Hypoxia-Responsive Root Hydraulic Conductivity Influences Soybean Cultivar-Specific Waterlogging Tolerance

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Abstract

Excess soil moisture induces hypoxic conditions and causes waterlogging injury in soybean [*Glycine max* (L.) Merr.]. This study investigated the mechanism underlying the development of waterlogging injury. Nine Japanese soybean cultivars with varying degrees of waterlogging tolerance were grown in a hydroponic system for 14 days under hypoxic conditions. Shoot and root biomasses and root hydraulic conductivity were measured at an early vegetative stage for plants under control and hypoxic conditions. Root morphological traits and intramembrane aquaporin proteins were also analyzed. The tolerance of each cultivar to field waterlogging was based on biomass changes induced by the hypoxia treatment. Root hydraulic conductivity responses to hypoxia were associated with changes in total dry weight, leaf dry weight, and leaf area. The effects of hypoxic conditions on root hydraulic conductivity were also represented by the changes in root morphology, such as total root length, thick-root length, and number of root tips. Additionally, a 32.3 kDa aquaporin-like protein seemed to regulate root hydraulic conductivity. Our results from a hydroponic culture suggest that the soybean cultivar-specific responses to hypoxic conditions in the rhizosphere reflect fluctuations in hydraulic conductivity related to root morphological or qualitative changes.

Keywords

Aquaporin, Hypoxia, Root Hydraulic Conductivity, Root Morphology, Soybean, Waterlogging Tolerance

1. Introduction

Prolonged heavy rain is generally a hindrance to agricultural production as it results in waterlogged soils [1]. Waterlogging can severely limit crop production

in agricultural fields, especially in regions with high water tables and poor drainage because of clay-like soils. This is a problem during the rainy season in Japan in fields that have been converted from paddy fields. In 1970, the Japanese government introduced a policy to reduce the acreage under rice cultivation and promoted the conversion of paddy fields. Approximately 82.9% of the current domestic soybean cultivation occurs in such converted fields [2].

Converted fields generally produce high crop yields because of their fertile soil. However, paddy fields are designed to maintain wet conditions for rice production. Thus, the converted fields are prone to retain water because of their high water table and fine soil consisting of clay and silt particles. Therefore, crop production in converted fields can become unpredictable after heavy or prolonged rainfall. Technical modifications to minimize soil water fluctuations, such as high ridge cultivation [3] or inter-row strip-tillage [4], have been applied to avoid waterlogging injury. A new method to irrigate and drain soils was recently developed. The farm-oriented enhancing aquatic system uses underdrain and sub-irrigation systems to control the water table in converted fields and decrease water damage [5]. Although these modifications reduce the impact of water damage, waterlogging injury to crops produced in converted fields continues to be reported. Therefore, the development of waterlogging-tolerant soybean cultivars is still warranted.

For soybean cultivation in converted fields, excess water negatively affects germination and the subsequent post-emergence growth [6]. Waterlogging is associated with characteristic injuries. For example, during germination, rapid water uptake causes the seed structure to collapse [7] [8], while in plantlets, it interferes with physiological characteristics such as leaf water potential, nitrogen fixation, and mineral absorption [9] [10] [11]. However, soybean plantlets adapt to waterlogged soils by forming adventitious roots [12] [13] and secondary aerenchyma [14], as well as by undergoing other non-morphological changes to their root system. Although there is clear evidence of waterlogging tolerance in soybean plants, the mechanism underlying the development of waterlogging injury and tolerance has not been fully elucidated.

Information regarding variations in waterlogging tolerance among soybean cultivars is limited because of the poor reproducibility of the induced injuries. Many researchers have investigated the response of single cultivars to low-oxygen conditions or the phenotypic consequences of waterlogging stress across multiple cultivars [9] [12] [15] [16]. Although the use of a comparative physiological approach might provide new insights into soybean waterlogging tolerance, there are no available reports describing studies involving several cultivars under normal and oxygen-stress conditions in the root zone. The responses of eight Japanese soybean cultivars to hypoxic conditions were compared in a short-term hydroponic culture system [17] [18]. The results revealed a cultivar-specific waterlogging tolerance during the immature growth stage without the development of adventitious roots or aerenchyma. Additionally, some susceptible cultivars exhibited decreased shoot and root biomasses due to hypoxia, with some plants

suddenly withering, possibly because of reduced root hydraulic conductance [18]. The present study involved a modified hydroponic culture system that enabled the creation of two different oxygen environments. Seedlings of nine soybean cultivars were grown in these hydroponic systems. Their responses to hypoxic conditions were determined by comparing shoot and root biomasses, hydraulic conductance, and root aquaporin-like protein production, which is related to root water permeability. This experimental design was used to clarify the mechanisms underlying the development of waterlogging injury in soybean plants.

2. Materials and Methods

2.1. Plant Materials

Most of the nine soybean [*Glycine max* (L.) Merr.] cultivars used in this study originated in Hokkaido (northern Japan) (Table 1). With the exception of "Suzumaru" ("SMa") and "Tanbakuro" ("TKu"), these cultivars have been used in studies of field waterlogging tolerance at the Hokkaido Central Agricultural Experiment Station (HCAES) and elsewhere [15] [17] [18] [19]. Some of the cultivars have exhibited unique waterlogging tolerance [15]. "Enrei" ("Ere"), which is widely grown in Japan, is insensitive to hypoxia, and has been used as a standard soybean cultivar [17]. "Toyomusume" ("TMu") seed responses to flooding and plant responses to hypoxia have been investigated [8] [18]. "Toyomusume" plants are insensitive to hypoxic [18] and waterlogged [20] conditions. "Shoku-kei32" ("S32") plants exhibit extreme field waterlogging tolerance [19] and hypoxia tolerance [18]. "Toyohomare" ("THo") plants are tolerant to flooding [20] and hypoxia [18], in contrast to "Toyoharuka" ("THa") plants [19]. "Suzumaru" plants produce extremely small seeds, and are thought to be susceptible to waterlogging stress. "Tanbakuro" plants, which are native to Hyogo prefecture,

Table 1. Field waterlogging tolerance and hypoxia tolerance of the nine analyzed soybean cultivars.

Cultivar[a]	Abbreviation	Field waterlogging tolerance	Hypoxia tolerance[b]
Enrei	ERe	Susceptible[c]	Tolerant/Susceptible
Koganejiro	KJi	Tolerant[d]	Enhanced/Tolerant
Shoku-kei32	S32	Tolerant[e]	Enhanced/Tolerant
Suzumaru	SMa	-	-
Shirotsurunoko	STs	Susceptible[d]	Susceptible
Toyoharuka	THa	Susceptible[e]	Susceptible
Toyohomare	THo	Tolerant[e]	Tolerant
Tanbakuro	TKu	-	-
Toyomusume	TMu	Moderate[e]	Tolerant

[a]Cultivars are arranged in alphabetical order (based on their abbreviations); [b]Hypoxia tolerance of seven soybean cultivars was determined by Jitsuyama (2015) [18]. [c]Jung *et al.* [52]; [d]Matsukawa *et al.* [15]; [e]HCAES [20].

produce large seeds surrounded by a black coat.

Before planting, all seeds were coated with the Cruiser MAXX™ fungicidal paste (22.6% Thiamethoxam, Syngenta Japan K.K., Tokyo, Japan). This formulation provides seeds surface sterilization and protection against soybean diseases, including Phytophthora root and stem rot, which can occur following the development of waterlogging injury.

2.2. Hydroponic Culture System

Soybean seeds were germinated on wet paper towels in an EYELA MTI-202 incubator (Tokyo Rikakikai Co, Ltd., Tokyo, Japan) at 25˚C in darkness [17] [18]. Soybean seedlings at the vegetative emergence growth stage (*i.e.*, straight hypocotyl and unfolded cotyledons) were transferred to the hydroponic culture system in a separate LH-220S incubator (NK System Ltd., Osaka, Japan). Seedlings were maintained at a density of one plant per 20 cm^2. The roots of 100 plantlets were immersed in a 10-L hydroponic solution [500-fold dilution of the Hyponica solution consisting of 80 $mg \cdot L^{-1}$ nitrate, 76 $mg \cdot L^{-1}$ phosphate, 178 $mg \cdot L^{-1}$ potassium; Kyowa Co., Ltd., Osaka, Japan], while the stems were supported by a Bio-silico® N-type stopper (Hirschmann, Inc. Louisville, KY, USA) placed in the container lid holes. The plantlets were grown to the V2-V3 growth stages [21] without changing the hydroponic solution for 14 days.

To evaluate hypoxia tolerance, treated plantlets were maintained in a low-oxygen environment. After 2 weeks, growth under hypoxic and normal (aerobic) conditions was compared. In previous studies, an oxygen absorber was used to reduce oxygen levels [17] [18]. However, in the present study, nitrogen gas was used to maintain a stable hypoxic condition. The loading speed of the nitrogen gas was 0.05 $L \cdot min^{-1}$ per 10 L hydroponic solution. For the control treatment, the hydroponic solution was aerated using an air pump to achieve an oxygen concentration of 18.7% - 19.0%. The oxygen concentrations and temperatures of the hydroponic solutions for both treatment groups were monitored using DO METER ID-100 dissolved oxygen analyzers (Iijima Electronics Co., Aichi, Japan). The average oxygen concentrations under hypoxic and control conditions were 0.78 ± 0.12 $mg \cdot L^{-1}$ and 6.86 ± 0.12 $mg \cdot L^{-1}$, respectively. Thus, the oxygen concentrations were significantly different between the two treatments ($p < 0.001$; n = 14, Student's *t*-test). The air temperature of the incubator was 25˚C $\pm$ 0.05˚C, while the culture solution temperature was 22.1˚C $\pm$ 0.13˚C; there was no significant difference between the treatment groups ($p > 0.1$). During the experiment, the pH and electrical conductivity changed from 7.6 to 7.0 and from 1.36 $mS \cdot cm^{-1}$ to 1.19 $mS \cdot cm^{-1}$, respectively. No differences were observed between the two treatments regarding pH or electrical conductivity ($p > 0.1$; n = 14, Student's *t*-test). Thus, the two treatment groups differed only in plant materials, and samples were analyzed under equivalent conditions except for oxygen concentrations. Plants were grown under a 15.5-h photoperiod (light intensity: 190 - 200 μmol $m^{-2} \cdot s^{-1}$) in an incubator set at 25˚C (dark period: 15˚C) and relative humidity of approximately 73%. Each treatment group comprised

10 plantlets per cultivar.

2.3. Measurements

Plants were harvested after a 14-day culturing period. The shoots were separated from the roots, and the leaves were collected. The leaf area was then measured using an AAM-7 automated area meter (Hayashi Denko Co. Ltd., Tokyo, Japan). Measurements were completed using three plants (*i.e.*, one plant per replicate).

Roots were carefully removed from the hydroponic solution, after which their hydraulic conductance (*i.e.*, inverse of hydraulic resistivity) was measured according to a pressure chamber method [22]. A pressure chamber was prepared using a pump-up chamber (PMS Instrument Company, Albany, OR, USA) and an AM02-04N air compressor (Fujiwara Sangyo Co., Ltd., Hyogo, Japan) to automatically pressurize the chamber [8]. A root was fixed to the lid of a pressure chamber and the root system was immersed in the hydroponic solution. The internal pressure of the chamber was increased step-wise by 50 kPa at 30-s intervals up to 600 kPa (0.6 MPa). The water discharged through the roots was trapped every 30 s using a pre-weighed cotton swab. The cotton swab was weighed before and after use with a VIC212 electric balance (sensitivity: 1 mg) (ACCULAB Sartorius AG, Göttingen, Germany). Hydraulic conductance was estimated as a "flow ratio" [*i.e.*, water volume per unit time and root length ($\mu L \cdot m^{-1} \cdot min^{-1}$). The relationship between hydraulic conductance and loading pressure was determined. The linear increase in hydraulic conductance was defined as the index of root hydraulic conductivity [*i.e.*, Lp ($\mu L \cdot MPa^{-1} \cdot m^{-1} \cdot min^{-1}$)], which is represented by the slope of the linear phase of the curve [23]. Measurements were completed with three plants (*i.e.*, the roots of one plant per replicate).

The roots were rinsed gently with tap water, after which various root morphological characteristics were analyzed using WinRhizo (Regent Instruments Inc., Canada; Supplementary Figure 1(A)-(D)). Roots with a diameter of 0.4 - 0.7 mm were designated "thick roots", while those with a diameter smaller than 0.4 mm were defined as "fine roots" (Supplementary Figure 1(E)). The analyses were completed with three plants (*i.e.*, the roots of one plant per replicate). Thus, three plants from each cultivar were used for leaf area, root conductivity, and root morphology measurements. Additionally, four plants from each cultivar were separated into leaves, stems, and roots. The plant tissue samples were dried at 80°C for 3 days, after which the dry mass of each organ from each plant was measured.

The H/A ratio, which represents the effect of hypoxic (H) and aerobic (A) conditions on plant biomass, was used to evaluate plant responses to hypoxia. An H/A ratio greater than 1.0 indicated that the trait was enhanced by hypoxia (*i.e.*, the cultivar is tolerant), and a ratio less than 1.0 indicated that the cultivar was damaged by hypoxia (*i.e.*, the cultivar is sensitive). Ratios close to 1.0 suggested that hypoxic conditions had limited effects on plants.

2.4. Protein Extraction and Immunoblotting Analysis

Proteins (including aquaporins) were extracted from plant tissues essentially as

described by Hanba *et al.* [24]. Frozen soybean roots (approximately 0.3 g FW) from plants in the two treatment groups were ground to a powder in liquid nitrogen and placed in 0.9 mL extraction buffer [50 mM Tris-HCl (pH 7.8), 0.3 M sucrose, 8 mM ethylenediamine tetraacetic acid, 4 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride] at 4°C. Samples were centrifuged at 10,000 rpm for 10 min at 4°C. A 100-μL aliquot of the supernatant was diluted with 100 μL Laemmli's buffer [500 mM Tris-HCl (pH 6.8), 3% (w/w) sodium dodecyl sulphate (SDS), 30% (w/v) glycerol, 9.3% (w/w) dithiothreitol, and 1.2% (w/w) bromophenol blue] and then heated at 70°C for 10 min. The solution was used as a soluble protein fraction, and its protein content was determined using the Bradford assay [25]. Proteins were separated by one-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) [26] using 5.1 μg protein per lane. The SDS-PAGE was conducted with a 4% stacking gel and a 12.5% running gel at a constant current of 20.5 mA. The proteins were then electrotransferred to an AE-6668 polyvinylidene difluoride membrane (ATTO Co., Tokyo, Japan) for 30 min at 80 mA in a transfer solution [25 mM Tris base and 5% (v/v) methanol]. The proteins were probed with the following four rabbit polyclonal anti-aquaporin antibodies (1:1000 dilution; Cosmo Bio Co., Ltd., Tokyo, Japan): anti-PIP1s (PIP1;1, 1; 2, and 1; 3; epitope: KDYNEPPPAPLFEPGELSSWS-C), anti-PIP2s (PIP2;1, 2;2, and 2; 3; epitope: Cys-QFVLRASGSKSLGSFRSA), anti-TIP1s gamma (TIP1;1 and TIP1; 2; epitope: GVQEEVTHPSALRA-Cys), and anti-TIP2;1 delta (epitope: Cys-MTSEHVPLASEF). Antibodies were visualized using an alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (1:500 dilution; Bio-Rad Laboratories Inc., CA, USA) and an AP Conjugate Substrate Kit, which included a 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium reagent system (Bio-Rad Laboratories Inc.). Because the density of the resulting bands was insufficient for photographs, membranes were scanned and analysed using the UN-SCAN-IT gel image analysis software (Silk Scientific, Inc., UT, USA). The protein quantifications were repeated three times (*i.e.,* one frozen root sample per replicate) for each cultivar. The densities of the detected bands were converted to values that represented the relative protein abundance. These values were then compared among cultivars and treatments. Some partial SDS-PAGE gels were stained with Coomassie brilliant blue, and the location of specific bands was determined immunologically.

2.5. Statistical Analysis

One plantlet was defined as a replicate, and three or four replicates were analyzed using a randomized block design. A two-way analysis of variance was used to compare the average values of all plant biomass data among treatments (*i.e.,* aerobic vs hypoxic conditions) and cultivars. Additional statistical analyses included the Tukey-Kramer and Student's *t*-test, which were used to separate means, and Pearson's correlation analysis, which was used to determine the relationship between two traits. Data analyses were conducted using SPSS 20.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Effect of Hypoxia on Soybean Biomass

The dry weight data for leaves, stems, and roots for each cultivar under the two treatment conditions are summarized in Table 2. The leaves and roots were the heaviest and lightest parts of the soybean plants, respectively. The hypoxic treatment significantly decreased the average stem dry weight ($p < 0.05$). Among the nine cultivars, "TKu" and "SMa" had the largest and smallest biomasses, respectively (Table 2). The cultivars with the largest leaf biomass were "TKu", "Shirotsurunoko" ("STs"), and "TMu". "Koganejiro" ("KJi") plants produced the largest root biomass. Although there were significant differences in biomass for the different components among the cultivars ($p < 0.001$), significant interactions between treatment and cultivar ($p < 0.05$ - 0.001) were also detected for all plant components. These results indicated that the effects of hypoxia on biomass differed among the cultivars (Table 2). A comparison of the mean square values revealed they were generally higher among cultivars (C) than between treatments (T) or for the interaction (C × T). However, for root dry matter, the mean square value for the interaction was higher than that between treatments or among cultivars. This suggests that the effect of hypoxia was different among cultivars regarding root biomass. Because the effect was significant, the characteristics of each cultivar were examined separately in the following analysis.

Table 2. Average dry weights of leaves, stems, and roots. Analysis of variance was used to compare the variables among cultivars under different conditions.

		Leaf dry weight (mg)			Stem dry weight (mg)			Root dry weight (mg)			Total dry weight (mg)		
Average		252.6	(17.0)[a]		179.0	(15.7)		88.8	(6.7)		519.9	(32.7)	
Treatment (T)	Aerobic	300.5	(24.4)		225.3	(23.8)		105.8	(7.4)		631.5	(47.4)	
	Hypoxic	204.6	(21.1)		132.8	(17.8)		71.8	(10.5)		408.2	(36.9)	
Cultivar (C)	ERe	146.7	(14.6)	bc[b]	136.6	(32.9)	bc	51.5	(15.0)	b	330.6	(61.6)	cd
	KJi	249.9	(18.9)	ab	205.9	(36.5)	abc	146.2	(29.3)	a	601.9	(49.8)	abc
	S32	239.2	(44.7)	ab	121.8	(21.3)	bc	72.3	(7.5)	ab	433.3	(43.4)	bcd
	SMa	53.3	(20.9)	c	65.5	(18.5)	c	50.5	(3.7)	b	169.3	(32.5)	d
	STs	337.7	(46.5)	a	247.6	(38.4)	ab	110.0	(21.6)	ab	695.3	(97.2)	ab
	THa	295.9	(48.4)	ab	144.9	(37.8)	bc	90.8	(22.3)	ab	531.1	(103.5)	abc
	THo	240.4	(45.7)	ab	107.8	(36.9)	bc	74.3	(13.1)	ab	422.2	(61.7)	bcd
	TKu	340.9	(67.8)	a	354.5	(71.9)	a	96.2	(26.5)	ab	791.6	(133.6)	a
	TMu	369.3	(22.0)	a	226.6	(31.4)	abc	107.8	(7.5)	ab	703.6	(22.4)	ab
ANOVA	df	MS		Sig[c]	MS		Sig	MS		Sig	MS		Sig
T	1	45733		ns	27968		*	485		ns	12757		ns
C	8	81811		***	62679		***	7495		***	320977		***
T × C	8	56327		***	16606		*	12352		***	172607		***

The mean square and significance values are provided; [a]Values in parentheses denote standard error; [b]Different letters within each row indicate significant differences at the 0.05 probability level according to the Tukey-Kramer test (n = 8). [c] * and *** in each row represent significance at 0.05 and 0.001 probability levels, respectively; ns, not significant according to ANOVA (n = 4); ANOVA, analysis of variance; df, degree of freedom; MS, mean square; Sig., significance.

Leaf, stem, root, and total dry weights were compared between the two treatment groups for each cultivar (Table 3). The leaf dry weights of "Ere" ($p < 0.01$), "SMa" ($p < 0.01$), "STs" ($p < 0.01$), "THa" ($p < 0.01$), "THo" ($p < 0.001$), and "TKu" ($p < 0.01$) plants were significantly decreased by hypoxia, whereas that of "S32" plants was significantly increased ($p < 0.001$). The stem dry weights of "Ere" and "THa" plants were significantly decreased by hypoxia ($p < 0.001$). Additionally, the root dry weights of "Ere" ($p < 0.001$), "STs" ($p < 0.01$), "THa" ($p < 0.001$), and "TKu" plants ($p < 0.01$) were significantly decreased by hypoxia, whereas that of "KJi" plants was increased ($p < 0.05$). The total dry weights of the cultivars decreased under hypoxic conditions in the order "Ere" ($p < 0.001$), "SMa" ($p < 0.01$), "STs" ($p < 0.01$), "THa" ($p < 0.001$), "THo" ($p < 0.05$), and "TKu" ($p < 0.001$). In contrast, the dry weight of "S32" plants increased after the hypoxia treatment (Table 3). The H/A values were used to estimate the waterlogging tolerance of each cultivar. The "S32" and "KJi" plants exhibited waterlogging tolerance, and tended to have high H/A ratios for each biomass.

3.2. Changes in Root Hydraulic Conductivity Due to Hypoxia

The flow ratio (per total root length, per minute) of sap solution from cut stems after roots were pressurized was plotted for each cultivar and treatment. The analyses revealed differences in loading pressures among cultivars (Figure 1). An increase in pressure resulted in increased sap production for all cultivars.

Table 3. Leaf, stem, root, and total dry weights as well as the ratio between hypoxic (H) and aerobic (A) conditions for each cultivar.

Cultivar	Leaf dry weight (mg)			Stem dry weight (mg)			Root dry weight (mg)			Total dry weight (mg)		
	A	H	H/A	A	H	H/A	A	H	H/A	A	H	H/A
ERe	180.0	113.3 ** ▽	0.63	219.6	53.6 *** ▽	0.24	90.3	12.8 *** ▽	0.14	489.9	171.3 *** ▽	0.34
KJi	222.5	277.3 ns	1.25	207.8	203.9 ns	0.98	89.0	203.3 * ▲	2.28	519.3	684.5 †	1.32
S32	128.0	350.3 *** ▲	2.74	160.7	83.0 †	0.52	61.3	83.3 ns	1.36	349.9	516.6 * ▲	1.48
SMa	104.0	2.6 ** ▽	0.03	94.6	36.4 ns	0.38	50.3	50.8 ns	1.01	248.9	89.8 ** ▽	0.36
STs	441.0	234.3 ** ▽	0.53	315.8	179.4 †	0.57	158.0	62.1 ** ▽	0.39	914.8	475.8 ** ▽	0.50
THa	407.3	184.6 ** ▽	0.45	240.9	49.0 *** ▽	0.20	147.8	33.8 *** ▽	0.23	795.9	266.4 *** ▽	0.33
THo	357.8	123.1 *** ▽	0.34	88.0	127.6 ns	1.45	93.0	55.6 ns	0.60	538.1	306.2 * ▽	0.57
TKu	493.8	188.1 ** ▽	0.38	465.5	243.6 ns	0.52	160.5	31.8 ** ▽	0.20	1119.7	463.5 *** ▽	0.41
TMu	370.3	368.3 ns	0.99	234.8	218.3 ns	0.93	102.5	113.1 ns	1.10	707.6	699.7 ns	0.99

†, *, **, and *** represent significance at 0.1, 0.05, 0.01, and 0.001 probability levels, respectively; ns, not significant according to Student's *t*-test (n = 4). ▲ and ▽ indicate significant increases and decreases in dry weight, respectively.

There was a significant variation in the linear part of the curves representing responses to hypoxia among cultivars. The "SMa", "STs", "THa", and "TKu" cultivars exhibited a significant decrease in the flow ratio in response to hypoxic conditions, whereas the opposite trend was observed for "S32" and "THo" cultivars.

The variation in the linear range of increasing flow was calculated and defined as the hydraulic root conductivity [*i.e.*, Lp ($\mu L \cdot MPa^{-1} \cdot m^{-1} \cdot min^{-1}$)]. Higher Lp values corresponded to greater root permeability per unit length. The H/A ratiofor the Lp values are indicated as an italicized number after the abbreviated cultivar names in Figure 1. If the Lp value under aerobic conditions decreased relative to that of the hypoxia treatment, the H/A ratio for the Lp value also decreased, indicating limited hydraulic conductivity due to hypoxia.

To identify the morphological characteristics that responded to hypoxic conditions similarly to hydraulic root conductivity, the correlation coefficients between root hydraulic conductivity and the other analysed characteristics were determined (Table 4). The H/A ratios for the total dry weights of hypoxia-treated and control plants were positively related (r = 0.78; $p < 0.05$) to the corresponding ratio for root hydraulic conductivity. This was also true for the leaf dry weight H/A ratio (r = 0.85; $p < 0.01$). This positive relationship was attributed to leaf area (r = 0.89; $p < 0.01$) rather than leaf thickness (r = 0.09; $p > 0.1$) (Table 4, Figure 2(A)). Although there was no correlation between the H/A ratios for root dry weight and root conductivity, the H/A ratios for root length (r = 0.67; $p < 0.05$) [especially thick-root length (r = 0.73; $p < 0.05$) (Figure 2(B))] and number of root tips (r = 0.68; $p < 0.05$) (Figure 2(C)) were significantly correlated with the H/A ratio for root conductivity (Table 4).

3.3. Changes in Root Aquaporin-Like Proteins Due to Hypoxia

The preceding sections have described the effects of morphological characteristics on root hydraulic conductivity. To assess the effects of non-morphological characteristics on root hydraulic conductivity, the hypoxia-induced responses of water channels, which are related to root water permeability, were examined. Digital image analyses of SDS-PAGE gels stained with Coomassie brilliant blue indicated that band densities were not significantly influenced by exposures to hypoxic conditions for all cultivars (data not shown).

An immunoblot analysis revealed variations in the abundance of root aquaporin-like proteins in hypoxia-treated plants. Although four different polyclonal anti-aquaporin antibodies were used, bands were only detected by the anti-PIP1s antibody. This antibody generated five different bands (*i.e.*, 24.3, 27.1, 32.3, 41.3 - 50.8, and 67.7 - 108.5 kDa), which were presumably derived from a single band or a group of bands (Figure 3).

The intensity of the 24.3 kDa band representing PIP1s-like proteins differed significantly between treatments and cultivars ($p < 0.01$; Supplementary Table 1). The intensity of the 24.3 kDa band decreased significantly for the "Ere" ($p < 0.05$), "SMa" ($p < 0.01$), and "THo" ($p < 0.05$) cultivars exposed to hypoxic conditions. In contrast, the intensity of this protein band increased considerably
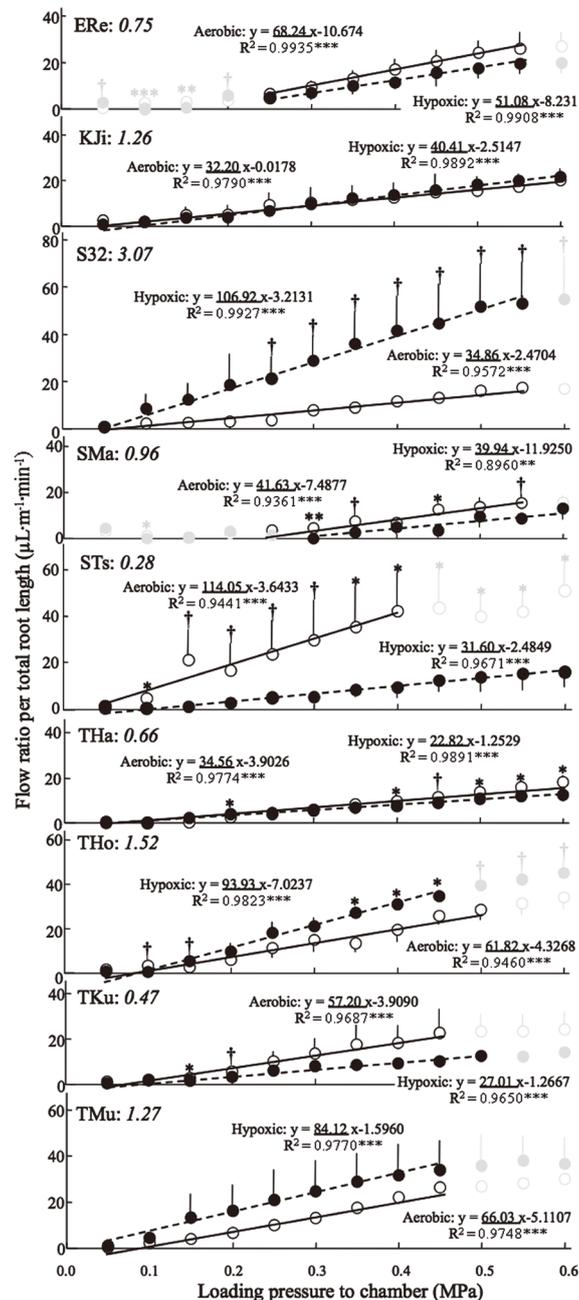
Figure 1. Relationship between the loading pressure of the chamber and the flow ratio per root length, which corresponds to the root hydraulic conductivity (Lp: μL MPa^{-1} m^{-1} min^{-1}). Data are provided as the means from three replicates, and bars indicate standard errors. †, *, and ** represent significant differences between the control (open circles) and hypoxia-treated (closed circles) samples at 0.1, 0.05, and 0.01 probability levels, respectively, according to Student's *t*-test. The regression lines were associated with formulae (** and *** represent significance at the 0.01 and 0.001 probability levels, respectively) with correlation coefficients based on Pearson's correlation analysis. The formula for each regression line is as follows: Treatment: $y = a \times x + b$; where a = Lp and b = constant. The Lp value is defined by the slope of the linear phase of the curve (Emery and Salon 2002) [23]. The values indicated by half tone symbols were not used for calculating the regression line because they were not included in the linear relationship between the loading pressure and flow ratio per total root length. The values in italics next to the abbreviated cultivar names indicate the Lp value ratios between hypoxic and aerobic conditions.
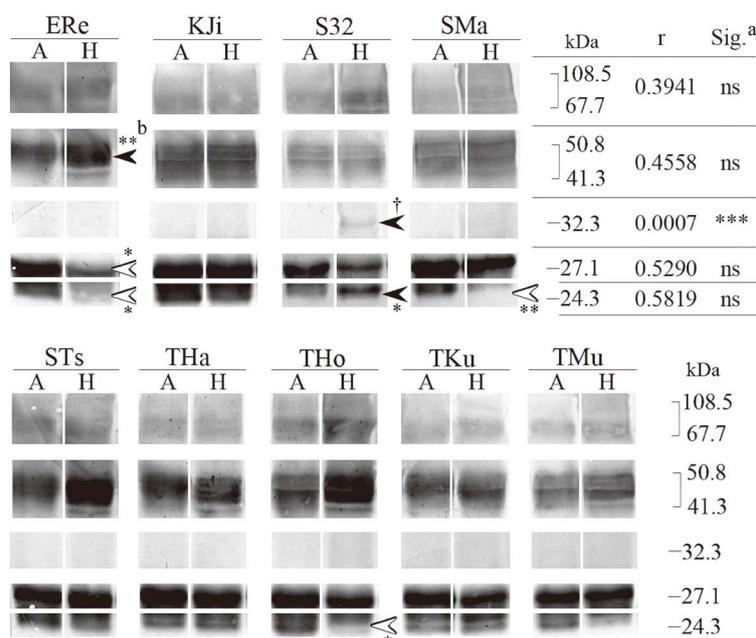
Table 4. Significance of the correlation coefficient for the relationship between the hypoxic (H)/aerobic (A) ratio of root hydraulic conductivity and the H/A ratio of various characteristics.

Characteristic	r	Sig.[a]
Dry weights		
Leaf	0.8467	**
Stem	0.2643	ns
Root	0.5275	ns
Total	0.7791	*
Leaf traits		
Area	0.8856	**
Thickness	0.0899	ns
Root traits		
Average diameter	0.3120	ns
Number of tips	0.6864	*
Total length	0.6678	*
Thick-root length[b]	0.7326	*
Fine-root length[b]	0.5804	ns

[a] * and ** represent significance at 0.05 and 0.01 probability levels, respectively; ns, not significant according to Pearson's correlation analysis; [b] Thick and fine roots were defined as those having a diameter of 0.4 - 0.7 mm and 0.0 - 0.4 mm, respectively; r, correlation coefficient; Sig., significance.
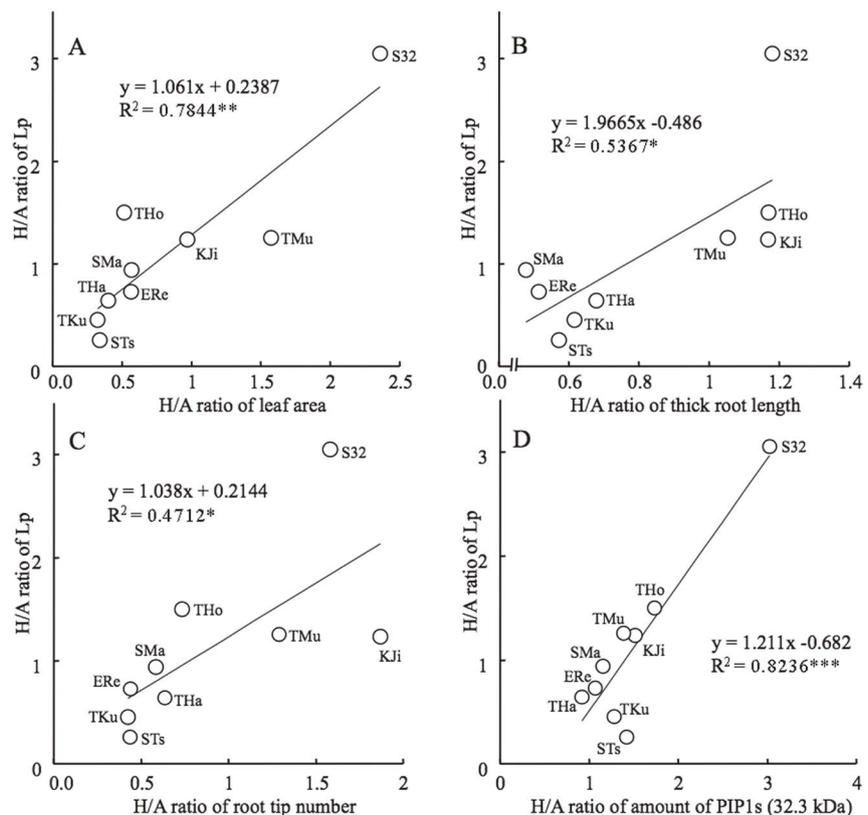
Figure 2. Relationships between the hypoxic (H)/aerobic (A) ratio of root hydraulic conductivity (Lp) and the H/A ratios of leaf area (A), thick-root length (B), number of root tips (C), and abundance of PIP1s (32.3 kDa) (D). Abbreviated cultivar names accompany each symbol. Regression lines marked by *, **, and *** indicate significance at the 0.05, 0.01, and 0.001 probability levels, respectively, according to Pearson's correlation analysis (n = 9).

Figure 3. Immunoblot analysis of soluble proteins extracted from soybean roots, and the correlation coefficient (r) and significance (Sig.) of the relationship between the hypoxic (H)/aerobic (A) ratio of root hydraulic conductivity (Lp) and the H/A ratio of protein abundance. An anti-aquaporin (PIP 1;1, 1;2, and 1;3) antibody was used. Closed and open arrowheads indicate proteins whose abundance significantly increased and decreased, respectively, under hypoxic conditions in a hydroponic culture. Each lane contained 5.1 μg total protein. a *** represents significance at the 0.001 probability level, while ns means not significant according to Pearson's correlation analysis (n = 9). b †, *, and ** above arrowheads represent significant differences in the band densities between the control and hypoxia-treated samples at 0.1, 0.05, and 0.01 probability levels, respectively, according to Student's *t*-test.

for the "S32" plants under the same conditions ($p < 0.05$; **Figure 3**). For the "Ere" plants under hypoxic conditions, the intensity of the 27.1 kDa band decreased and that of the 41.3 - 50.8 kDa bands increased. There were no differences in the intensities of the 67.7 - 108.5 kDa bands between treatments for any cultivar.

The relationship between the H/A ratio for root hydraulic conductivity and the H/A ratio for PIP1s band sizes in the two treatments was analyzed (**Figure 3**). The interaction between the H/A ratios was significant for only the 32.3 kDa band ($p < 0.001$). Furthermore, the relationship was positive (**Figure 2(D)**), implying that the increase in the abundance of the aquaporin-like protein enhanced root hydraulic conductivity.

4. Discussion

4.1. Analysis of the Mechanism Underlying Field Waterlogging Tolerance Based on Responses to Hypoxic Conditions

The main cause of waterlogging injury in soybean plants is reportedly the low respiration levels in the roots because of a lack of oxygen in the rhizosphere. This in turn limits nutrient uptake and eventually decreases photosynthetic activities [27]. Soybean plantlets require more oxygen than other field crops be-

cause the nitrogen-fixing bacteria in their root nodules need a considerable oxygen supply. Because the nitrogen supply from nodules is substantially reduced at lower partial pressures of oxygen, rhizospheric hypoxia is a primary factor triggering waterlogging injury in field crops [28]. The present study involved a hydroponic culture system to avoid having to consider the effects of nodule bacteria. Additionally, the duration of the experiment was insufficient for the hypoxia treatment to induce the formation of aerenchymatous cells, as reported by Thomas *et al.* [16] and Shimamura *et al.* [14]. Therefore, the observed responses of immature soybean plantlets to hypoxic conditions were unaffected by nodulation or the aerenchyma.

Hypoxia significantly influenced all biomass traits investigated in this study. The degree of influence varied significantly among different cultivars. Thus, the cultivars could be categorized according to their specific hypoxic responses (Table 3). The nine cultivars were grouped into the following three hypoxic-response categories: cultivars exhibiting stimulated growth (enhanced type: "S32" and "KJi"), those unaffected by hypoxia (tolerant type: "THo" and "TMu"), and those exhibiting decreased growth (susceptible type: "Ere", "SMa", "STs", "THa", and "TKu"). The oxygen diffusion rate in the soil is closely related to the rate of plant root development [29]. This characteristic might also apply to the hypoxic responses of the susceptible cultivars examined in the present study. Thus, cultivar-specific hypoxic effects may be associated with the soybean waterlogging response. Some researchers have indicated that high carbon dioxide levels [30] [31] [32] or impaired mineral diffusion [33] in the rhizosphere are key factors influencing crop waterlogging injury. Additionally, low partial pressure of oxygen around the roots [17] [34] should be considered an important factor in the development of waterlogging injury in soybean plants.

4.2. Cultivar-Specific Response to Hypoxia Related to Root Hydraulic Conductivity

Plants that have been exposed to anoxic conditions exhibit decreased respiratory activities and impaired mitochondrial functions [35]. Growth rates and yields have been reported to decrease in these plants because of limited water and nutrient uptake [36]. The cultivation of soybean in wet soil inhibits root development and nodulation [37], ultimately resulting in low yields [1]. The hypoxic conditions used in the present study tended to decrease plant biomass and shrink the root system of susceptible cultivars. In addition to light, a suitable temperature, and carbon dioxide, plants depend on water and nutrients for growth. In our experimental environments, all soybean plantlets were exposed to the same light conditions, temperatures, and carbon dioxide levels. Although both treatment groups were supplied with equal amounts of water, plant water usage may have differed depending on the plant status under specific conditions. The differences in growth and biomass due to hypoxia treatments implied that water usage is likely a key factor affecting the development of waterlogging injury.

The analysis of the effects of hypoxia on root water permeability using the pressure chamber method revealed that the level of hypoxia required to affect the sap flow ratio for cut stems with pressurized roots differed significantly among cultivars. These results suggest that hypoxia affected root hydraulic conductivity in specific cultivars. In "S32", "THo", "KJi", and "TMu" plants, the H/A ratio of Lp was greater than 1.0, indicating that root water permeability was enhanced by hypoxia. In contrast, the H/A ratio of Lp for "Ere", "SMa", "STs", "THa", and "TKu" plants was less than 1.0, indicating that the root water permeability for these cultivars decreased in response to hypoxia. This separation of cultivars based on differences in root hydraulic conductivity under hypoxic conditions suggests a close relationship between this response and hypoxia tolerance.

4.3. Root Hydraulic Conductivity Fluctuates with Changes to Leaf and Root Systems

Water flow in a plant vessel is regulated by transpiration pressure and modulated by hydraulic resistance, which is influenced by the length and diameter of the vessel as well as the flow rate. The relationship between water pressure and resistance is analogous in many respects to an electrical circuit (*i.e.*, the voltage and electrical resistance of Ohm's law) [38]. In the soil-plant-atmosphere system, which specifies the hydraulic sequence from root uptake to leaf transpiration, water behaviour is also analogous to an electrical circuit. The soybean root system has many lateral fine roots and exhibits synthetic resistance, but not serial resistance. This needs to be considered when calculating total hydraulic resistance. If a large leaf area generates more transpiration pressure, and if a root system develops more branches or tips, then the total hydraulic resistance would be expected to decrease.

The correlation analyses of the H/A ratio for root hydraulic conductivity and the H/A ratios of morphological characteristics indicated that leaf area, thick-root length, and number of root tips had a close relationship with root hydraulic conductivity. This finding suggests that hypoxia-induced changes to leaf area, thick-root length, and number of root tips are associated with root hydraulic conductivity. Thus, if the leaf area decreases because of hypoxic injury, the root hydraulic conductivity also decreases. This is likely because of reduced transpiration, although this parameter was not assessed in this study. Regarding root traits, when the thick-root length and number of root tips decreased because of exposure to hypoxic conditions, the root hydraulic conductivity also decreased (Figure 2(B) Figure 2(C)), as expected according to Ohm's law.

Root system morphology might regulate root hydraulic conductivity, in agreement with Ohm's law, in many soybean cultivars. However, root hydraulic conductivity must be regulated not only by root quantity, but also by root water permeability. In radish, water absorption depends mostly on the water channels of the root tissue cells [39]. Furthermore, in soybean roots, the production of an aquaporin, which is a plasma membrane intrinsic protein, varies under hypoxic

conditions [40]. Consequently, the changes in the abundance of aquaporin-like proteins in response to hypoxia were studied for each cultivar.

4.4. Cultivar-Specific Changes to the Plasma Membrane Intrinsic Proteins in the Water Channels of Soybean Roots

Plant water channels consist primarily of two kinds of membrane intrinsic proteins, namely PIP and TIP. In the present study, the abundance of the following four aquaporin proteins were examined immunologically: PIP1s (PIP1;1, 1;2, and 1;3), PIP2s ('PIP2;1, 2;2, and 2;3), TIP1s (TIP1;1 and TIP1;2), and TIP2 (TIP2;1). The PIP2s and TIP2 proteins exhibit greater hydraulic activity than PIP1s [41] [42], although PIP1; 1 considerably enhances PIP2; 1 hydraulic activities [43]. The PIP1s aquaporins may affect a regulator of heteromerization and fine-tuning of the channel activity of the aquaporin complex along with PIP2 [44].

The anti-PIP1s antibodies detected five bands in immunoblots (*i.e.*, 24.3, 27.1, 32.3, 41.3 - 50.8, and 67.7 - 108.5 kDa) (Figure 3). The monomeric form of the *Arabidopsis thaliana* aquaporin is 28.0 kDa (Cosmo Bio Co., Ltd.). Thus, the 27.1 kDa band may represent the original size of the PIP1s monomers, while the 41.3 - 50.8- and 67.7 - 108.5 kDa bands might correspond to the dimeric and trimeric/tetrameric forms, respectively. The 32.3 kDa band is likely a chemically modified aquaporin-like protein, and not a multimeric form.

A correlation between the H/A ratio of the 32.3 kDa PIP1s and the H/A ratio of root hydraulic conductivity was observed (Figure 2(D) and Figure 3), indicating the possibility of a close relationship between hypoxia tolerance and aquaporin-like protein production. The suppressed expression of the tobacco aquaporin gene *NtAQP1* was associated with decreased transpiration activity and root hydraulic conductivity [45]. The abundance of the 32.3 kDa PIP1s significantly increased in the hypoxia-tolerant "S32" plants under hypoxic conditions. Additionally, root hydraulic conductivity may also have increased in these plants because of the influence of abundant aquaporin-like proteins in addition to root morphology changes. The field waterlogging tolerance of "S32" plants was not attributed to the formation of aerenchymatous cells [19], which is in contrast to many other soybean cultivars [14] [16]. A relationship between hypoxia tolerance and aquaporin-like protein production was still observed even if the "S32" data were not considered. Thus, responses to hypoxia depend on the regulation of PIP1s, possibly by different mechanisms among cultivars. Matsuo *et al.* described a relationship between hypoxia and changes to mRNA levels for the "Ere" soybean root aquaporin PIP2 gene *GmPIP2*; 2 [40]. The expression of this gene was downregulated in roots after a 3-h exposure to hypoxic conditions. Although the aquaporins and durations of hypoxia treatment were different from those used in the present study, the fact that the external hypoxic conditions influenced the production of PIP proteins and consequently affected root water uptake was consistent in both studies.

The production of PIP1s aquaporin-like proteins did not differ between aero-

bic and hypoxic conditions for five cultivars (*i.e.*, "KJi", "STs", "THa", "TKu", and "TMu"). In these cultivars, the changes in the membrane water channels of the root system had considerable effects on root hydraulic conductivity. This suggests that PIP proteins are not the main factor responsible for the quantitative changes caused by hypoxia in some cultivars. In *A. thaliana* roots subjected to anoxic conditions, the aquaporin gating activity is regulated by cytosolic pH [46]. This implies that the hydraulic activity of water channels should be investigated even if there is no change in aquaporin production. Moreover, the importance of root hydraulic conductivity for water channel-induced permeability needs to be investigated among cultivars with specific hypoxic responses (e.g., examining the effects of aquaporin inhibitors such as silver nitrate or mercury compounds) [47]. Furthermore, hypoxia-inducible root-related transcription factors [48], fibrillins [49], nuclear-related proteins [50], and the special fermentative metabolic system in soybean roots [51] [52] may affect cultivar-specific waterlogging tolerance and will also need to be studied to further characterize the mechanism underlying waterlogging tolerance.

Acknowledgements

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Abbreviations

ANOVA, analysis of variance; **Ere**, Enrei; **H/A ratio**, ratio of hypoxic (H) and aerobic (A) values; **KJi**, Koganejiro; **Lp**, root hydraulic conductivity; **PIP**, plasma membrane intrinsic protein; **S32**, Shoku-kei32; **SMa**, Suzumaru; **STs**, Shirotsurunoko; **THa**, Toyoharuka; **THo**, Toyohomare; **TIP**, tonoplast intrinsic protein; **TKu**, Tanbakuro; **TMu**, Toyomusume.

Supplementary
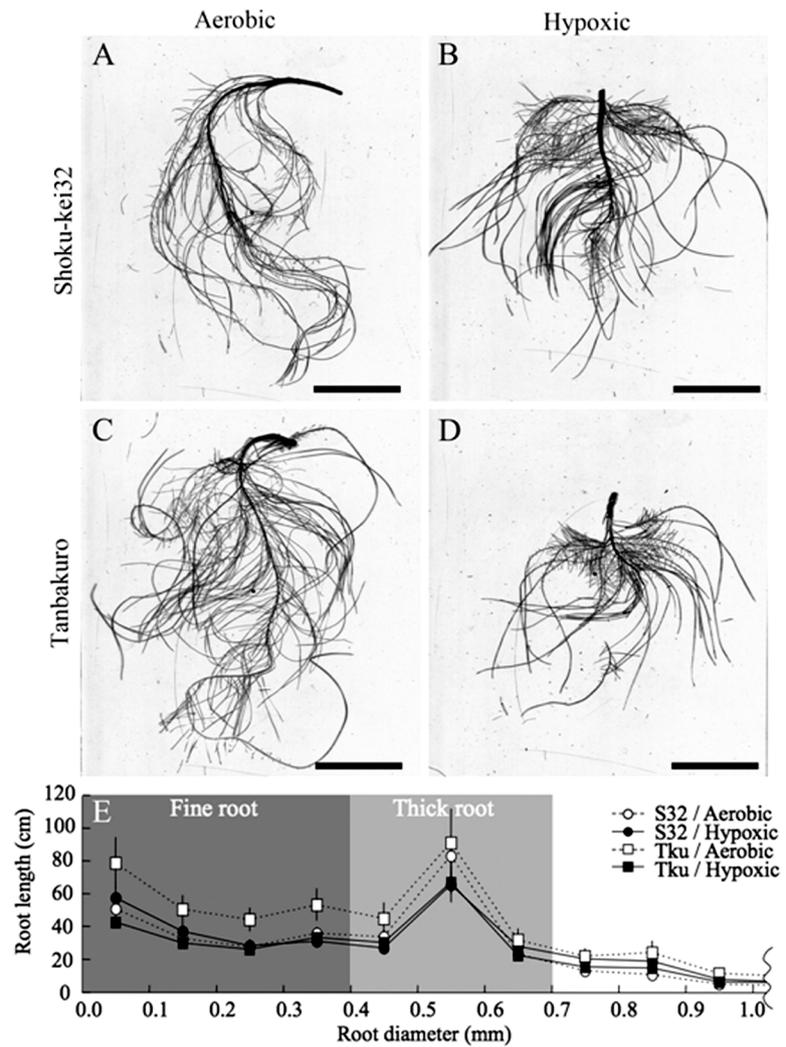
Supplementary Figure 1. Typical images of "Shoku-kei32" (A and B) and "Tanbakuro" (C and D) roots under aerobic (A and C) and hypoxic (B and D) conditions. Scale bars represent 5 cm. Root length frequencies for fine roots (diameter: 0.0 - 0.4 mm) and thick roots (diameter: 0.4 - 0.7 mm) (E). Vertical bars indicate standard errors.

Supplementary Table 1. Average band intensities for the immunoblot analysis of soluble proteins extracted from soybean roots.

ANOVA	df	Molecular size of PIP1s proteins (kDa)									
		67.7 - 108.5		41.3 - 50.8		32.3		27.1		24.3	
		MS	Sig.[a]	MS	Sig.	MS	Sig.	MS	Sig.	MS	Sig.
T	1	253	*	779	**	119	†	688	**	1487	**
C	8	333	***	253	**	63	†	277	**	379	†
C × T	8	40	ns	124	ns	26	ns	127	ns	578	**

Analysis of variance was used to compare the variables among cultivars under different conditions; The mean square and significance values are provided; [a] †, *, **, and *** in each row represent significance at 0.1, 0.05, 0.01, and 0.001 probability levels, respectively; ns, not significant according to ANOVA (n = 3); ANOVA, analysis of variance; df, degree of freedom; MS, mean square; Sig., significance.

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