PCR results. Although *Glyma.10g050300* had considerable amplification in both leaf and root tissues, the gene expression was contrasting only in the root tissues. On the other hand, the amplification of *Glyma.12g030900* was satisfactory only in leaf tissues but not in root tissues. So, the gene expression analysis of *Glyma.10g050300* was conducted using the root tissues alone and that of *Glyma.12g030900* was carried out only in the leaf tissues.

Two genes *Glyma.10g050300* and *Glyma.12g030900*, identified as potential candidate genes for flooding tolerance, reside in QTLs *qSFT_10-43* and *qSFT_12-8* on chromosomes 10 and 12, respectively (Dhungana et al. 2020a). Lipid phosphate phosphatase (LPP), the product of *Glyma.10g050300*, is reported to be involved in biotic and abiotic stresses. The regulation of an LPP gene in *Arabidopsis* (*AtLPP1*) was in accordance with the radiation stress supporting the hypothesis that its

encoded LPP enzyme might attenuate the signaling functions of phosphatidate (PA) and/or diacylglycerol pyrophosphate (DGPP) that form in response to gamma or UV-B irradiation stress in plants (Pierrugues et al. 2001). The accumulation of PA and DGPP after elicitor treatment or stress in different plant systems (Munnik et al. 1998, Pical et al. 1999, Frank et al. 2000, van der Luit et al. 2000) suggests their role in response to various stresses such as salinity, hyperosmotic, or dehydration. LPP is also associated with pathogenesis of the rice blast fungus for the regulation of cellular diacylglycerol (Sadat et al. 2014), indicating its role in biotic stresses as well. Disruption of *LPP2* (*Atlg15080*) gene in *Arabidopsis* caused hypersensitivity to abscisic acid (ABA) and significant phospholipase (PA) accumulation during seed germination, indicating the involvement of PA in ABA signaling (Katagiri et al. 2005). Endogenous ABA regulates shoot elongation under flooding stress (Chen et al. 2010). ABA is a key signaling regulator in several vital plant processes, including response to various abiotic and biotic stresses (Lee & Luan 2012, Wang et al. 2018). ABA plays a role in regulating plant water balance under drought and flooding stresses (Olivella et al. 2000, Nan et al. 2002).

Although the product of *Glyma.12g030900*, auxin influx carrier LAX12, has not been reported to be associated with flooding stress tolerance in soybean so far, other members of auxin influx carrier AUX/LAX families are found to play a role in several functions, including salinity and drought stress tolerance in plants. AUX/LAX are the major auxin influx carriers and are found to mediate auxin-related developmental functions in different organs and tissues (Swarup & Bhosale 2019). AUX/LAX along with other auxin transporter gene families were associated with phytohormone and abiotic stresses such as salinity and drought (Shen et al. 2010). Auxin-responsive genes have roles in flooding tolerance in tomato (Bouzroud et al. 2018) and soybean (Ye et al. 2018). Auxin modulates different hormone levels (Woodward & Bartel 2005) and regulates adventitious root formation in response to flooding (Visser et al. 1995), possibly to alleviate negative effects of flooding stress. The auxin influx carrier, *OsAUX3* regulates rice root development and responses to aluminium stress (Wang

et al. 2019). Another auxin transporter *OsAUX1* is involved in primary root and root hair elongation and in cadmium stress responses in rice (Yu et al. 2015). The analysis of the promoter cis-element shows *BnAUX/LAX* genes may participate in drought stress tolerance (Bao et al. 2019). Cis-acting elements are involved in various functions in plants, such as stress response, growth, and development (Ibraheem et al. 2010).

In the QTL mapping study (Dhungana et al. 2020a), QTLs *qSFT_10-43* and *qSFT_12-8* harboring two candidate genes *Glyma.10g050300* and *Glyma.12g030900* accounted for 11.2 and 16.5% phenotypic variation, respectively, for flooding tolerance. High (>10%) phenotypic variations explained by the QTLs containing the candidate genes and contrasting expression of the genes found in the present study indicate their potential role in flooding tolerance. A few potential candidate genes, other than *Glyma.10g050300* and *Glyma.12g030900* found in the present study, have been reported for flooding tolerance in soybean. Ali et al. (2020), Sharmin et al. (2020), and Yu et al. (2019) identified several candidate genes for seed-flooding tolerance in the germinated seeds after a few hours to 7 d of stress and Casarotto et al. (2019) found some candidate genes in the flood-stressed plants at the V1 stage that were stressed for up to 4 d. The candidate gene identified in one study was not detected in other reports, even for similar seed-flooding tolerance experiments (Yu et al. 2019, Ali et al. 2020, Sharmin et al. 2020). Possible reasons for the result discrepancies among different studies might be due to the disparity in the expression pattern of some genes in various tissues at varying durations of flooding stress (Casarotto et al. 2019) and/or due to differences in genotypes because the expression of some genes may be higher in some tolerant genotypes but lower in other tolerant as well as in susceptible genotypes (Yu et al. 2019).

Abiotic stress such as flooding tolerance is a complex phenomenon. Although various physiological and molecular mechanisms are involved in specific stress responses, a vast number of genes and pathways are common across various stress conditions (Tuteja & Sopory 2008, Deshmukh et al. 2014). Although the expression of two candidate genes was not consistently higher in all tolerant genotypes, possibly they play a role in flooding tolerance because the outcomes of flooding

tolerance is supposed to be the interactions among different genes belong to many metabolic pathways (Loreti et al. 2016). In addition, the expression of some potential candidate genes for flooding tolerance might be genotype-specific. Similar results with higher expression of a few potential candidate genes under flooding stress were observed even in susceptible genotype (Casarotto et al. 2019). So, it can be considered that these two genes, *Glyma.10g050300* and *Glyma.12g030900*, are potential candidate genes for flooding tolerance in soybean. The consistently higher CCI of tolerant genotypes than the susceptible ones over two years also supports this result.

Conclusion

A number of potential candidate genes for flooding tolerance in soybean was screened and their expression in the tolerant and susceptible genotypes were evaluated. Out of the 19 potential genes with considerable amplification, *Glyma.12g030900* and *Glyma.10g050300* showed differential expression in leaf and root tissues, respectively, among tolerant and susceptible genotypes. Consistently greater chlorophyll index values of the tolerant lines than the susceptible ones over two years also supported the gene expression results. The finding of this study provide valuable information for future studies on flooding tolerance and could be useful in breeding programs.

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