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Research Paper

Genetic variation and QTLs related to root development in upland New Rice for Africa (NERICA) varieties

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To understand variation in the root development traits (total root length (TRL), maximum root length (MRL) and root number) of 18 New Rice for Africa (NERICA) varieties, seedlings were hydroponically grown under deficient and sufficient concentrations of two forms of nitrogen, NH_4^+ and NO_3^-. The donor African rice variety, 'CG14' (*Oryza glaberrima* Steud.), showed greater TRL and MRL than three background Asian rice varieties (*Oryza sativa* L.). Wide distribution was observed in all traits of the 18 NERICAs. The 18 NERICAs and parental varieties were classified into three cluster groups by cluster analysis. Cluster Ia included only 'CG14'. Comparative analysis characterized cluster Ib (including 'NERICA7') as an active root elongation group, and cluster II (including 'WAB56-104') as an active primordia development group. QTL analysis of F_2 plants developed from a cross between 'WAB56-104' and 'NERICA7' detected two putative quantitative trait loci (QTLs) for root elongation on chromosome 1. Of these, a major QTL, designated as *qRL1.4-NERICA7*, was an NH_4^+-responsive QTL, which was narrowed down to a 0.7-Mbp region through progeny testing using F_7 lines. *qRL1.4-NERICA7* should help us understand genetic control in NERICAs, and improve root elongation in rice breeding programs.

Key Words: genetic variation, upland New Rice for Africa (NERICA), nitrogen, quantitative trait locus/loci (QTL), rice (*Oryza sativa* L.), root development.

Introduction

Unlike *O. sativa* which has been widely cultivated in Asia, *O. glaberrima*, which has been cultivated in West Africa, is a potentially useful gene source for African insect pests and diseases, weed competition, and seedling vigor (Jones *et al.* 1997a, 1997b). For upland conditions in Africa, 18 New Rice for Africa (NERICA) varieties have been developed to introduce useful traits of *O. glaberrima* in the genetic background of *O. sativa*, using interspecific hybridization, at the Africa Rice Center (AfricaRice; former name: Western Africa Rice Development Association) (Jones *et al.* 1997b). Over the last decade, these 18 NERICAs have been characterized for their agronomical performance, nutrient (nitrogen or phosphorus) use efficiency, and weed competition (Saito *et al.* 2012). However, there are very few reports on genetic control in the 18 NERICAs, and these only concern aboveground, not underground plant components (Fukuta *et al.* 2012, Koide *et al.* 2013, 2015). It is widely accepted, with regard to breeding programs, that the accumulation of knowledge on genetic control in developed lines leads to genetic improvement through marker-assisted breeding. For breeding beyond the NERICA varieties, for upland conditions, it is necessary to understand the genetic control of critical traits among the 18 NERICAs. Additionally, although direct seeding of rice has been spreading into tropic regions (Pandey and Velasco 2002), 18 NERICAs have potential of gene source for direct seeding of rice in uplands, because they have been developed and selected with direct seeding in upland condition differing from many varieties that have been developed and selected with transplanting in lowland conditions. Roots are the sole organ for the uptake of water and nutrients from surrounding soil, owing to their direct contact with the soil; and it is widely known that root plasticity responds to numerous environmental factors, such as drought, nutrient concentration, and soil redox conditions (de Dorlodot *et al.* 2007, Lynch 1995). For example, the plasticity was found in direct seeding of rice (Kumar and Ladha 2011) indicating that root development are important

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traits in not only physiological process but also breeding targets. However, it is difficult to investigate such plasticity, because root development traits are complex, defined both by morphological traits such as elongation and distribution (de Dorlodot *et al.* 2007), and physiological traits such as sensing the status of water content and soil nutrient concentrations (Schachtman and Shin 2007). Additionally, unlike in the case of above-ground plant components, it is difficult to precisely determine root development and distribution in field conditions. To solve these problems, reliable methods have been developed to examine novel genetic effects of dissected root traits, such as elongation and distribution, in controllable conditions (Obara *et al.* 2010, Oyanagi *et al.* 1993, Tomita *et al.* 2017, Wang *et al.* 2013). Some quantitative trait loci (QTLs) concerned with root traits have been successfully detected and identified by these reliable methods. Further, it has been proposed that root improvements are potential trait targets for enhancing stable crop production through a second green evolution (Gewin 2010), after the 1960 discovery of the use of semi-dwarf genes to achieve higher cereal yields. In fact, two genes or allelic genes capable of improving the stability of rice production have been identified in the genetic background of the shallow-rooting IR64: *DEEPER ROOTING 1*, promoting downward root distribution, increased drought avoidance (Uga *et al.* 2013); and phosphorus-starvation tolerance 1, promoting root primordia development, enhanced phosphorus uptake activity (Gamuyao *et al.* 2012).

Nitrogen is the most essential nutrient for plant growth, including root development, and thus the form and concentration of nitrogen in soil greatly affects the dissected traits of root development, such as elongation. There are two forms of inorganic nitrogen available to incorporate into plants: NH_4^+ and NO_3^- (Yamaya and Oaks 2004). When *Arabidopsis thaliana* was grown with these different nitrogen forms as the sole nitrogen source, the QTLs for root elongation were detected at different chromosomal regions, indicating that the trait is regulated by specific genes, depending on the form of nitrogen (Rauh *et al.* 2002). Inhibition of root elongation is the general response to an increase in exogenous NH_4^+ concentration in rice (Tanaka *et al.* 1993). This dependence of root elongation on the form and concentration of nitrogen suggests that it is important to determine the character of and variation in varieties or ecotypes which have adapted to multiple environmental conditions such as water conditions and soil nutrient concentration. Our previous studies clearly revealed genetic variation in the maximum root length of seedlings, after the successful establishment of reliable methods for growing rice seedlings in hydroponic conditions, with a range of deficient to sufficient NH_4^+ concentrations (Obara *et al.* 2010, 2011, 2014). Furthermore, a number of QTLs for root elongation, identified by means of nearly-isogenic line (NIL) characterization, were categorized into two types based on their dependence on NH_4^+ concentrations. One type was an NH_4^+-constitutive QTL, including *qRL6.1* and *qRL6.4-YP5* on chromosome 6 (Obara *et al.* 2010, 2014); and the other was an NH_4^+-responsive QTL, *qRL1.1* on chromosome 1 (Obara *et al.* 2011). In this study, we aimed to characterize the genetic variation of 18 NERICAs based on the root development traits of seedlings grown hydroponically in deficient and sufficient nitrogen concentrations, with each of two forms of nitrogen (NH_4Cl and KNO_3) as the sole nitrogen source. Based on the variation between *O. sativa* 'WAB56-104' and 'NERICA7', a major QTL for root elongation (*qRL1.4-NERICA7*) was detected and identified as an NH_4^+-responsive QTL for root elongation on chromosome 1; and subsequent fine-mapping analysis narrowed down the QTL region to a 0.7 Mbp-long section.

Materials and Methods

Plant materials

A total of 18 NERICA varieties and their parents (Rodenburg *et al.* 2006) were used in this study. The NERICAs were introduced to Japan International Research Center for Agricultural Sciences in 2005 by the Japan International Cooperation Agency (Fukuta *et al.* 2012). They were used to evaluate the root development traits, including total root length (TRL), maximum root length (MRL), and root number (RN), of seedlings grown with different forms and concentrations of nitrogen; and to develop related genetic materials. The NERICA varieties were inbred to adapt to upland conditions by selecting progeny developed through repeated recurrent backcrossing between a line of *O. glaberrima* 'CG14' and three varieties of *O. sativa*: 'WAB56-104', 'WAB56-50', and 'WAB181-18' (Rodenburg *et al.* 2006). The recurrent parent was 'WAB56-104' in NERICAs 1 to 11, 'WAB56-50' in NERICAs 12 to 14, and 'WAB181-18' in NERICAs 15 to 18.

'WAB56-104' was crossed with 'NERICA7' as a pollen parent, and the resulting F_1 plant was grown in a paddy field to obtain F_2 plants for detection of QTLs associated with the TRL, MRL, and RN of seedlings grown in 500 μM NH_4Cl conditions. The other F_2 plants were self-pollinated to obtain F_5 plants, using single descent methods. A total of 90 F_6 lines originating from an F_5 plant with marker segregation at the candidate region of the target QTL were self-pollinated to obtain an F_7 line, in order to characterize and finely map the target QTL.

Plant growth in hydroponic conditions

Seedlings were hydroponically grown for 8 days in a greenhouse maintained at 28°C under natural day conditions. Well-filled seeds were germinated at 30°C for 2 days after two-step seed stabilization, by soaking the seeds for 10 min in water at 60°C and then for 20 min in a 2% (v/v) sodium hydrochloride solution, as described in Obara *et al.* (2011, 2014). Germinated seeds were sown on four nylon nets (160 × 220 mm) floated on 40 L of nutrient solution. The same basal nutrient solution as in Obara *et al.* (2010), containing 5 mM 2-(N-morpholino)ethanesulfonic acid,

was employed, but in this case without nitrogen. To characterize the genetic variations depending on the form and concentration of nitrogen, seedlings were grown with 5 or 500 μM NH_4Cl or KNO_3 as the sole nitrogen source. Approximately 80 ml of these respective nutrient solutions were fed to the individual seedlings on a two-day cycle. The pH of the nutrient solutions was maintained at around 5.3 to 5.6 during the period of plant growth.

Evaluation of root development traits

Three root development traits (TRL, MRL, and RN) were evaluated in all the experiments except for the fine-mapping analyses. We evaluated only the MRL of seedlings, to finely map the target QTL. After evaluating the MRL of 8-day-old seedlings using a ruler, seedlings were stored in plastic bags at –28°C for measurement of the length of individual roots and the root count (Obara *et al.* 2014). At least duplicate analyses were performed in all the experiments except for the QTL analyses.

Genetic variation among NERICA varieties, based on the root development traits of seedlings grown with different forms and concentrations of nitrogen

The TRL, MRL, and RN of 22 varieties (18 NERICAs and four parental varieties) were evaluated using 8-day-old seedlings grown with 5 or 500 μM NH_4Cl or KNO_3. The value of 12 traits in each variety were used for cluster analysis by Ward's hierarchical analysis (Ward 1963) using JMP7.0 (SAS Institute Inc., USA). A comparison of differences in all the traits was performed among the cluster groups, under the same nitrogen conditions, to characterize the respective groups.

Genotypic analyses

DNA was extracted by precipitation with isopropanol, as in Obara *et al.* (2004, 2014). A total of 833 simple sequence repeat (SSR) markers (International Rice Genome sequencing Project 2005, McCouch *et al.* 2002) were employed to detect polymorphism between 'WAB56-104' and 'NERICA7' across the whole genome (**Supplemental Table 1**). The amplification of specific fragments by polymerase chain reaction with Quick Taq HS DyeMix (TOYOBO, Osaka, Japan), separation of the fragments by electrophoresis, and detection of the fragments stained with ethidium bromide were all as in Obara *et al.* (2014).

QTL analyses of TRL, MRL, and RN

A total of 184 F_2 plants developed from a cross between 'WAB56-104' and 'NERICA7' were employed to detect QTLs affecting the TRL, MRL, or RN of seedlings grown in 500 μM NH_4^+ conditions. A probability of less than 0.01% was used to detect putative QTLs by single marker analysis, using Windows QTL Cartographer v. 2.5 (Wang *et al.* 2012). The genotypic data of individual F_2 seedlings were determined, for single marker analysis, at the loci of 57 SSR markers which showed distinct polymorphism between parental varieties under the study conditions. The position of each SSR marker was determined through *in silico* mapping with basic local alignment search tool investigation of the primer sequences or the SSR flanking sequences on the genomic sequence of *O. sativa* 'Nipponbare' [International Rice Genome Sequencing Project (IRGSP)-1.0], based on the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/).

Progeny test

A total of 44 F_7 plants were used for progeny testing to characterize and finely map the target QTL. To characterize the target QTL based on the responses of root traits to changes in the form and/or concentration of nitrogen, an F_7 line with marker segregation at the candidate region of the target QTL was grown for 8 days in 5 or 500 μM NH_4Cl or KNO_3 as the sole nitrogen source. To evaluate the MRL, appropriate F_7 lines occurring through recombination between flanking markers of the target QTL were grown for 8 days in 500 μM NH_4Cl. On the final day, part of a leaf blade tip was harvested to extract DNA from the individual seedlings, and genotypic analysis was performed with appropriate DNA markers.

Statistical analyses

In order to verify whether the study's hydroponic growth conditions for irrigated varieties were suitable for upland varieties, the broad-sense heritability (h_b^2) of each trait of the 18 NERICAs was estimated using one-way analysis of variance (ANOVA) with the following formula, $h_b^2 = \sigma^2_g / \{(\sigma^2_e/r) + \sigma^2_g\}$, where σ^2_g is the genetic variance, σ^2_e is the environmental variance, and r is the number of data items employed (Kobayashi and Koyama 2002). Differences in the mean value between two lines were statistically analyzed by ANOVA, and among multiple lines by Tukey's test.

Results

Genetic variation in root development traits among 18 NERICAs

First, h_b^2, which is the ratio of total genetic variance to total phenotypic variance, was estimated for the TRL, MRL, and RN of the 18 NERICAs varieties, under the specified conditions. The representative h_b^2 values for TRL, MRL, and RN, for all combinations of form and concentration of nitrogen, are shown in **Supplemental Table 2**. The h_b^2 value is greater than 0.950 for TRL, 0.994 for MRL, and 0.908 for RN, under all conditions. These results indicate strongly that the study's growth conditions were reliable for evaluating these traits, for upland as well as lowland rice varieties.

The TRL of the donor parent ('CG14') was significantly greater than that of recurrent parents ('WAB56-104', 'WAB56-50', and 'WAB181-18') for all nitrogen applications (**Fig. 1A**). A wide distribution was observed in the TRL of the 18 NERICAs for all nitrogen applications, ranging from 294 to 468 mm for 5 μM NH_4Cl, 304 to 563 mm

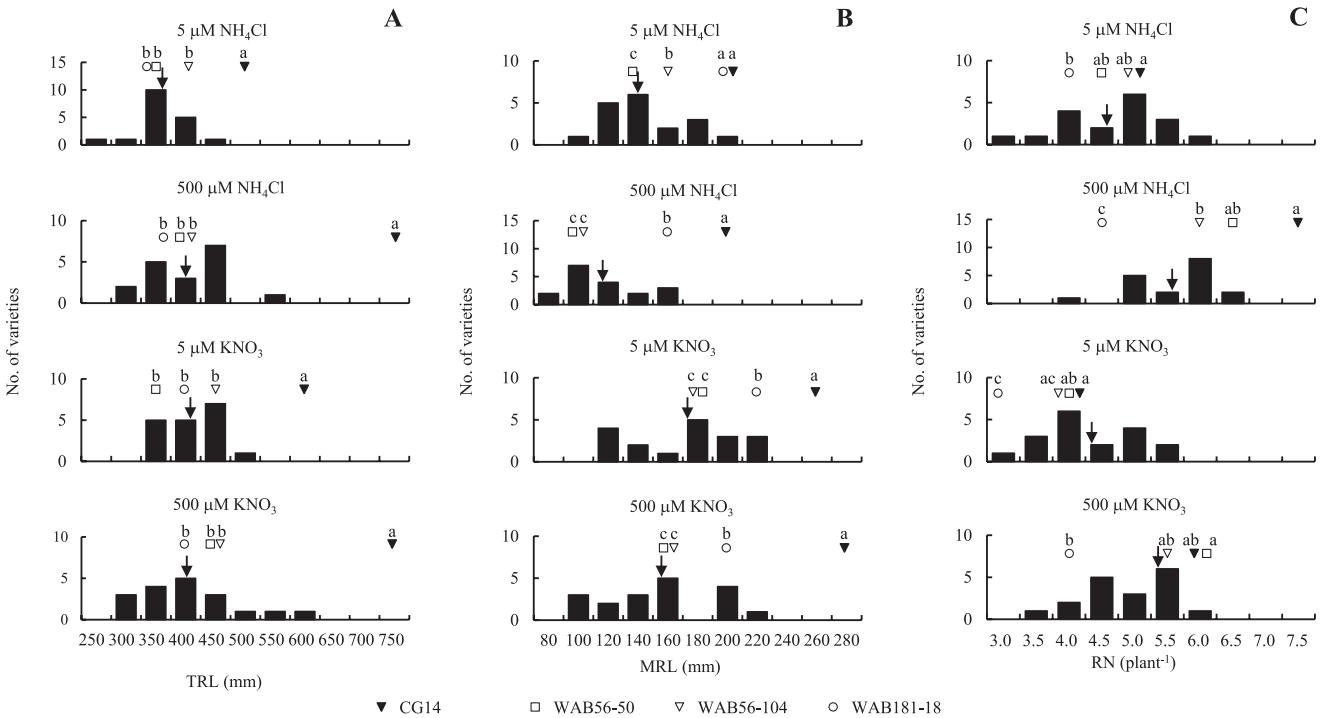

Fig. 1. Frequency distribution of the root development traits of 18 NERICAs, compared with their parental varieties. Arrows indicate mean value of 18 NERICAs. Root development trait is dissected into three traits: TRL (A), MRL (B) and RN (C). Letters above the symbols indicate significant differences among multiple applications (Tukey's test, $P < 0.05$).

for 500 μM NH_4Cl, 354 to 500 mm for 5 μM KNO_3, and 318 to 622 mm for 500 μM KNO_3. Similarly, larger MRL values were also observed in the case of 'CG14' for almost all nitrogen applications (**Fig. 1B**). The MRL of the 18 NERICAs showed a wide distribution, ranging from 113 to 201 mm for 5 μM NH_4Cl, 99 to 169 mm for 500 μM NH_4Cl, 125 to 230 mm for 5 μM KNO_3, and 110 to 222 mm for 500 μM KNO_3. Significant differences were observed in the RN for 'CG14' versus 'WAB56-104' in 500 μM NH_4Cl, and 'WAB181-18' in all conditions except 500 μM KNO_3 (**Fig. 1C**). The RN of the 18 NERICAs was widely distributed, ranging from 3.4 to 6.2 for 5 μM NH_4Cl, 4.4 to 6.6 for 500 μM NH_4Cl, 3.4 to 5.8 for 5 μM KNO_3, and 3.8 to 6.0 for 500 μM KNO_3.

Classification of 18 NERICAs based on the responses of root development traits to changes in the form and concentration of nitrogen

A total of 22 varieties (18 NERICAs and their parental varieties) were classified into three clusters (Ia, Ib and II), based on the responses of root development traits to changes in the form and/or concentration of nitrogen (**Fig. 2**). Cluster Ia included only 'CG14'. Cluster Ib included one recurrent parent, 'WAB181-18', and 6 NERICAs: 1, 6, 7, 8, 9, and 11. The remaining 14 varieties were classified into cluster II, which included two recurrent parents, 'WAB56-104' and 'WAB56-50', and 12 NERICAs. Compared to cluster Ib, cluster Ia showed longer TRL for all applications, longer MRL at a 500 μM concentration of both nitrogen forms,

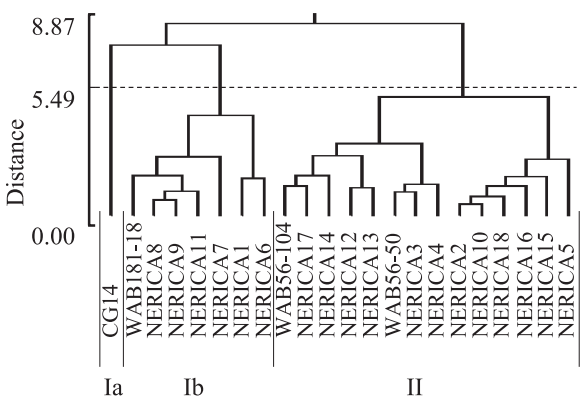

Fig. 2. Classification of 18 NERICAs and their parental varieties based on root development traits. Seedlings were grown for 8 days under four different nitrogen conditions (5 μM NH_4Cl, 500 μM NH_4Cl, 5 μM KNO_3, and 500 μM KNO_3) to evaluate three traits: TRL, MRL, and RN. These values were used for cluster analysis by Ward's hierarchical analysis (Ward 1963) using with JMP7.0 (SAS Institute Inc., USA).

and greater RN at both concentrations of NH_4^+ (**Table 1**). Compared to cluster II, cluster 1a showed longer TRL and MRL for all applications, and greater RN at 500 μM NH_4Cl. Cluster Ib showed longer MRL and lesser RN compared to cluster II. These results indicate that genetic factor(s) for root elongation and root primordia development were introduced from 'CG14' into NERICAs.

Table 1. Variation in root development traits of 8-day-old seedlings grown with deficient and sufficient concentrations of two forms of inorganic nitrogen, among three cluster groups comprising 18 NERICAs and four parental varieties

Trait	Cluster group	No. of varieties	Nitrogen treatment			
			5 μM NH_4Cl	500 μM NH_4Cl	5 μM KNO_3	500 μM KNO_3
TRL	Ia	1	539.6 a	764.4 a	637.8 a	796.6 a
	Ib	7	377.5 b	439.9 b	420.0 b	471.5 b
	II	14	392.2 b	410.8 b	434.4 b	400.4 b
MRL	Ia	1	203.8 a	210.2 a	260.0 a	283.2 a
	Ib	7	183.6 a	157.8 b	210.3 a	201.5 b
	II	14	141.7 b	110.3 c	172.0 b	148.5 c
RN	Ia	1	5.4 a	7.6 a	4.2 ab	6.0 ab
	Ib	7	4.1 b	5.1 c	3.8 a	4.6 a
	II	14	5.2 a	6.2 b	4.7 b	5.4 b

Different letters indicate significant differences among cluster groups under the same nitrogen conditions (Tukey's test, $P < 0.05$).
TRL: total root length, MRL: maximum root length, RN; root number.

Characterization of root development traits in 'NERICA7'

Among the 18 NERICAs, 'NERICA7', belonging to cluster Ib, was selected as the donor for detection of QTLs for root development traits because 'NERICA7' is one of the long-rooted varieties in cluster Ib. The respective TRLs of 'NERICA7' were significantly (33.6% and 26.3%) greater than those of 'WAB56-104' in 500 μM NH_4Cl and KNO_3 (**Fig. 3A**). There were no significant differences in TRL between 'WAB56-104' and 'NERICA7' in 5 μM NH_4Cl or KNO_3. Compared to the respective nitrogen-deficient conditions, the TRL of 'NERICA7' significantly increased in NH_4^+ (47.8%) and NO_3^- (29.0%) in nitrogen-sufficient condition. No such increases in TRL were observed in the case of 'WAB56-104'. A significant increase in TRL in NO_3^- versus NH_4^+ was only found in 'NERICA7' grown in 5 μM conditions. The MRL of 'NERICA7' was significantly greater than that of 'WAB56-104' in almost all conditions except 5 μM NH_4Cl (**Fig. 3B**). The length of the longest root of 'NERICA7' increased by 58.3%, 19.8%, and 37.5% in 500 μM NH_4Cl, 5 μM KNO_3, and 500 μM KNO_3, respectively, compared with 'WAB56-104'. In both forms of nitrogen, inhibition of elongation of the longest root in a dose-dependent manner was significant in 'WAB56-104' but not in 'NERICA7'. A significant increase in the MRL in NO_3^- versus NH_4^+ was found in 'WAB56-104' grown in 5 μM condition and 'NERICA7' grown in both concentrations, respectively. There was no significant difference in RN between 'WAB56-104' and 'NERICA7' in all tested conditions (**Fig. 3C**). In 'NERICA7', the increase in RN in a dose-dependent manner was significant only in NH_4Cl conditions.

Distribution of root development traits in F_2 seedlings

A total of 184 F_2 plants developed from a cross between 'WAB56-104' and 'NERICA7' were grown in 500 μM NH_4Cl conditions, because the greatest difference between 'WAB56-104' and 'NERICA7' was observed in the TRL and MRL of seedlings grown in 500 μM NH_4Cl among all the tested conditions (**Fig. 3**). In the 184 F_2 seedlings grown in 500 μM NH_4Cl conditions, all the root development traits showed transgressive distributions, either in the negative direction (some F_2 trait values less than those of the parent; open triangle), or in the positive direction (some F_2 trait values greater than those of the parent; closed triangle) (**Fig. 4**). The F_2 populations showed a wide continuous distribution ranging from 235 to 634 mm in TRL (**Fig. 4A**), 88 to 171 mm in MRL (**Fig. 4B**), and 3.0 to 7.0 in RN (**Fig. 4C**).

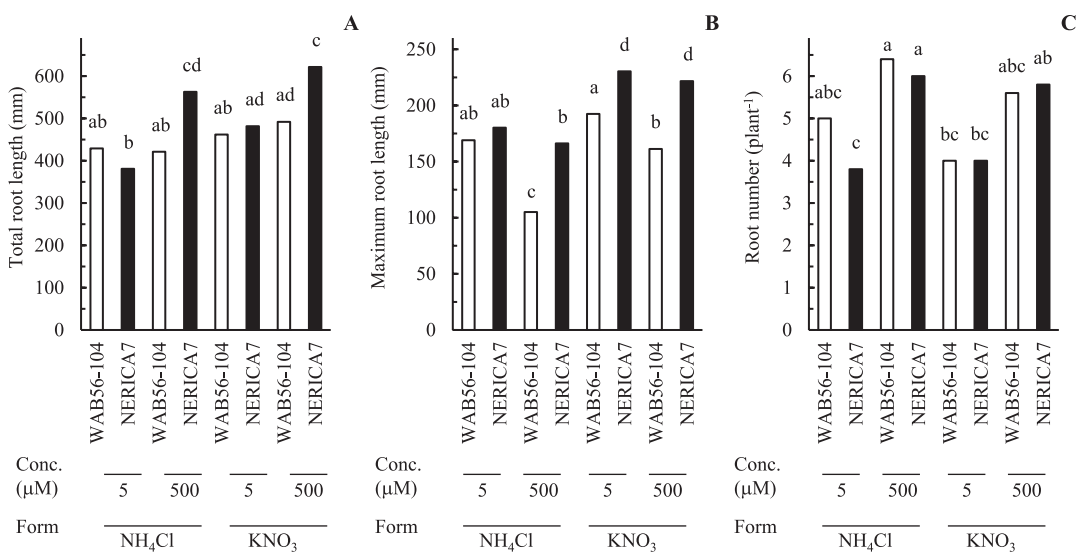

Fig. 3. Responses of root development traits in 'WAB56-104' and 'NERICA7' to different forms and concentrations of nitrogen. Seedlings were hydroponically grown with 5 or 500 μM of NH_4Cl or KNO_3. Columns represent mean values (n = 5) of TRL (A), MRL (B), and RN (C). Letters above the columns indicate significant differences among multiple applications (Tukey's test, $P < 0.05$).

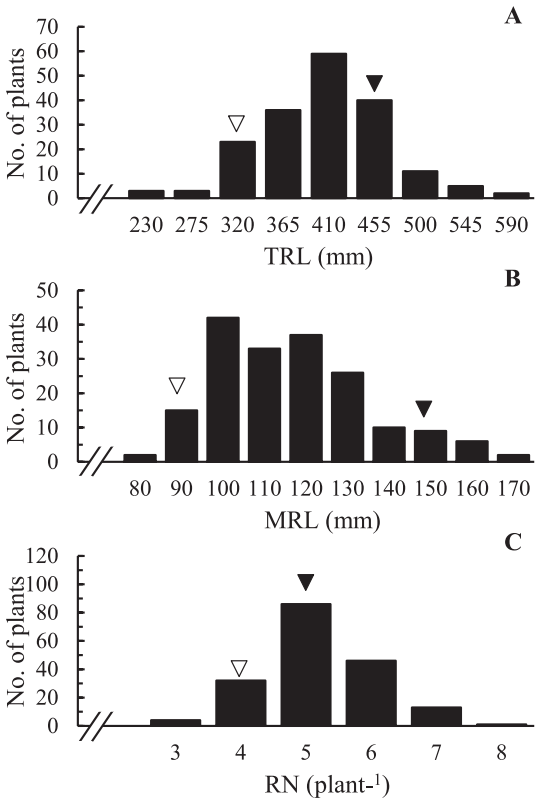

Fig. 4. Frequency distribution of the TRL (A), MRL (B), and RN (C) of 182 F_2 plants developed from a cross between 'WAB56-104' and 'NERICA7'. Seedlings were hydroponically grown for 8 days with 500 NH_4Cl. Open triangle indicates a mean-value group for 'WAB56-104'. Closed triangle indicates a mean-value group for 'NERICA7'.

Detection of QTLs affecting root development traits under 500 μM NH_4Cl conditions

Among 833 SSR markers tested, 57 showed distinct polymorphism between 'WAB56-104' and 'NERICA7' under the study conditions (**Supplemental Table 1**). A graphical genotype of 'NERICA7' is shown in **Fig. 5**. Introgression of chromosomal segments was found in almost all chromosomes except for chromosome 9 (**Fig. 5**).

Based on single marker analysis, significant markers were detected only for MRL, not TRL or RN (**Fig. 5**, **Table 2**). A total of two putative QTLs associated with MRL were detected on chromosome 1, and respectively

Table 2. Putative QTLs affecting the MRL of 8-day-old seedlings grown in hydroponic conditions with 500 μM NH_4Cl as the sole nitrogen source

QTL	Chr.	Position (Mbp)[a]	Marker[b]	*F* score	Additive effect[c]	R^2 (%)
qRL1.3-NERICA7	1	6.27	RM8111	25.3	–8.7	12.7
		7.41	RM10464	32.1	–9.5	15.6
qRL1.4-NERICA7	1	31.95	RM3709	42.6	–11.7	19.0
		32.99	RM302	41.2	–11.9	19.1
		34.55	RM5501	43.0	–12.2	20.5

[a] Physical position: markers were identified at the positions of corresponding primers in IRGSP-1.0.
[b] Indicates marker associated with the trait. A probability of less than 0.01% was used to detect significant difference between the genotypes of 'NERICA7' and 'WAB56-104'.
[c] Negative value indicates an increase in the trait (unit of length: mm). MRL: maximum root length.

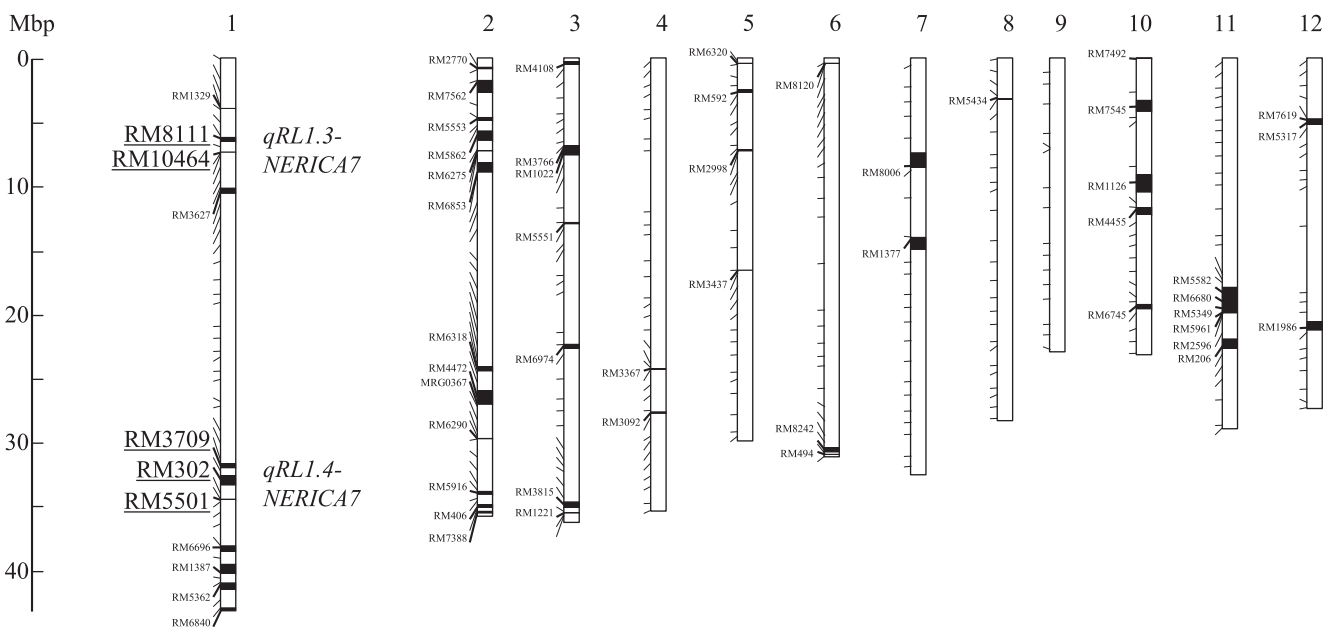

Fig. 5. Graphical genotype of 'NERICA7', and map position of two putative QTLs affecting MRL. Open bars indicate chromosomes based on a physical map of the 'Nipponbare' genome sequence. Closed columns represent regions differing from 'WAB56-104' segments. Straight and oblique lines on the left of chromosomes represent SSR marker positions used in the study. Specific SSR markers on the left indicate polymorphic markers between 'WAB56-104' and 'NERICA7' under the study conditions. Underlined SSR markers indicate linked markers of putative QTLs, which are represented on the right side of chromosome 1.

Table 3. Verification of the effect of *qRL1.4-NERICA7* on the MRL of 8-day-old seedlings through progeny testing using F_7 plants

Nitrogen form	Conc. (μM)	Genotype[a]	No. of plants	TRL (mm)	MRL[b] (mm)	RN (plant^{-1})
NH_4Cl	5	WAB56-104	13	497.3 ± 46.6	230.8 ± 11.3	4.5 ± 0.6
		NERICA7	10	483.4 ± 31.9	242.5 ± 13.2	4.2 ± 0.4
	500	WAB56-104	10	404.4 ± 45.0	175.1 ± 12.4	4.3 ± 0.6
		NERICA7	7	443.9 ± 52.8	203.9 ± 12.2**	4.4 ± 0.9
KNO_3	5	WAB56-104	11	484.0 ± 24.7	254.8 ± 10.6	3.6 ± 0.5
		NERICA7	13	482.1 ± 30.0	263.2 ± 12.0	3.5 ± 0.6
	500	WAB56-104	9	506.9 ± 30.3	250.0 ± 17.4	4.4 ± 0.4
		NERICA7	11	510.6 ± 38.7	258.9 ± 9.2	4.1 ± 0.3

[a] Genotypes in the candidate region of *qRL1.4-NERICA7* were determined using three DNA markers: RM3709, RM302 and RM5501.
[b] Asterisks indicate significant difference in the trait in genotypes between 'WAB56-104' and 'NERICA7' (ANOVA, $P < 0.01$).
TRL: total root length, MRL: maximum root length, RN; root number.

designated as *qRL1.3-NERICA7* linked to RM811 and RM10464, and *qRL1.4-NERICA7* linked to RM3709, RM302, and RM5501. 'NERICA7' allele increased root elongation at each QTL (**Table 2**). In terms of phenotypic variance, 15.6% was explained by *qRL1.3-NERICA7*, as determined by the R^2 score of the higher *F*-score marker, RM10464, and 20.5% was explained by *qRL1.4-NERICA7*, as determined by the R^2 score of the higher *F*-score marker, RM5501.

Verification and characterization of qRL1.4-NERICA7 by progeny testing

F_7 plants with marker segregation at the candidate region of *qRL1.4-NERICA7* were subjected to progeny testing to verify and characterize *qRL1.4-NERICA7*, which showed higher phenotypic variance compared to *qRL1.3-NERICA7*. The MRL of homozygous F_7 seedlings for 'NERICA7' at RM3709, RM302, and RM5501 was significantly (16.4%) higher than that of homozygous F_7 seedlings for 'WAB56-104' at these markers, in 500 μM NH_4Cl (**Table 3**); whereas, there was no such significant increase in the case of the other nitrogen applications. No significant difference was observed in the TRL or RN, under any test conditions, between homozygous F_7 seedlings for 'WAB56-104' and 'NERICA7' at these markers. Thus, the presence of *qRL1.4-NERICA7* was confirmed in the vicinity of RM3709, RM302, and RM5501. Similar tendencies were observed in two spatial replications.

Mapping qRL1.4-NERICA7 through progeny testing

In seven F_6 plants carried recombinant points around the flanking markers (designated as R1 to R7) of the 90 F_6 plants, recombination occurred between the flanking markers, RM3709 and RM5501, of the candidate region for *qRL1.4-NERICA7* (**Fig. 6**). Three new markers (RM11725, RM11744, and RM 11772) showing distinct polymorphism between 'WAB56-104' and 'NERICA7' were established between the candidate regions. Progeny testing for the MRL of their self-pollinated F_7 seedlings grown with 500 μM NH_4Cl was conducted, with two replications. One line (H1), of which the genotype was heterozygous at the candidate region, was used to verify the progeny test for segregation of MRL by *qRL1.4-NERICA7*.

Progeny testing of H1 showed MRL segregation between homozygous 'WAB56-104' and homozygous 'NERICA7' seedlings at the candidate region of *qRL1.4-NERICA7*, indicating that a genotype of *qRL1.4-NERICA7* is heterozygous in the H1 line of F_6 plant. The segregation of MRL was observed in five lines (R1, R2, R3, R4, and R7), with the remaining two lines (R5 and R6) showing no such segregation. Consequently, *qRL1.4-NERICA7* was mapped in the interval between RM11725 and RM11744, corresponding to a 0.7-Mbp region, based on the IRGSP-1.0 rice genome assembly. A recombination event, R1, was identified at the flanking marker, RM11725; and two events, R2 and R3, were identified at the flanking marker, RM11744.

Discussion

Genetic variations in root development traits (TRL, MRL, and RN) were clearly detected among 18 NERICA seedlings grown in hydroponic solution, with deficient or sufficient concentration of NH_4Cl or KNO_3 as the sole nitrogen source. To our knowledge, this is the first study to categorize the 18 NERICAs into two clusters, with cluster Ib characterized as an active root elongation variety, and cluster II as active root primordia development variety.

Various genetic factors would be relevant to the root development traits of the 18 NERICAs grown with different forms and concentrations of nitrogen. Wide distributions in all tested traits (TRL, MRL, and RN) were observed among the 18 NERICAs in this study (**Fig. 1A–1C**), and it was revealed that such distributions depended on the form and concentration of nitrogen. For example, a different distribution was clearly detected in the MRL of seedlings grown in NH_4Cl condition for nitrogen concentration, and in 500 μM condition for nitrogen form (**Fig. 1B**). Such MRL distribution dependence has, in fact, been previously reported with respect to *O. sativa* and *O. glaberrima* grown in hydroponic conditions (Obara *et al.* 2010, 2011, 2014, Ogawa *et al.* 2014). In these reports, two types of QTL (constitutive and NH_4^+-responsive) were detected through QTL analysis and NIL characterization; and the reports strongly support the supposition that various genetic factors were introduced

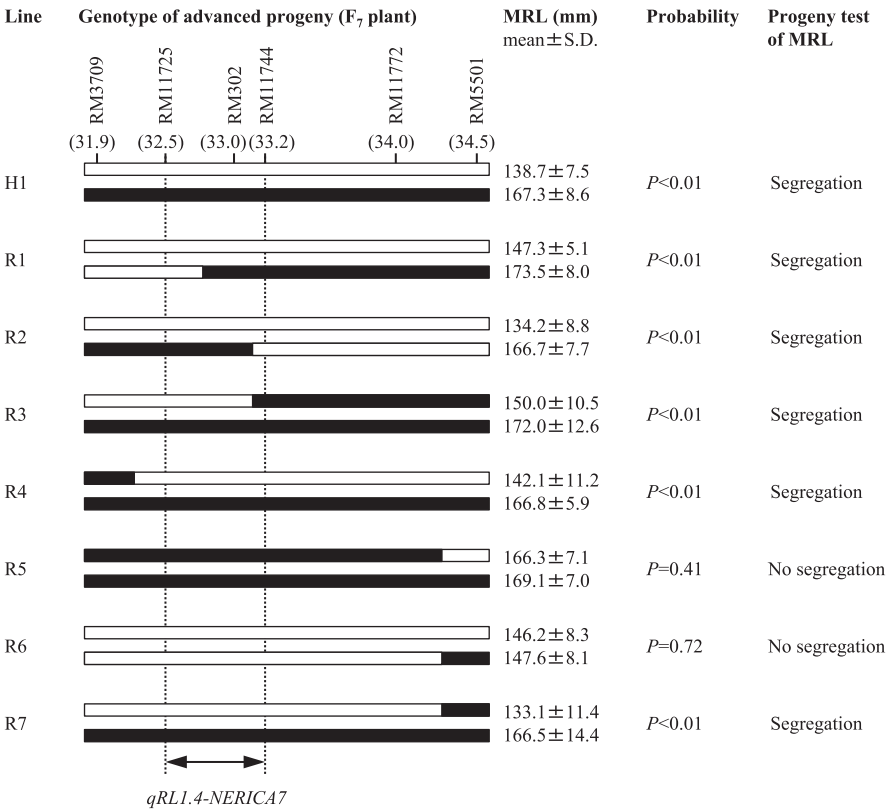

Fig. 6. Mapping of *qRL1.4-NERICA7*. Horizontal bars indicate the chromosome components locating *qRL1.4-NERICA7* in each F_7 line. Polymorphic markers between 'WAB56-104' and 'NERICA7' in the region are represented at the physical positions indicated in the parentheses. Region in bas indicate opened regions for homozygous segment of 'WAB56-104' and closed regions for homozygous segment of 'NERICA7'. Dotted vertical lines indicate flanking markers for *qRL1.4-NERICA7*. The double arrow indicates the region of *qRL1.4-NERICA7*.

into the 18 NERICAs in the present study. In this study, a total of 22 varieties, including 18 NERICAs and four parental varieties, were classified into three clusters based on cluster analysis of TRL, MRL, and RN with changes in the form and concentration of nitrogen (**Fig. 2**). Cluster Ia included only 'CG14'. Cluster Ib consisted of 7 varieties (NERICAs 1, 6, 7, 8, 9, and 11) and a recurrent parent ('WAB181-18'), and was characterized by longer MRL and lesser RN than the varieties in cluster II (**Table 1**). Cluster II consisted of 14 varieties, including the remaining 12 NERICAs and two recurrent parents ('WAB56-104' and 'WAB56-50'), and was characterized by shorter MRL and greater RN under all test conditions (**Table 1**). These results indicate that genetic factor(s) originating from 'CG14' are introduced into the NERICAs, promoting root elongation in cluster Ib varieties, and root primordia development in cluster II varieties.

Insertion of 'CG14' segments into individual NERICAs would result in variation in tested root development traits. Genetic variation in the pattern of root plasticity has been reported in the case of two NERICAs, 'NERICA1' and 'NERICA4', under moderate drought conditions (Menge *et al.* 2016). 'NERICA4', with greater deep root development, maintained dry matter production under these soil conditions, and the roots had little difficulty penetrating into the deeper layers; whereas, 'NERICA1', with greater lateral root development at shallow layers showed the highest shoot dry weight under these soil conditions, and the roots had difficulty penetrating to the deeper layers. Recently, the root angle distribution among 97 accessions, including 18 NERICAs and their four parental varieties, was evaluated in detail, and the genetic variation characterized, based on effective new methods for evaluating the distribution of individual roots (Tomita *et al.* 2017). A wide distribution was observed in the root vertical angle, which is one of the indicators for root angle distribution among the 18 NERICAs. Our present finding of wide genetic variation in TRL, MRL, and RN, among the 18 NERICAs, agreed with previous reports of genetic variation in root traits (Menge *et al.* 2016, Tomita *et al.* 2017), although the relevant genetic factors, such as QTLs, have not yet been fully identified. Using 243 SSR markers, Fukuta *et al.* (2012) demonstrated that inserted loci originating from 'CG14' and unknown parents ranged from 1.2% to 10.7%. Association analysis of the 18 NERICAs, using these polymorphic markers, enabled the detection of candidate regions for tested agronomic traits (Fukuta *et al.* 2012). However, no candidate regions for root development traits evaluated in the present study were detected in association analysis using the same genotypic data (data not shown).

We developed advanced progenies from a cross between 'WAB56-104' and 'NERICA7', to detect and verify relevant genetic factors. 'NERICA7' is one of the long-rooted varieties classified into cluster Ib, and 'WAB56-104', belonging to cluster II, is a recurrent parent for developing 'NERICA7'. The increase in the TRL of 'NERICA7' grown in two conditions, 500 μM NH_4Cl and KNO_3, was thought to be due to an increase in the root elongation of individual roots, because a significant difference between 'WAB56-104' and 'NERICA7' was only observed in MRL and not RN (**Fig. 3**). Thus, active root elongation (as expressed by MRL), rather than root primordia development (as expressed by RN), was the major factor in the greater TRL of 'NERICA7'.

The progenies developed from a cross between 'WAB56-104' and 'NERICA7' would be effective for identifying MRL-related QTLs responsive to changes in NH_4^+ concentration. A significant reduction in MRL was observed in 'WAB56-104' (but not 'NERICA7') in response to the increase in NH_4Cl concentration from 5 μM to 500 μM (**Fig. 3B**). Very few varieties have shown no reduction in seedling MRL in response to an increase in NH_4Cl as the sole nitrogen source; these include *O. sativa*, *O. glaberrima*, and *O. rufipogon* (Obara *et al.* 2011, Ogawa *et al.* 2014). The results of the present study suggested that 'NERICA7' was one of the rare varieties that have at least one segment capable of nullifying reduction in root elongation in response to an increase in NH_4^+ concentration. These results strongly support the inference that 500 μM NH_4Cl conditions offer a strong candidate for identifying MRL-related QTLs, in order to understand the genetic control in 'NERICA7'.

We detected two putative QTLs for MRL on chromosome 1, through QTL analysis using F_2 plants developed from a cross between 'WAB56-104' and 'NERICA7', under 500 μM NH_4Cl conditions (**Table 2**). One, detected at a region from 6.27 Mbp to 7.41 Mbp, was designated as *qRL1.3-NERICA7*; and the other, detected at a region from 31.95 Mbp to 34.55 Mbp, was designated as *qRL1.4-NERICA7*. QTL analysis of F_2 plants revealed that the effect of *qRL1.4-NERICA7* was greater than that of *qRL1.3-NERICA7*, and the positive allele in both regions is 'NERICA7', suggesting that *qRL1.4-NERICA7* is a major genetic factor for root elongation, at least in 500 μM NH_4Cl conditions. Thus, *qRL1.4-NERICA7* offers the first target for characterizing genetic control in NERICAs.

qRL1.4-NERICA7 would offer an NH_4^+-responsive QTL for root elongation in the genetic background of 'WAB56-104'. Verification progeny testing clearly revealed that the MRL of homozygous F_7 seedlings for 'NERICA7' at the candidate region of *qRL1.4-NERICA7* was significantly greater than that of homozygous F_7 seedlings for 'WAB56-104', in 500 μM NH_4Cl (**Table 3**). These results imply that root elongation in rice is controlled by different loci, depending on the form and concentration of nitrogen. In previous reports, a number of NH_4^+-responsive QTLs for root elongation were detected in the vicinity of *qRL1.4-NERICA7* (Obara *et al.* 2010, 2011, Ogawa *et al.* 2014). Among them, *qRL1.1* was also detected as an NH_4^+-responsive QTL for root elongation in mapping population developed from a cross between 'Taichung 65' (*O. sativa*) and 'IRGC 104038' (*O. glaberrima*), and its nature was confirmed through NIL characterization (Obara *et al.* 2011). Interestingly, an NH_4^+-responsive QTL for root elongation was detected in the same region in mapping population developed from a cross between 'Curinga' (*O. sativa*) and 'IRGC 105491' (*O. rufipogon*) (Ogawa *et al.* 2014). In both QTLs, segments coming from non-*O. sativa* varieties enhanced root elongation in the genetic background of *O. sativa*, in 500 μM NH_4Cl. This coincidence in the region of detection indicates the possibility that these three QTLs are allelic with respect to NH_4^+-responsive root elongation, across rice species. However, the present study could not determine whether the three QTLs for NH_4^+-responsive root elongation were allelic, because, though the respective candidate regions overlapped, the common region contained a variety of different genes. Our fine-mapping analysis narrowed the region for *qRL1.4-NERICA7* down to a 0.7-Mbp section (**Fig. 6**). Further analysis to identify the *qRL1.4-NERICA7* gene will aid in revealing allelic variation in the relevant QTLs, across rice species. It will be necessary for understanding genetic control in 'NERICA7', to characterize the response of *qRL1.3-NERICA7* (the putative QTL for root elongation in association analysis using F_2 populations) to different forms and concentrations of nitrogen.

In conclusion, 18 NERICA varieties were characterized and categorized into two cluster groups, one for active root elongation ('NERICA7') and the other for active primordia development ('WAB56-104'), based on genetic variation in the root development traits (TRL, MRL, and RN) of seedlings grown in hydroponic conditions. *qRL1.4-NERICA7*, detected in an F_2 mapping population developed from a cross between 'WAB56-104' and 'NERICA7', was characterized as an NH_4^+-responsive QTL for root elongation and narrowed down to a 0.7-Mb region on chromosome 1 through progeny testing using F_7 lines. *qRL1.4-NERICA7* should help us understand genetic control in NERICAs, and improve root elongation in rice breeding programs. Further analysis, to identify and characterize QTLs for root development traits under NO_3^- conditions, will be advanced to understand genetic control in NERICA and apply detected QTLs in breeding. Recombinant inbred lines originating from two NERICAs, one for 'NERICA7' (as a representative variety for active root elongation) and the other for 'NERICA4' (as a representative variety for active primordia development), will contribute to better understanding of genetic control in NERICAs, and improvement in precision rice breeding programs. Development of these genetic materials is ongoing.

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