

Mapping quantitative trait loci for root development under hypoxia conditions in soybean (*Glycine max* L. Merr.)

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Abstract

Key message Greatest potential, QTLs for hypoxia and waterlogging tolerance in soybean roots were detected using a new phenotypic evaluation method.

Abstract Waterlogging is a major environmental stress limiting soybean yield in wet parts of the world. Root development is an important indicator of hypoxia tolerance in soybean. However, little is known about the genetic control of root development under hypoxia. This study was conducted to identify quantitative trait loci (QTLs) responsible for root development under hypoxia. Recombinant inbred lines (RILs) developed from a cross between a hypoxia-sensitive cultivar, Tachinagaha, and a tolerant landrace, Iyodaizu, were used. Seedlings were subjected to hypoxia, and root development was evaluated with the

value change in root traits between after and before treatments. We found 230 polymorphic markers spanning 2519.2 cM distributed on all 20 chromosomes (Chrs.). Using these, we found 11 QTLs for root length (RL), root length development (RLD), root surface area (RSA), root surface area development (RSAD), root diameter (RD), and change in average root diameter (CARD) on Chrs. 11, 12, 13 and 14, and 7 QTLs for hypoxia tolerance of these root traits. These included QTLs for RLD and RSAD between markers Satt052 and Satt302 on Chr. 12, which are important markers of hypoxia tolerance in soybean; those QTLs were stable between 2 years. To validate the QTLs, we developed a near-isogenic line with the QTL region derived from Iyodaizu. The line performed well under both hypoxia and waterlogging, suggesting that the region contains one or more genes with large effects on root development. These findings may be useful for fine mapping and positional cloning of gene responsible for root development under hypoxia.

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Abbreviations

CARD	Change in average root diameter
Chr.	Chromosome
DAS	Days after sowing
HTI	Hypoxia tolerance index
LG	Linkage group
NIL	Near-isogenic line
QTL	Quantitative trait loci
RD	Root diameter
RDW	Root dry weight
RIL	Recombinant inbred lines
RL	Root length
RLD	Root length development
RSA	Root surface area
RSAD	Root surface area development

SDW Shoot dry weight

Introduction

Waterlogging is a major environmental stress limiting soybean yield in the monsoonal areas of Asia (Kokubun 2013), as well as in other parts of the world, such as the Mississippi Delta of the USA (Linkemer et al. 1998; VanToai et al. 2010) and the lowlands of Brazil's Rio Grande (Pedó et al. 2015). Soybean is vulnerable to waterlogging, which decreases photosynthesis (Oosterhuis et al. 1990; VanToai et al. 1994; Cho and Yamakawa 2006), reduces nitrogen fixation (VanToai et al. 1994; Shimamura et al. 2003; Henshaw et al. 2007a), and lessens biomass and grain yield (Bacanamwo and Purcell 1999; Henshaw et al. 2007b; Rhine et al. 2010). Waterlogging stress can happen during any growing stage, but waterlogging in the early stage results in substantial reduction of grain yield (Kokubun 2013). Therefore, soybean cultivars with strong waterlogging tolerance are desired in regions where rainfall is heavy during early plant growth.

Soil hypoxia (Hossain and Uddin 2011) is a major problem associated with waterlogging because of the slow diffusion of oxygen in water (Armstrong 1980; Wiengweera et al. 1997; Armstrong and Drew 2002). Hypoxia promotes root respiration (Drew 1992). The root system of plants usually responds to waterlogging by staying shallow (Sallam and Scott 1987). As the root system determines above-ground growth, both root and shoot growth are, therefore, inhibited by waterlogging (Sallam and Scott 1987; Shimamura et al. 2003; Araki et al. 2012; Sauter 2013). Therefore, the ability of roots to develop is an important indicator of hypoxia tolerance.

A common response of plants to a lack of oxygen is the production of adventitious roots (Colmer and Voesenek 2009), which can contribute to plant growth under waterlogging stress (Rich et al. 2012). Adventitious roots develop in wheat (Mano and Omori 2007), maize (Zaidi et al. 2003), tomato (McNamara and Mitchell 1990), and soybean (Thomas et al. 2005; Bacanamwo and Purcell 1999; Shimamura et al. 2003; Henshaw et al. 2007a) under waterlogging. However, another crucial aspect is the ability to maintain root development under hypoxia, as seen in seedlings of waterlogging-tolerant soybeans which were able to maintain root elongation and root branching under hypoxia (Sakazono et al. 2014).

Previous studies have measured root morphological traits under waterlogging (Bacanamwo and Purcell 1999; Shimamura et al. 2003; Henshaw et al. 2007a; Sakazono et al. 2014), but measurements at the seedling stage were not accurate because of differences in seed weight, which varies among soybean genotypes (Kaga et al. 2012), and in

plant size, which is significantly influenced by seed weight (Vandamme et al. 2016). Therefore, to assess the effects of hypoxia on soybean root traits correctly, we evaluated changes in root traits between before and after treatments.

Numerous studies have reported QTLs related to waterlogging tolerance in soybean (VanToai et al. 2001; Reyna et al. 2003; Cornelious et al. 2005; Githiri et al. 2006; Sayama et al. 2009; Nguyen et al. 2012), but most focused on indirect measures, such as injury score and tolerance index. For example, a QTL associated with better soybean growth and grain yield under waterlogging was identified on chromosome (Chr.) 18 near marker Sat_064 (VanToai et al. 2001). QTLs for flooding injury score and tolerance index were found and mapped on Chrs. 3, 5, 12, 13, and 16 (Cornelious et al. 2005), Chrs. 2, 6, 7, 10, 11, 14, and 19 (Githiri et al. 2006), and Chrs. 11 and 13 (Nguyen et al. 2012). Although many QTL studies of waterlogging tolerance in soybean have been reported, QTLs specific to root development characteristics under both hypoxia and waterlogging have not yet been reported.

Dr. Kohsei Tabuchi at Hokuriku National Agricultural Research Center evaluated waterlogging tolerance of soybean germplasm, mainly originated from Japan under field conditions (personal communication, 2001). Field-grown plants were waterlogged for 5 weeks beginning at 5 weeks after sowing. Tolerance was evaluated based on seed yield reduction in response to treatments, leaf color under treatments and observation at maturity. Most germplasm classified as tolerant were late-maturing probably because late maturity confers a longer growth period for recovery from flooding stress (Githiri et al. 2006). Among them, Iyodaizu (accession number of Genetic Resources Center, NARO: JP29488) was classified into tolerant group with medium maturity (maturity group IV). Iyodaizu was also hypoxia-tolerant under our capillary watering system without soil medium (Sakazono et al. 2014). Therefore, we conducted this study to identify QTLs for root development using $F_{8,9}$ recombinant inbred lines (RILs) derived from a cross between Iyodaizu and hypoxia-susceptible cultivars. In addition, we developed near-isogenic lines (NILs) carrying target QTLs from backcrossed lines using marker-assisted selection (MAS) and then examined their root development under both hypoxia and waterlogging.

Materials and methods

Plant materials

For QTL analysis, we used a population of 94 $F_{8,9}$ RILs derived from a cross between Iyodaizu (100-seed weight 13 g), and Tachinagaha (100-seed weight 36 g), the leading Japanese cultivar in the Kanto region (accession number:

JP67666). Iyodaizu is hypoxia-tolerant, and Tachinagaha is hypoxia-susceptible (Sakazono et al. 2014). RILs were developed without any selection by the single-seed descent method from the F_1 plant. To develop NILs carrying target QTLs, we conducted recurrent backcrossing to Tachinagaha using MAS. We genotyped the BC_3F_2 , BC_6F_1 , and BC_6F_2 generations with nine simple sequence repeat markers: Satt192, Satt442, Sat_334, Satt541, Satt469, Satt052, Sat_206, Satt676, and Satt302. A homozygous line designated NIL-9-4-5 (100-seed weight 26 g) carried the fixed target QTLs. The QTLs were validated under hypoxia and waterlogging conditions.

Plant culture and hypoxia treatment

The RILs were tested under hypoxia in 2014 and under both hypoxia and normoxia in 2015. To ensure a uniform rate

of germination, we incubated eight seeds of each genotype per Petri dish at 23 °C and 70% RH for 3 days (Fig. 1a). The seeds were then treated with a fungicide (benomyl, 1/2 of dry seed weight), sown in moist vermiculite (Midori-sangyou, Fukuoka, Japan) in plastic cups, and held at 23 °C for 2 days. Uniform seedlings of each genotype were then selected for treatment. The seedlings were inserted through holes punched in a 7-mm-thick polystyrene board and held upright with plugs of silicone rubber. Each board, holding 20 seedlings, was placed over an opaque plastic container (386 mm × 256 mm × 135 mm), with the roots in deionized distilled water continuously aerated at 1.0 L min⁻¹ by air pumps (Fig. 1b). The plants were first grown for 4 days at 23 °C in a growth chamber (220 μmol m⁻² s⁻¹ light density, 14 h light/10 h dark, 23 °C). Then at 6 days after sowing (DAS), the plants were grown for 7 days under either normoxia (control) or hypoxia. Under normoxia, seedlings

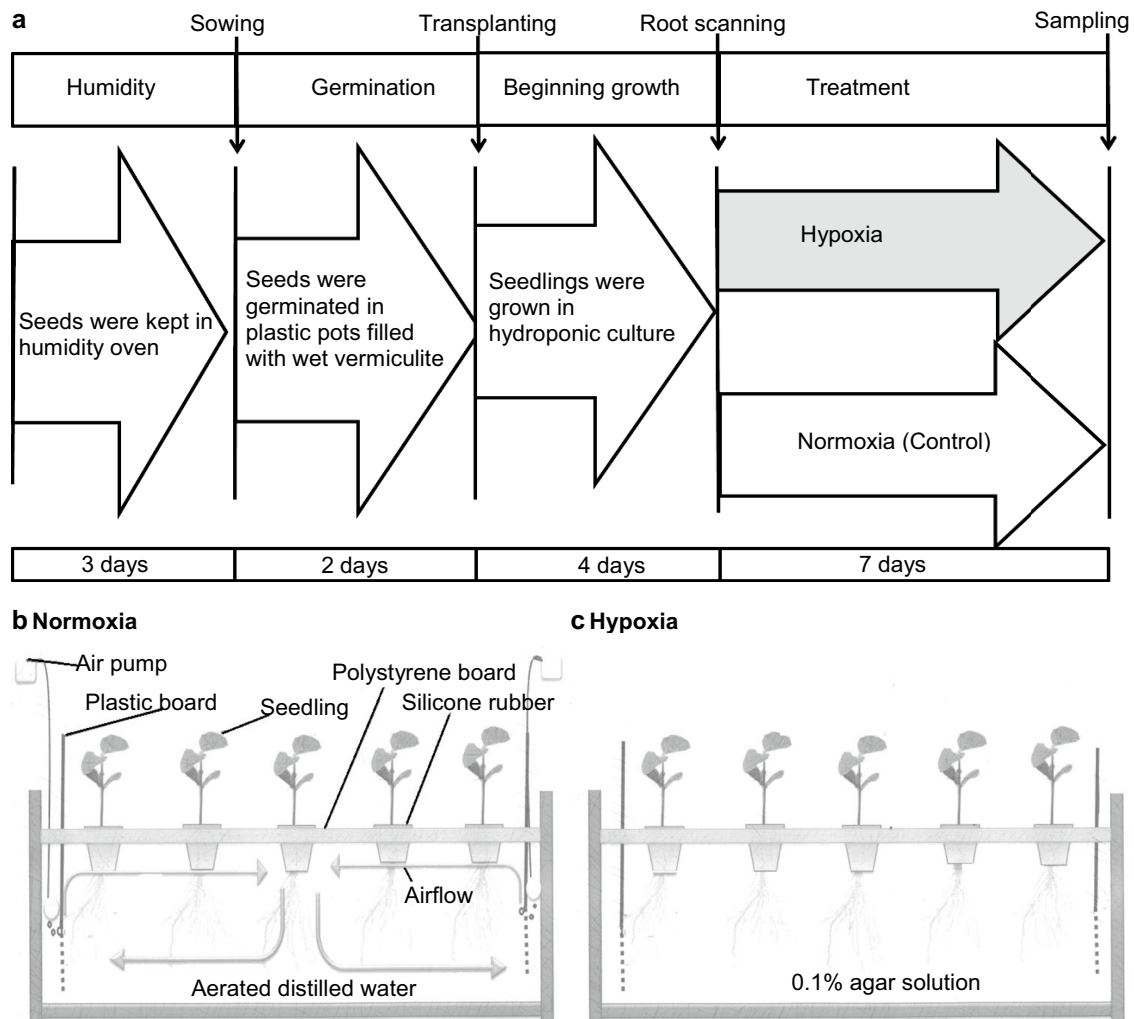


Fig. 1 **a** Culture schedule. **b**, **c** Plant culture under **(b)** normoxia (in aerated distilled water) and **(c)** hypoxia (in 0.1% agar solution deoxygenated with nitrogen gas)

were grown in deionized distilled water aerated by two air pumps at opposite ends of the container to maintain a dissolved oxygen (DO) concentration of $>7.0 \text{ mg L}^{-1}$ (Fig. 1b). Under hypoxia, seedlings were grown in 0.1% (w/v) agar solution in deionized distilled water that was deoxygenated with a continuous flow of nitrogen to maintain a DO concentration of $<1.0 \text{ mg L}^{-1}$ (Fig. 1c). DO was measured with a DO meter (CM-51, Horiba, Kyoto, Japan) at the start (6 DAS) and end of treatment (13 DAS). The experiments were conducted in 3 replications with the randomized complete block method. Five plants from each RIL were used as an experimental unit.

Target QTLs were validated under both hypoxia and waterlogging. The hypoxia and normoxia experiments were conducted in a growth chamber as the same method described above. The waterlogging experiments were conducted in a greenhouse at the Experimental Farm of Kyushu University, Fukuoka, Japan ($33^{\circ}37'N$, $130^{\circ}27'E$). The seeds of parents and NILs were germinated as above. Seedlings were transplanted into pots containing 0.5 kg of dried soil (20.7% clay, 23.2% silt, and 56.1% sand; sandy clay-loam) on 25 April 2015. The pots were then put in a tray (924 mm \times 610 mm \times 200 mm) in water maintained at a 2.0-cm depth. At 4 DAS, the seedlings were thinned to one representative seedling per pot. At 6 DAS, seedlings were grown for 7 days under either drained (control) or waterlogging conditions. Under control conditions, the water level was maintained at 2.0 cm depth. Under waterlogging conditions, it was maintained at 2.0 cm above the soil surface. All experiments had five replicates arranged in a randomized complete block design. Single plants were used as an experimental unit.

Measurement of root development traits

Root traits were measured before and after treatments. The root systems of individual plants were scanned with an image scanner (GTX-970 scanner, Epson, Nagano, Japan) under the control of WinRhizo software (Regent Instruments, Inc., Quebec, Canada). According to WinRhizo regent instruments, total root length (RL) of root system was measured on the so-called skeleton images. Root diameter (RD) was measured continuously along the root and classified into ten groups with unequal width (>0 and ≤ 0.5 , >0.5 and ≤ 1.0 , >1.0 and ≤ 1.5 , >1.5 and ≤ 2.0 , >2.0 and ≤ 2.5 , >2.5 and ≤ 3.0 , >3.0 and ≤ 3.5 , >3.5 and ≤ 4.0 , >4.0 and ≤ 4.5 , and >4.5 mm). Root surface area (RSA) was automatically calculated by software based on root length and root diameter of root in each class. Root length development (RLD), root surface area development (RSAD), and the change in average root diameter (CARD) were calculated as the change in value of traits between when treatments were started and when treatments ended. Roots and

shoots were dried in oven at 80°C until constant weight. Then root dry weight (RDW) and shoot dry weight (SDW) were determined. We calculated a “hypoxia tolerance index” (HTI, %) for each trait as hypoxia value/normoxia value $\times 100$.

Linkage map construction

DNA was extracted from fresh soybean leaves by the CTAB method (Murray and Thompson 1980). The simple sequence repeat (SSR) markers developed by USDA (Song et al. 2004) or by the Kazusa DNA Research Institute (Hisano et al. 2007) were used to evaluate polymorphism among parents. Polymorphic markers were used to construct a linkage map from the 94 RILs. The PCR mixture contained 10 ng of genomic DNA, 2.25 pmol of primer, 625 pmol of nucleotides, and 0.125 unit of ExTaq in $1 \times$ ExTaq buffer supplied by the manufacturer (Takara Bio) in a total volume of 5 μL . The PCR conditions consisted of an initial 5 min at 95°C ; 40 cycles of 30 s at 95°C , 30 s at 55°C , and 30 s at 72°C ; and a final 5 min at 72°C . The products were stored at 10°C and then separated in 8% polyacrylamide gels. The linkage map was constructed in MAPMAKER/EXP v.3 software (Lander et al. 1987).

QTL mapping

QTL analysis was performed by composite interval mapping in QTL Cartographer v. 2.5 software (Wang et al. 2007). Background cofactors with a 10-cM window were associated using forward and backward regression (0.05 confidence level). The LOD threshold for each trait was calculated using 1000 permutations corresponding to a genome-wide significance level of 0.05.

Statistical analysis

Statistics were analyzed in SYSTAT 13 software (SYSTAT Software, Inc., San Jose, CA, USA). The effects of genotype and hypoxia treatment on root development traits were analyzed by two-way ANOVA. The *t* test was used to compare the average values among treatments.

Results

Variance analysis

In 2014, genotype had a significant effect on RD, CARD, RDW, and SDW, but not on RL, RLD, RSA, or RSAD (Table 1). Hypoxia had a significant effect on all traits except SDW. The genotype by hypoxia interaction had a significant effect on all traits except CARD and SDW. In

Table 1 Results of two-way ANOVA of root growth traits of the parents in 2014 and 2015

Traits	F value					
	Genotype		Hypoxia treatment		Genotype × hypoxia treatment	
	2014	2015	2014	2015	2014	2015
RL	1.49	31.22**	19.95**	64.75***	47.44***	133.43***
RLD	0.42	17.94*	29.42***	162.55***	57.56***	214.81***
RSA	6.67	41.63**	9.42*	41.70**	31.21***	106.79***
RSAD	0.66	23.09**	13.66**	127.07***	18.60***	194.71***
RD	54.36**	8.26*	22.60**	11.89**	27.28***	5.28*
CARD	12.81**	0.54	47.44***	40.20***	1.94	7.85*
RDW	26.45***	8.19**	7.05*	4.66*	18.05**	8.74**
SDW	141.45***	187.37***	4.05	1.93	0.066	2.13

RL, RLD, RSA, RSAD, RD, CARD, RDW and SDW denote root length, root length development, root surface area, root surface area development, root diameter, change in average root diameter, root dry weight and shoot dry weight, respectively

Significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

2015, genotype had a significant effect on all traits except for CARD. Hypoxia and the genotype by hypoxia interaction had a significant effect on all traits except SDW.

Among measured traits, SDW was only affected by genotype but not by hypoxia or the genotype by hypoxia interaction in both years.

Root development traits

The root morphology (RL, RSA) and root development traits (RLD, RSAD) of the parents significantly differed between normoxia and hypoxia (Fig. 2; Table 2). Under normoxia, values of these traits of Tachinagaha were much higher than those of Iyodaizu. In contrast, under hypoxia, values of Iyodaizu were higher than those of Tachinagaha except for RSA in 2015. Hypoxia reduced RL and RSA of Tachinagaha by 54–56 and 47–50%, respectively, relative to normoxia, but increased those of Iyodaizu by 18–20 and 25–33%. RD of both tended to increase under hypoxia ($HTI > 1$). Hypoxia reduced RLD and RSAD of Tachinagaha by 74–75 and ~67%, respectively, but increased those of Iyodaizu by 11–21 and ~34% respectively. The frequency distributions of RLD and RSAD showed continuous variation in the RIL population under hypoxia; the values of Iyodaizu were the same as or slightly higher than the means of the RIL population, but those of Tachinagaha were much lower than the mean (Fig. 3).

Genetic linkage map

Of the surveyed SSR markers, 230 showed distinct polymorphism among parents and were used for linkage mapping. Among the 94 RILs, 3 RILs had markers with genotypes different from the parents and were considered to

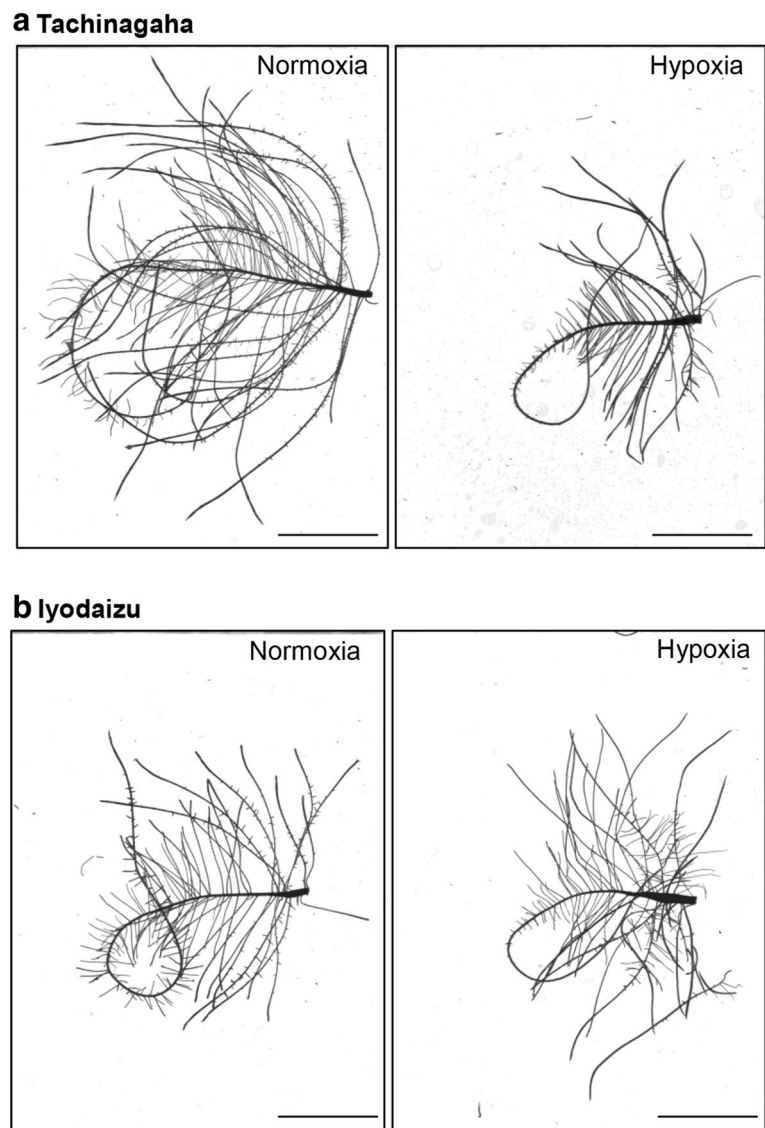
have been the result of outcrossing, so we excluded them from analysis. A genetic linkage map consisting of 20 linkage groups (LGs) spanning 2519.2 cM with an average distance of 10.95 cM between markers was constructed. The order of the markers was in line with the published soybean integrated genetic map (<http://soybase.org/>).

QTLs for root development traits

We performed QTL analysis for RL, RLD, RSA, RSAD, RD, and CARD in 2014 and 2015. We found a total of 11 QTLs on Chromosomes (Chrs.) 11 (*Qcard-11*), 12 (*Qrl-12*, *Qrld-12*, *Qrsad-12*, *Qrd-12*), 13 (*Qrl-13*, *Qrsa-13*), and 14 (*Qrl-14*, *Qrd-14*, *Qcard-14*) (Table 3; Fig. 4). Three QTLs were associated with RL under hypoxia; two of these were found on Chrs. 14 (LG B2) and 13 (LG F) in 2014, and the other on Chr. 12 (LG H) in 2015. A QTL for RLD was found on Chr. 12 (LG H) in 2014 and 2015. A QTL for RSA was found on Chr. 13 (LG F) in 2014. A QTL for RSAD was found on Chr. 12 (LG H) in 2014 and 2015. Three QTLs for RD were found on Chr. 12 (LG H) and Chr. 14 (LG B2), one of them in 2014 under hypoxia and the other two in 2015 under normoxia (Chr. 14) and hypoxia (Chr. 12). Two QTLs for CARD were found on Chrs. 11 (LG B1) and 14 in 2015.

Only the QTLs for RLD and RSAD were detected stably in both years (Table 3; Fig. 4). Both were located in the same marker interval on Chr. 12 (LG H). The single QTL associated with RLD under hypoxia had LOD scores of 3.97 in 2014 and 5.12 in 2015. The Iyodaizu allele had a positive effect at this locus (Table 3). The proportion of phenotypic variation explained by this QTL was 13% in 2014 and 19% in 2015. One QTL was detected for RSAD under hypoxia in the same region as *Qrld-12*, and the

Fig. 2 Root morphology of parents under normoxia and hypoxia at 13 DAS. Bars 4 cm



Iyodaizu allele again had a positive effect. The proportion of phenotypic variation it explained was 13% in 2014 and 11% in 2015.

QTLs for HTI of root traits

Seven QTLs were mapped for hypoxia-tolerant index (HTI) of root traits on chromosomes: Chr. 4 (*Qhti-4*), Chr. 9 (*Qhti-9*), Chr. 12 (*Qhti-12-1* and *Qhti-12-2*), and Chr. 14 (*Qhti-14-1*, *Qhti-14-2* and *Qhti-14-3*) in this study (Table 4). These QTL accounted for 10–18% of phenotypic variation. Chr. 12 and Chr.14 were more important than other of these, with greater additive effect and higher explanation of phenotypic variation as compared with other chromosomes detected.

Validation of root development QTLs under hypoxia and waterlogging

To verify the effects of root development QTLs (*Qrld-12* and *Qrsad-12*), we compared root development traits of NIL-9-4-5 with those of the recurrent parent Tachinagaha by *t* test (Fig. 5). Under hypoxia, RLD and RSAD of Tachinagaha were greatly decreased, but those of Iyodaizu and NIL-9-4-5 showed no decrease. Under waterlogging, RLD and RSAD of Tachinagaha were reduced by more than those of Iyodaizu. NIL-9-4-5 maintained root development under waterlogging.

Table 2 Root trait (means \pm SD) and hypoxia tolerance index (HTI) of the parents under normoxia and hypoxia

Traits	Iyodaizu											
	Tachinagaha				Normoxia				HTI			
	Normoxia		Hypoxia		2014		2015		2014		2015	
RL (cm)	4518 \pm 57	580 \pm 73	209 \pm 28	256 \pm 40	0.46	0.44	300 \pm 27	242 \pm 21	355 \pm 37	289 \pm 18	1.18	1.20
RLD (cm)	335 \pm 43	441 \pm 71	82 \pm 15	115 \pm 31	0.25	0.26	216 \pm 30	165 \pm 21	261 \pm 32	185 \pm 12	1.21	1.12
RSA (cm ²)	79 \pm 13	108 \pm 13	42 \pm 6	54 \pm 11	0.53	0.50	47 \pm 3	41 \pm 3	58 \pm 5	55 \pm 1	1.25	1.33
RSAD (cm ²)	56 \pm 10	80 \pm 13	18 \pm 4	26 \pm 8	0.33	0.33	32 \pm 4	27 \pm 3	42 \pm 4	36 \pm 1	1.34	1.34
RD (mm)	0.55 \pm 0.03	0.60 \pm 0.00	0.63 \pm 0.02	0.67 \pm 0.04	1.15	1.12	0.49 \pm 0.01	0.54 \pm 0.01	0.52 \pm 0.01	0.60 \pm 0.03	1.06	1.12
CARD (mm)	-0.06 \pm 0.02	-0.04 \pm 0.00	0.05 \pm 0.01	0.04 \pm 0.03	-0.82	-0.79	-0.08 \pm 0.02	-0.07 \pm 0.01	-0.02 \pm 0.03	0.03 \pm 0.02	0.26	-0.44
RDW (g)	0.08 \pm 0.02	0.12 \pm 0.01	0.05 \pm 0.01	0.07 \pm 0.01	0.65	0.60	0.05 \pm 0.00	0.05 \pm 0.00	0.05 \pm 0.01	0.07 \pm 0.00	1.09	1.32

RL, RLD, RSA, RSAD, RD, CARD, and RDW denote root length, root length development, root surface area, root surface area development, root diameter, change in average root diameter and root dry weight, respectively

Discussion

Waterlogging hampers both shoot and root growth (Sallam and Scott 1987; Araki et al. 2012), reducing root growth first (Shimamura et al. 2003; Sauter 2013). Bacanamwo and Purcell (1999) concluded that the shoot biomass of a soybean plant was unaltered during the first 7 days of waterlogging, but decreased relative to the control by 21 days. Sakazono et al. (2014) reported the same trend in shoot and root responses to hypoxia. Our results show that the effects of hypoxia on RDW were significant, whereas the effects of hypoxia and the genotype by hypoxia interaction on SDW were not (Table 1). These results indicate that root responses to hypoxia might reflect hypoxia tolerance in soybean under short-term stress.

There are some main parallels between hypoxia under agar solution and soil waterlogged environment. Wiengweera et al. (1997) used 0.1% agar solution to simulate the low diffusion of oxygen in waterlogged soil. The results showed that oxygen concentration rapidly decreased below 1.0 mg L⁻¹, while ethylene increased during treatment; such changes were similar to those described for waterlogged soil in a field environment by Trought and Drew (1980). This suggests that hypoxia tolerance of plants under hydroponic culture reflected hypoxia tolerance under waterlogged soil in a field environment. Under waterlogging, RL and RDW of soybean plant are inhibited (Sallam and Scott 1987; Shimamura et al. 2003; Henshaw et al. 2007a; Yamane and Iijima 2016). RL in maize also reflected its waterlogging tolerance under field conditions (Souza et al. 2012). These symptoms were also observed in other species (Visser et al. 2000; Colmer 2003; Yamauchi et al. 2014). Under hypoxia, root traits of soybean, especially RL and RSA, were dramatically inhibited and RD increased (Sakazono et al. 2014). Tachinagaha and Iydaizu were used in both of hypoxia and waterlogging studies. Iyodaizu was reported with tolerance (Sakazono et al. 2014; Jitsuyama 2015) and Tachinagaha was a moderately/susceptible cultivar (Sayama et al. 2009; Sakazono et al. 2014; Jitsuyama 2015). Furthermore, the relationship between cultivar-specific hypoxia response and waterlogging tolerance was reported by Jitsuyama (2015). The results showed that the strong positive correlation between RL and field waterlogging-tolerant index was found among 12 studied genotypes including Tachinagaha and Iydaizu, while other traits, such as dry weight, root diameter, number of root tips were not observed (Jitsuyama 2015). In this paper, our results show that RD and CARD of the parents tended to increase under hypoxia. Root development (RLD and RSAD) of Tachinagaha decreased by 59–74%; in contrast, root traits and their development of Iyodaizu tended to increase under hypoxia (Table 2). The effects of hypoxia on the RIL population were similar to those on the parents: RLD and RSAD

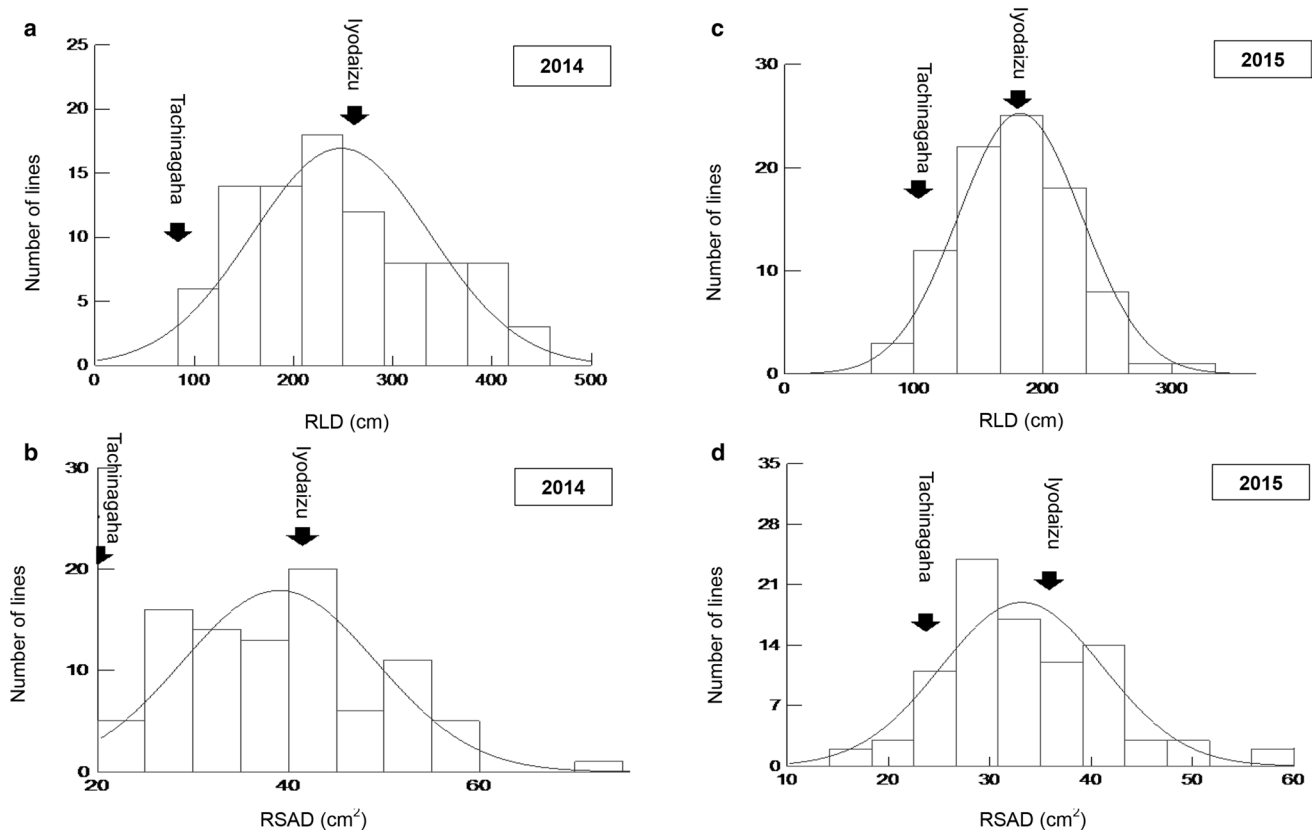


Fig. 3 Phenotypic distributions of root length development (RLD) and root surface area development (RSAD) of the RIL population under hypoxia in 2014 (**a, b**) and 2015 (**c, d**)

showed the same responses to hypoxia in the parents and the RIL population, indicating that root development is a mechanism of hypoxia tolerance.

Size of soybean seed depends on cultivars, and seedling growth is positively correlated with seed size (Kaga et al. 2012; Vandamme et al. 2016). To avoid this autocorrelation, we measured the changes in root traits between before and after treatments. This method showed that under hypoxia, RLD and RSAD of Tachinagaha were reduced by 67–75%, but RL and RSA were reduced by 47–56% (Table 2). Whereas QTLs for root traits at the end of hypoxia treatment were unstable between years or showed positive effects of the intolerant parent, detected QTLs for root development traits (*Qrld-12*, *Qrsad-12*) contributed by the hypoxia-tolerant parent were stable between years (Table 3).

QTLs for waterlogging injury score and tolerance index were found and mapped on Chrs. 2, 3, 5, 6, 7, 10, 11, 12, 13, 16 and 19 (Cornelious et al. 2005; Githiri et al. 2006; Nguyen et al. 2012). These indexes were proven to be strongly correlated with RL (Jitsuyama 2015). In this study, QTLs were mapped for HTI of root traits on Chrs. 4, 9, 12, and 14; two new of chromosomes 4 and 9 were detected

(Table 4). Among these, *Qhti-12-1* and *Qhti-12-2* were co-localized with *Qrld-12* and *Qrsad-12*, and contributed by Iyodaizu (Tables 3, 4), indicating that root development QTLs could explain for hypoxia tolerance index in soybean.

RL was strongly correlated with RSA under hypoxia (Henshaw et al. 2007a). In our results, RLD and RSAD showed the same responses to treatments (Fig. 5) in agreement with the mapping of the QTLs for these traits together on Chr. 12. These QTLs, contributed by Iyodaizu, were located between markers between Satt052 and Satt302, and did not appear under normoxia (Table 3; Fig. 4). Many QTLs for soybean seed weight, including seed weight 23-2, 35-4, 13-8, 36-4, 41-1, and 34-4 were found in our introgression region of *Qrld-12* and *Qrsad-12* on chr. 12 (<http://soybase.org/>). In this study, seed weight of NIL-9-4-5 was smaller than recurrent parent Tachinagaha. Thus, a negative effect of target QTLs on root development under normoxia conditions may be relevant to seed weight QTLs. Sayama et al. (2009) detected four QTLs for flooding tolerance of soybean plant at germination stage which were named *Sft1*, *Sft2*, *Sft3* and *Sft4*. Interestingly, one of these QTLs (*Sft1*) was identified in the marker interval containing *Qrld-12* and *Qrsad-12*. Effective targeting of QTL alleles

Table 3 QTLs for root and root development traits of RIL population

Trait	QTL name	Environment	Chromosome	Linkage group	Marker interval	LOD ^a		Additive effect ^b		R ^{2c}	
						2014	2015	2014	2015	2014	2015
RL	<i>Qrl-14</i>	Hypoxia	14	B2	Satt342-Satt126	4.52 (3.2)		60.42		0.23	
	<i>Qrl-13</i>	Hypoxia	13	F	Satt663-Satt362	3.32 (3.2)		34.67		0.12	
	<i>Qrl-12</i>	Hypoxia	12	H	Satt052-Satt302	–	6.03 (3.0)	–	–25.7	–	0.19
RLD	<i>Qrld-12</i>	Hypoxia	12	H	Satt052-Satt302	3.79 (3.2)		–33.89	–21.44	0.13	0.19
	<i>Qrsa-13</i>	Hypoxia	13	F	Satt663-Satt362	4.2 (3.1)		5.1		0.19	
RSAD	<i>Qrsad-12</i>	Hypoxia	12	H	Satt052-Satt302	3.64 (3.2)		–3.75	–2.8	0.13	0.11
	<i>Qrd-14-1</i>	Hypoxia	14	B2	Sat_342-Satt126	3.36 (3.3)		–0.03		0.15	
RD	<i>Qrd-14-2</i>	Normoxia	14	B2	Sat_342-Satt126		3.64 (3.2)		0.02		0.14
	<i>Qrd-12</i>	Hypoxia	12	H	Satt469-Satt302		6.15 (3.2)		0.02		0.22
CARD	<i>Qcard-11</i>	Hypoxia	11	B1	Set_026-Sat_364		3.19 (2.1)		0.01		0.11
	<i>Qcard-14</i>	Hypoxia	14	B2	Sat_177-Satt126		2.6 (2.1)		–0.01		0.11

^aLOD: Likelihood of odds score calculated by composite interval mapping. The numbers in brackets indicate the threshold of LOD values

^bAdditive effects: positive, increased by Tachinagaha allele; negative, increased by Iyodaizu

^cR² value explained by putative QTL. RL, RLD, RSA, RSAD, RD, and CARD denote root length, root length development, root surface area, root surface area development, root diameter and change in average root diameter, respectively

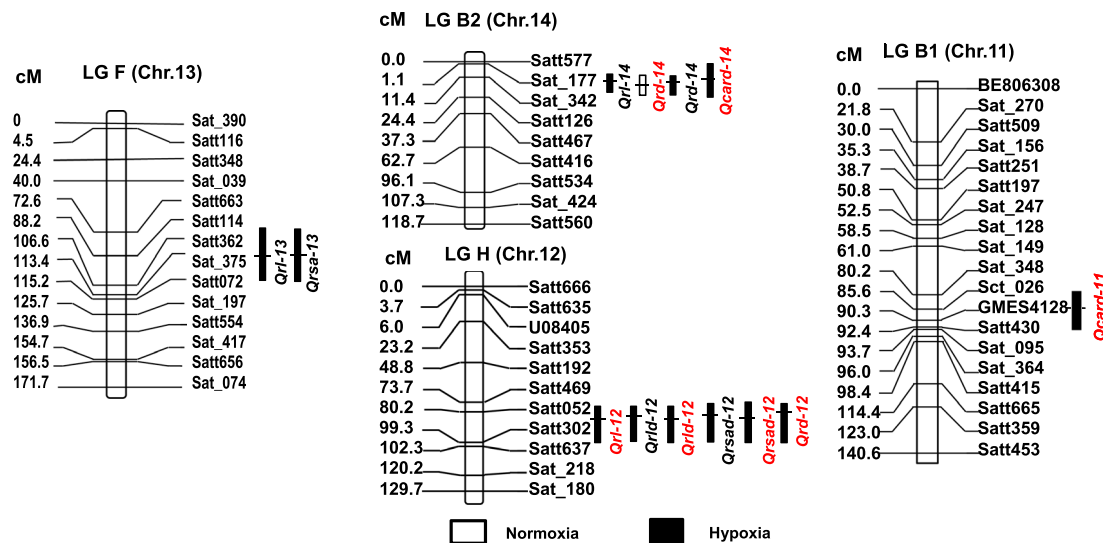


Fig. 4 Molecular linkage map of the RIL population derived from Tachinagaha × Iyodaizu, and summary of QTLs for all traits responsive to hypoxia in the mapping population. *Q* QTL, *rl* root length, *rl*d root length development, *rsad* root surface area development, *rd* root diameter, *card* change in average root diameter. In all QTL names, the first number represents the chromosome location; black, QTLs found

in 2014; red, QTLs found in 2015. Markers are listed to the right and the distances from the top of each linkage group are listed to the left. Length of vertical bars is equal to the 1-LOD likelihood confidence interval. Horizontal lines in the middle of the bars indicate the positions of the QTL peaks. (Color figure online)

Table 4 QTLs for hypoxia tolerance index (HTI) of root traits

Trait	Chromosome	Linkage group	Marker interval	LOD ^a	Additive effect ^b	<i>R</i> ^{2c}
RL						
<i>Qhti-14-1</i>	14	B2	Sat_177-Sat_342	5.47 (3.0)	0.19	0.10
RLD						
<i>Qhti-12-1</i>	12	H	Satt052-Satt302	3.28 (3.1)	−0.10	0.11
RSA						
<i>Qhti-14-2</i>	14	B2	Sat_177-Sat_342	5.86 (3.2)	0.11	0.18
RSAD						
<i>Qhti-12-2</i>	12	H	Satt052-Satt302	4.26 (3.1)	−0.09	0.18
RD						
<i>Qhti-14-3</i>	14	B2	Satt126-Satt467	3.65 (3.2)	−0.03	0.11
CARD						
<i>Qhti-4</i>	4	C1	AW277661-Satt399	2.65 (1.4)	0.97	0.10
<i>Qhti-9</i>	9	K	GMES1693-CRRS100	3.00 (1.4)	1.12	0.12

^aLOD: Likelihood of odds score calculated by composite interval mapping. The numbers in brackets indicate the threshold of LOD values

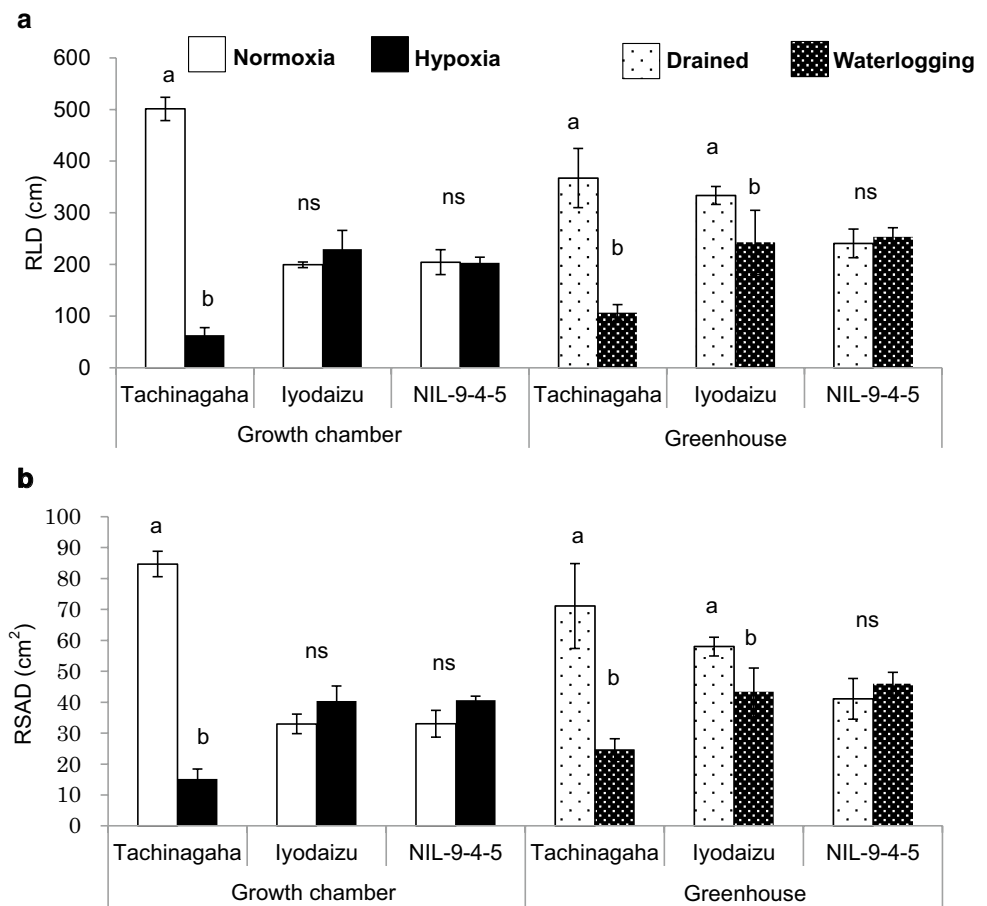
^bAdditive effects: positive, increased by Tachinagaha allele; negative, increased by Iyodaizu

^c*R*² value explained by putative QTL. RL, RLD, RSA, RSAD, RD, and CARD denote root length, root length development, root surface area, root surface area development, root diameter and change in average root diameter, respectively

in breeding programs requires QTLs to be stably expressed among years and environments. NIL-9-4-5, carrying both QTLs, showed stable root performance under both hypoxia and waterlogging (Fig. 5). These results indicate that QTLs for root development have the greatest potential for breeding for both hypoxia and waterlogging tolerance in soybean.

In conclusion, we identified QTLs that contribute to root development under hypoxia conditions. QTLs for RLD and RSAD (*Qrld-12*, *Qrsad-12*) on Chr.12 (LG H) were stable across years. NIL9-4-5, carrying the target QTLs, performed well under both hypoxia and waterlogging conditions, confirming the effect of these QTLs. Markers proximal to the QTLs may be useful for the development

Fig. 5 **a** Root length development (RLD) and **b** root surface area development (RSAD) of parents and NIL-9-4-5 under hypoxia and waterlogging. Vertical bars indicate SEM. Bars with the same letter within a genotype are not significantly different at $P < 0.05$ by *t* test ($n = 5$)



of cultivars tolerant to waterlogging. Fine mapping and positional cloning may reveal gene(s) responsible for root development under hypoxia conditions and help elucidate the mechanism of waterlogging tolerance in soybean. Effects of the QTLs should be verified under field conditions.

Author contribution statement TM, RT, KH and LVN designed the methodology. TM, RT, LVN, TOR, SK and RT developed experimental materials. TM, RT and TA oversaw and guided experiments, data analysis, writing and editing. LVN, NT, SK, TOR, SMG, TS and MI and KS conducted the experiments and analyzed the data. LVN wrote the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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