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ORIGINAL PAPER

Fine-mapping of *qRL6.1*, a major QTL for root length of rice seedlings grown under a wide range of NH_4^+ concentrations in hydroponic conditions

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Abstract Root system development is an important target for improving yield in cereal crops. Active root systems that can take up nutrients more efficiently are essential for enhancing grain yield. In this study, we attempted to identify quantitative trait loci (QTL) involved in root system development by measuring root length of rice seedlings grown in hydroponic culture. Reliable growth conditions for estimating the root length were first established to renew nutrient solutions daily and supply NH_4^+ as a single nitrogen source. Thirty-eight chromosome segment substitution lines derived from a cross between 'Koshihikari', a *japonica* variety, and 'Kasalath', an *indica* variety, were used to detect QTL for seminal root length of seedlings grown in 5 or 500 μM NH_4^+. Eight chromosomal regions were found to be involved in root elongation. Among them, the most effective QTL was detected on a 'Kasalath' segment of SL-218, which was localized to the long-arm of chromosome 6. The 'Kasalath' allele at this QTL, *qRL6.1*, greatly promoted root elongation under all NH_4^+ concentrations tested. The genetic effect of this QTL was confirmed by analysis of the near-isogenic line (NIL) *qRL6.1*. The seminal root length of the NIL was 13.5–21.1% longer than that of 'Koshihikari' under different NH_4^+ concentrations. Toward our goal of applying *qRL6.1* in a molecular breeding program to enhance rice yield, a candidate genomic region of *qRL6.1* was delimited within a 337 kb region in the 'Nipponbare' genome by means of progeny testing of F_2 plants/F_3 lines derived from a cross between SL-218 and 'Koshihikari'.

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Abbreviations

CSSL	Chromosome segment substitution lines
LOD	Logarithm (base 10) of the odds
MES	2-(*N*-morpholino)ethanesulfonic acid
NIL	Near-isogenic lines
PCR	Polymerase chain reaction
QTL	Quantitative trait loci/locus
RAP-DB	Rice Annotation Project Database
RGP	Rice Genome Research Program
RSA	Root system architecture

Introduction

Many important agronomic traits are controlled by the complex interaction of multiple genes and by a possibly more complex regulation of gene function. Additionally, most agricultural traits, such as heading day or yield, are influenced by many environmental factors. Quantitative trait locus (QTL) analysis is one of the most efficient ways to identify loci or genes for quantitative traits (Salvi and Tuberosa 2005; Yamamoto et al. 2009; Yano 2001). Recently, many QTL genes associated with yield-related traits have been identified by a positional cloning strategy in rice; for example, grain number (Ashikari et al. 2005), grain size (Fan et al. 2006; Huang et al. 2009; Shomura et al. 2008; Song et al. 2007; Weng et al. 2008), or heading day (Doi et al. 2004; Kojima et al. 2002; Takahashi et al. 2001; Yano et al. 2000; Xue et al. 2008). Many QTL controlling root morphological and physiological traits have been identified during the last decade. Recently, Courtois et al. (2009) summarized root QTL consisting of 29 traits and performed a meta-QTL analysis from a drought QTL database. This database will benefit the molecular identification of QTL or putative candidate genes for root traits; however, no QTL genes for root traits have been cloned from rice. Molecular identification of genes controlling root traits will contribute to discovery of new functional alleles and to marker-assisted selection by the introgression of target genes (Collins et al. 2008). Furthermore, such an achievement could be exploited in pyramid breeding by combining with other useful genes to improve rice grain yield (Ashikari et al. 2005).

Plant roots are important organs for water and nutrient uptake from the surrounding soil. Crop breeders accept that an active root system contributes to enhanced nutrient uptake, resulting in improved grain yield (Chloupek et al. 2006; de Dorlodot et al. 2007). Thus, the improvement of root system architecture (RSA) is an important breeding target for producing higher yields (de Dorlodot et al. 2007), together with improvement of plant type such as plant height (Sasaki et al. 2002) and erect leaves (Sakamoto et al. 2006). RSA improvement is, however, less frequent than that for plant type. The reduced attention to RSA improvement is simply caused by two problems: roots in soil are difficult to access and difficult to handle as experimental materials. Additionally, RSA shows plasticity in adapting to environmental factors, such as limitation of water (Li et al. 2005; Tuberosa et al. 2002) and concentration of nutrients (Kirk and Du 1997; Shimizu et al. 2004; Zhang and Forde 1998). RSA is generally defined by length, weight, number, thickness and density of primary, lateral and adventitious roots or root to shoot dry weight ratio, deep root to shoot dry weight ratio and root weight per tiller, etc. Genetic variation of maximum root length was distinctly observed in a hydroponic culture compared with soil culture in rice (Price et al. 1997). Hence, root length of plants grown in a hydroponic culture has been used widely as a target trait to detect QTL for improvement of root systems in both non-stressed and stressed fields of rice (Price and Tomas 1997; Shimizu et al. 2004) and maize (Tuberosa et al. 2002). In maize, a coincidence of positive alleles for primary root length of seedlings grown in hydroponic culture and grain yield in well-watered fields was found only for the long-arm region of chromosome 1 (Tuberosa et al. 2002). These results strongly suggest that some QTL for root length of seedlings grown in hydroponic culture may potentially enhance grain yield in the field.

Nitrogen is quantitatively the most essential nutrient for plant growth, including development of the root system. There are two forms of inorganic nitrogen available to rice plants, i.e., NO_3^- in well-drained fields and NH_4^+ in paddy fields. Rice plants mainly depend on a form of NH_4^+ that is the major form of nitrogen in paddy fields (Yamaya and Oaks 2004). Most NH_4^+ is assimilated into amino acids in rice roots, whereas NO_3^- absorbed by the roots is transported to the shoots and assimilated there (Tabuchi et al. 2007). In *Arabidopsis*, all QTL for root length were detected at different chromosomal regions depending on the nitrogen source, i.e., NH_4^+, NO_3^-, NH_4NO_3 (Rauh et al. 2002). Thus, regulatory genes associated with plant growth are likely to be different between the two forms of nitrogen in rice.

In rice, elongation of the seminal root is inhibited by increases in exogenous NH_4^+ concentration. Genetic variation of the response was clearly observed among four varieties (Tanaka et al. 1993). In previous reports describing QTL mapping for root length of seedlings grown in hydroponic conditions, the nutrient solution was replaced every week (Price et al. 1997) or every 2 weeks (Xu et al. 2004) for experiments conducted with rice. Reductions in nutrient concentration are generally observed in hydroponic nutrient solutions, and unstable nutrient concentrations can affect gene expression or accumulation of gene products associated with root elongation. In maize, QTL for axial root length were detected only under low NO_3^- concentrations, not but under high NO_3^- concentrations (Liu et al. 2008). Thus, regulatory genes associated with root elongation are likely to be different in rice depending on the nitrogen concentration. Despite the suggestion that different genetic controls of root elongation may be related to nitrogen source or nitrogen concentration, QTL for rice seedling root length have been identified only under conditions where plants were supplied with a combination of the NH_4^+-form and the NO_3^--form at a single nitrogen concentration (Price and Tomas 1997; Price et al. 1997; Xu et al. 2004). Based on the current knowledge, it must be noted that an appropriate growth condition for root elongation should be applied in the genetic analysis.

The objectives of this study were to (1) establish growth conditions to reliably score root length of rice seedlings grown in hydroponic culture, (2) map the constitutive QTL to NH_4^+ concentration in a population of chromosome segment substitution lines (CSSLs) derived from a cross between 'Koshihikari', a *japonica* elite variety, and 'Kasalath', an *indica* local variety, and (3) fine-map *qRL6.1*, a major QTL on the long-arm of chromosome 6.

Materials and methods

Plant materials

Five plants of 39 CSSLs and their parents were planted in a field to obtain large quantities of seeds as described previously (Obara et al. 2004). Mature seeds of SL-208 were not obtained, since its heading date is delayed about 20 days compared to 'Koshihikari' under our field growth conditions at Kashimadai, Miyagi, Japan. Therefore, a total of 38 CSSLs were employed for QTL detection (Ebitani et al. 2005). The substituted chromosome segments in the set of 38 CSSLs covered almost all of the genome, except for a region 93.7 cM (maximum region) from the end of the long-arm of chromosome 3, a region 1.7 cM (maximum region) from the end of the short-arm of chromosome 8 and a region 2.9 cM (maximum region) from the end of the long-arm of chromosome 12 (Ebitani et al. 2005).

'Koshihikari' was backcrossed to SL-218 in which a chromosome segment was substituted with a 'Kasalath' segment spanning a 89.6 cM region (maximum region) from the end of the long-arm of chromosome 6 in the 'Koshihikari' genetic background. The resulting F_1 plant was self-pollinated to obtain F_2 plants. A total of 197 F_2 plants were used for linkage analysis of target QTL and self-pollinated to obtain F_3 lines. One plant from the F_2 plants was selected for producing a near-isogenic line (NIL) that was designated SL-218 F_2 A. The line is least heterozygous within a region between two DNA markers, C11635 and P3B2, on chromosome 6. A total of 48 F_3 plants of the SL-218 F_2 A were used for NIL selection with DNA markers C11635, P3A2 and P3B2. One NIL, NIL-A, fixed the 'Kasalath' segment between C11635 and P3B2 on chromosome 6 and was planted in a field to obtain large quantities of seed.

Evaluation of root length

The seeds of all lines used in this study were pre-selected to ensure their potential for root elongation before germination. Seed selection with NaCl solution is known to provide a stable germination rate for rice. Well-filled seeds were selected by soaking in a NaCl solution ($d = 1.13$) with gentle shaking. Submerged seeds were washed 20 min with running tap water, drained, and spread on filter paper at room temperature until they were completely dry. Seeds were sterilized in a three-step process. First, seeds were soaked 10 min in distilled water with gentle shaking at 60°C, and then washed 20 min with distilled water. Secondly, the wet seeds were soaked 30 s in a 70% (v/v) ethanol solution and then washed 2 min with distilled water. Finally, the seeds were soaked 20 min in a 1% (v/v) sodium hypochlorite solution and then washed four times with large amounts of distilled water. The seeds of each line were imbibed in distilled water at 30°C in the dark until the tip of the plumule had barely emerged.

Plants in this study were hydroponically grown in greenhouse conditions where the temperature was controlled at 25°C and natural sunlight was supplemented with 14 h artificial light (MLBOC400C-U:Mitsubishi Electric Osram Ltd, Yokohama, Japan). The basal nutrient solution was the same as described by Mae and Ohira (1981) with minor modifications, (1) distilled water was used instead of tap water to prevent any contamination with additional nutrients and (2) a Good's buffer, MES, was used to maintain the pH of the nutrient solution. Germinated seeds were sown on a nylon net that floated on quarter-strength nutrient solution without nitrogen or supplied with the appropriate amount of NH_4Cl as a single source of nitrogen and containing 5 mM MES at pH 5.5. Approximately 80 mL of nutrient solution was supplied to individual plants. The nutrient solution was renewed every day at approximately 10:00 starting at 2 days after sowing to harvest. On the final day, seminal root length was measured with a ruler and a leaf blade tip was harvested for DNA extraction for genotype analysis (linkage and fine-mapping).

Growth conditions in hydroponic culture

For comparison of seminal root length of parental lines, ten seedlings of parental lines were grown for 8 days after planting in small-scale (1.6 L solution) hydroponic culture in 0, 5, 50, or 500 μM NH_4^+ concentrations only once. The germinated seeds of parental lines were placed on a nylon net (6 × 9 cm) supported by polystyrene and floated for 30 h after starting imbibition.

For QTL detection, ten seedlings of each CSSL and parental line were grown for 8 days in large-scale (40 L solution) hydroponic culture supplied with 5 or 500 μM NH_4Cl with two temporal replications. The plumule tip of most CSSL lines and parental lines emerged at 30 h after starting imbibition, whereas those of the CSSL lines SL-215, SL217, SL-218 and SL-224 emerged at 37 h after imbibition. The germinated seeds of each CSSL and parental line were placed on a nylon net (16 × 22 cm)

supported by polystyrene and floated for 30 or 37 h after imbibition, respectively. Four nylon nets were floated on a large-scale tank (42 L) in a 2 × 2 configuration. Each nylon net included 6 rows with 20 seeds placed on each row. Ten seedlings of each line were grown on the neighboring two rows by placing five seeds of four lines on one row. Seeds were spaced at least 4 mm within rows and 10 mm between rows. A single nylon net included 120 seedlings of 12 lines. For checking differences of seminal root length among planting location, parental lines were grown at the side-edge, corner or center across four nylon nets.

For linkage analysis, a total of 197 F_2 seedlings self-pollinated from a F_1 plant between SL-218 and 'Koshihikari' were grown in large-scale hydroponic culture supplied with 5 μM NH_4Cl for 12 days with two spatial replications. The germinated seeds of these F_2 plants were placed on a nylon net supported by polystyrene and floated for 30 h after imbibition as described above. Ten seedlings of 'Koshihikari', 'Kasalath' and SL-218 were also grown on the side-edge, corner or center of one nylon net to validate the genetic effect of *qRL6.1*.

For verification of QTL, ten seedlings of NIL-A and 'Koshihikari' were grown in small-scale hydroponic culture supplied with 5, 50 or 500 μM NH_4Cl for 8 days with three temporal replications. The germinated seeds of these lines were placed on a nylon net supported by polystyrene and floated for 30 h after starting imbibition as described above.

For fine-mapping analysis, 48 seedlings of each F_3 line were grown in large-scale hydroponic culture supplied with 5 μM NH_4Cl for 10 days with one or two temporal replications as described above. The plumule tip of most F_3 line seeds emerged by 30 h after starting imbibition, whereas the seeds of the remaining F_3 lines emerged at 37 h after imbibition. F_3 line seeds were placed on a nylon net supported by polystyrene and floated for 30 or 37 h after imbibition, as appropriate. A total of nine F_3 lines were grown in one large-scale tank. Ten seedlings of Koshihikari' and SL-218 were also grown to validate the genetic effect of *qRL6.1*.

Linkage analysis of *qRL6.1*

For detecting the precise location of target QTL controlling root length localized to the long-arm region of chromosome 6, a total of 197 F_2 plants from the F_1 plant were subject to linkage analysis with two spatial replications. Linkage maps with six DNA markers were constructed from genotype data with MAPMAKER/EXP 3.0 with Kosambi's mapping function (Lander et al. 1987). Linkage analysis was performed using composite interval mapping with QTL Cartographer version 2.0 with model 6 in the forward and backward methods (Basten et al. 2002). A significant threshold was determined using the 1,000 permutation test at the 5% level of significance. An additive effect was estimated as half of the difference between homozygotes of 'Koshihikari' ($n = 45$) and 'Kasalath' ($n = 36$) within the region between C11635 and P3A2. A dominance effect was estimated as the difference between the heterozygote ($n = 83$) and the middle-point value for the homozygote of 'Koshihikari' and 'Kasalath' within the region between C11635 and P3A2.

Fine-mapping by using advanced progeny

We selected 27 F_2 plants with recombination in the vicinity of the QTL on the basis of the genotypes of the DNA markers among 197 F_2 plants used. The self-pollinated F_3 plants were used for phenotyping to determine the genotypes at the QTL. Five F_3 lines with recombination between flanking markers *qRL6.1*, MID06024 and MID06029, were used for progeny testing with two temporal replications. The remaining F_3 lines in Fig. 7 were used for progeny testing only once. A total of 48 seedlings of F_3 lines were used for progeny testing in each line. Genotype classes of F_2 plants for the QTL were determined by observing segregation patterns of root length in their self-pollinated F_3 plants. A probability of <0.001 (t test) was used as a threshold for estimating segregation pattern. In all F_3 lines, more than five seedlings were homozygous for 'Koshihikari' or 'Kasalath' at the QTL, based on genotypic analysis.

QTL detection and statistical analysis

QTL for seminal root length were detected based on the t test of the difference between the mean value of each CSSL and 'Koshihikari', the recurrent parent of the CSSL. QTL detections were performed with two temporal replications. A probability of <0.001 was used as a threshold for QTL detection in this study to avoid false-positives. The data in this study were analyzed using the Excel Statistical Analysis 97 package (SSRI Co., Tokyo, Japan), and the differences in mean values between appropriate lines were determined by Student's t test.

DNA marker analysis

A total of 12 DNA markers were used for genotyping analysis of backcrossed plants originating from SL-218. Two sequence-tagged site markers, R3879 and C11635, were obtained from a database established by the Rice Genome Research Program (http://rgp.dna.affrc.go.jp/E/index.html). The remaining ten DNA markers (Supplemental Table 1 in ESM) were constructed based on

sequence information annotated in the Rice DNA Polymorphisms database (Shen et al. 2004). DNA preparation from leaves, PCR conditions, and electrophoresis conditions were the same as previously described (Obara et al. 2001, 2004).

Results

Establishment of growth condition for root elongation

We tried to establish the most appropriate growth conditions for the parental line and CSSLs in order to estimate the effect of genes on root length. When NH_4Cl is fed to rice plants as a single source of nitrogen, the pH of the nutrient solution shifts to the acidic region, around pH 3.0–4.0. To avoid the pH change, a Good's buffer, MES at 5 mM, was added to maintain the pH at around 5.2–5.5 for 24 h after every solution renewal. Stable pH values were observed for all concentrations of NH_4^+ tested throughout the 12-day growth period. There was no physical contact of roots to the side or bottom of the container during the growth period. There was no significant difference in the mean value for seminal root length of 'Koshihikari' among plants grown on the side-edge, corner or center of a square nylon net (16 × 22 cm) supported by polystyrene floats under all concentrations of NH_4^+ tested (data not shown). This observation indicated that all plants can be used to determine the root length of plants grown with the hydroponic culture conditions established in this study. These precise growth conditions were used in the following experiments to estimate gene effects on seminal root length under a wide range of NH_4^+ concentrations.

High potential of root system development in 'Kasalath'

Seminal root length of 'Koshihikari' and 'Kasalath' plants grown in small-scale hydroponic culture with various NH_4^+ concentrations was measured as an indicator of genetic variation in the potential for root system development. Significant differences in the mean values of seminal root length were observed for both 'Koshihikari' and 'Kasalath' plants grown in either 0–5 μM NH_4^+ and 50–500 μM NH_4^+ with a probability of <0.001 (Fig. 1). The wide range of NH_4^+ concentrations used, e.g., 0, 5, 50 and 500 μM NH_4^+, covered deficient to sufficient status because a gradual reduction in root length was observed in both 'Koshihikari' and 'Kasalath' as a function of increasing NH_4^+ concentration (Fig. 1). Seminal roots of 'Kasalath' were significantly longer than those of 'Koshihikari' grown in the presence or absence of NH_4^+ with a probability of <0.001 (Fig. 1). The increased lengths of seminal roots of 'Kasalath' compared with 'Koshihikari' were: 19.5% without NH_4^+, 21.9% at 5 μM NH_4^+, 29.9% at 50 μM NH_4^+, and 26.0% at 500 μM NH_4^+, respectively. These results indicated that 'Kasalath' is constitutively capable of elongating roots at all NH_4^+ concentrations. Genetic variation in root elongation was clearly observed between 'Koshihikari' and 'Kasalath'.

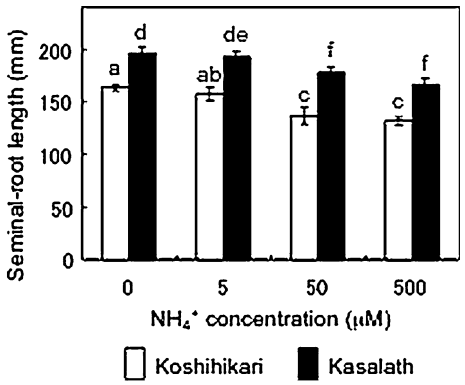

Fig. 1 Comparison of seminal root length of 'Koshihikari' and 'Kasalath' grown in hydroponic culture under a wide range of NH_4^+ concentrations. *Vertical bars* indicate the 95% confidence interval ($n = 10$). *Different letters* above the *columns* represent significant differences in seminal root length between 'Koshihikari' or 'Kasalath' plants across NH_4^+ concentrations NH_4^+ at the 0.1% level (paired Student's *t* test)

Chromosomal regions affecting seminal root elongation

A total of 38 CSSLs were used to define QTL (chromosomal regions) controlling seminal root length in the presence of 5 or 500 μM NH_4^+. The seminal root length of 'Koshihikari' was 158.5 ± 5.8 mm and that of 'Kasalath' was 201.5 ± 6.4 mm, when the seedlings were grown in large-scale hydroponic culture conditions supplemented with 5 μM NH_4^+. The seminal root length of the 38 CSSLs ranged from 134.7 to 196.7 mm. Significant differences ($P < 0.01$) in seminal root length were detected between 21 CSSLs and 'Koshihikari' (Fig. 2a). Seminal root lengths of lines SL-215, SL-216, SL-218, SL-224, SL-225, SL-231, SL-233 to 235, SL-237, and SL-238 were longer than 'Koshihikari', whereas a reduction in root length was seen in lines SL-201 to 206, SL-209, SL-210, SL-219, and SL-226 (Fig. 2a). A similar trend was observed in all replicates.

When plants were grown in 500 μM NH_4^+, the seminal root length of 'Koshihikari' was 114.8 ± 6.7 mm and that of 'Kasalath' was 166.8 ± 6.7 mm. Seminal root lengths of the 38 CSSLs ranged from 92.4 to 133.2 mm (Fig. 2b). Significant differences ($P < 0.01$) in seminal root length were detected between 20 CSSLs and 'Koshihikari' (Fig. 2b). Seminal roots of lines SL-215, SL-216, SL-218, SL-219, SL-223, SL-233 to 235, SL-237, and SL-238, were

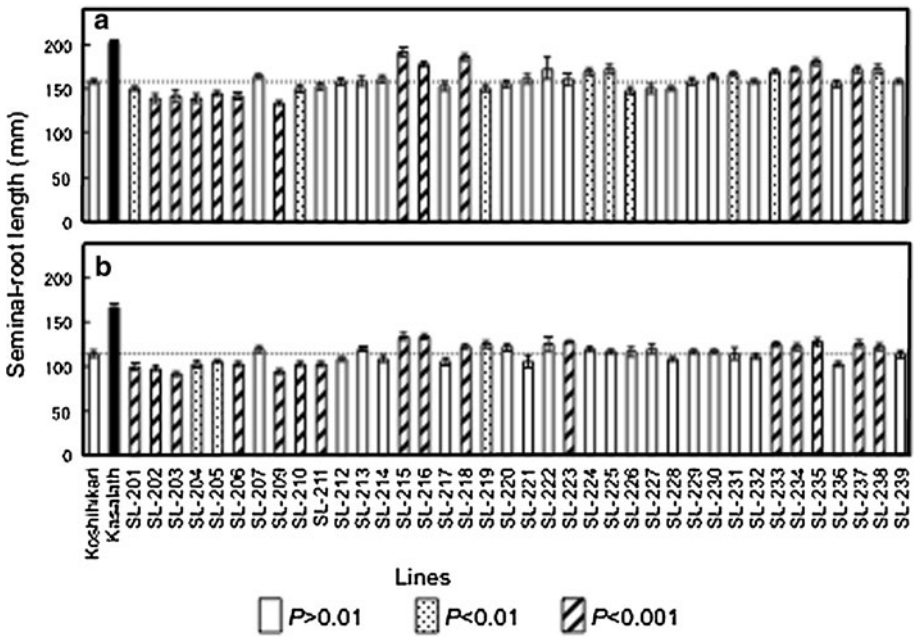

Fig. 2 Comparison of seminal root length of parental lines and CSSLs grown in hydroponic culture with 5 μM NH_4^+ (**a**) and 500 μM NH_4^+ (**b**). *Columns* indicate the mean values of seminal root length with a 95% confidence interval ($n = 10$). *Dotted horizontal bars* indicate the mean value of seminal root length of 'Koshihikari'. *Hatched bars* significant difference at 0.1%; *shaded bars* significant difference at 1% level; *open bars* not significant. A result from duplicate analyses is illustrated

longer than 'Koshihikari', and shorter in lines SL-201 to 206, SL-209 to SL-211 (Fig. 2b). A similar trend was observed in all replicates.

Based on the comparison between seminal root elongation patterns and genotypes among the CSSLs (Ebitani et al. 2005), at least eight putative QTL are likely to be located on the long-arm of chromosome 1 (2 regions), the long-arm of chromosome 2, chromosome 4, the long-arm of chromosome 6, the short-arm of chromosome 8, the long-arm of chromosome 11 and the short-arm of chromosome 12 (Fig. 3; Table 1). Only the QTL located on the long-arm of chromosome 6 was common across NH_4^+ treatments. This result indicates that the 'Kasalath' allele enhances root elongation at four QTL in chromosomes 6, 8 and 11. Conversely, the 'Kasalath' allele inhibits root elongation at four QTL in chromosomes 1, 2 and 4. Among them, the putative QTL in chromosome 6 exhibited the biggest effect on root elongation, because SL-218 showed a significant difference in seminal root length in a concentration-dependent manner with the most effective promotion of root elongation occurring in 5 μM NH_4^+. Therefore, we focused on chromosome 6 to characterize and verify the detected QTL.

Verification of QTL on chromosome 6

F_2 plants were hydroponically grown in 5 μM NH_4^+, since large differences in seminal root length were observed between SL-218 and 'Koshihikari' with this concentration of NH_4^+ (Fig. 2a). The frequency distribution of seminal root length in F_2 plants showed continuous variation with two peaks (Fig. 4a). Based on linkage analysis for seminal root length, a single LOD peak (above 60) was clearly detected between DNA markers C11635 and P3A2 on chromosome 6 in two spatial replications (Fig. 4b). The 'Kasalath' allele at this QTL promoted root elongation. The additive effect of the 'Kasalath' allele was 12.7 mm, and the dominance value was 0.4 mm. The phenotypic variance explained by this QTL was 76.4% (Table 2). With clear verification of this QTL, named *qRL6.1*, we performed progeny testing to map it as a single Mendelian factor.

Allele effect at *qRL6.1* was further confirmed by developing near-isogenic lines

A NIL, NIL-A, was selected among the F_3 plants originating from SL-218 F_2 A by using marker-assisted selection. A chromosomal segment of NIL-A between C11635 and P3B2 was substituted with a 'Kasalath' segment and covered a region of the LOD peak of *qRL6.1* between intervals (Fig. 5). The seminal root length of NIL-A was 21.1% longer than that of 'Koshihikari' when plants were grown in 5 μM NH_4^+ (Fig. 5). Also, root length of NIL-A was 13.5% longer than that of 'Koshihikari' when grown in 50 μM NH_4^+ and 14.2% longer than those of 'Koshihikari' when grown in 500 μM NH_4^+ (Fig. 5). The effect of *qRL6.1* on root elongation was observed in three temporal replications. These results indicate that *qRL6.1* resides within the 'Kasalath'-segment substituted region of NIL-A and is effective in a wide range of NH_4^+ concentrations.

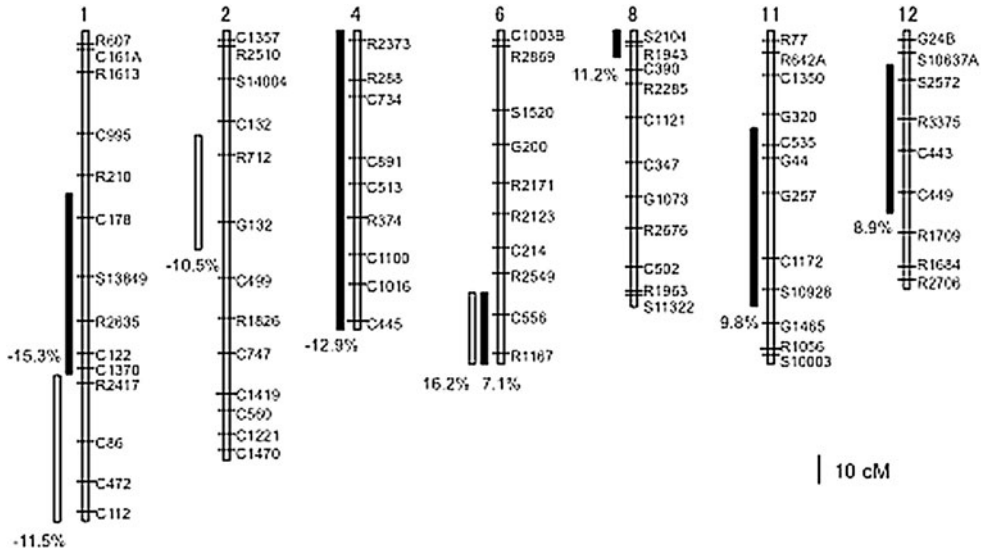

Fig. 3 Chromosomal regions affecting seminal root elongation of seedlings grown in hydroponic culture with 5 or 500 μM NH_4^+. The *vertical open bars* indicate chromosomes in which QTL were examined for seminal root length by substitution mapping. The number above the *vertical open bars* indicates chromosome number. The names of the restriction fragment length polymorphism marker and their chromosomal positions are indicated on the *right* side of each *bar*. *Opened* or *closed column* on the *left* side of each *bar* indicates the most likely chromosomal regions including putative QTL for seminal root length in 5 or 500 μM NH_4^+, respectively. *Values* under each *column* indicate the substitution effect of the 'Kasalath' allele to the 'Koshihikari' allele in seminal root length. A *positive* value indicates that the 'Kasalath' allele enhances root elongation, whereas a *negative* value indicates that the 'Kasalath' allele inhibits root elongation

Fine-mapping of *qRL6.1* as a single Mendelian factor

In order to determine genotype classes at *qRL6.1* for F_2 plants, we performed progeny testing of F_3 lines for root elongation. All F_3 lines were classified into the following three types based on the segregation of seminal root length (Fig. 6). One type showed clear segregation for root length (Fig. 6a) and other two types did not show segregation and were likely to correspond to root length of the two lines, 'Koshihikari' (Fig. 6b) and SL-218 (Fig. 6c). These three classes are likely to correspond to three genotype classes of *qRL6.1* in each F_2 plant. Consequently, *qRL6.1* was mapped in the interval between MID06024 and MID06029 with 2 and 3 recombination events, respectively (Fig. 7). The repeatable results of progeny testing on seminal root length were observed among five F_3 lines with recombination between flanking markers *qRL6.1*, MID06024 and MID06029. Based on the genome sequence in IRGSP build 4 (2007), the interval defined by MID06024 (30,821,420 bp) and MID06029 (31,157,985 bp) was 337 kb.

Discussion

Recent progress in rice genome sequencing has provided valuable resources and tools for rice molecular genetics. In particular, a large number of genetic markers, such as simple sequence repeats (SSR), has facilitated the genetic dissection of complex traits in rice by means of QTL analysis (Yamamoto et al. 2009). In this study, to enhance understanding of root development, we established reliable growth conditions for root elongation. Using this growth condition, we could identify at least eight QTL for root elongation in rice. Furthermore, one QTL, *qRL6.1* was finely mapped as a single Mendelian factor.

In this study, we aimed to establish reliable growth conditions in hydroponic culture for estimating genetic control of root elongation in paddy fields. In general, root development is often affected by several environmental conditions, such as soil moisture, nutrient concentrations, etc. It is necessary to maintain nutrient concentrations in hydroponic culture for precise measurement of root elongation. Additionally, it is necessary to handle large number of plants for fine-mapping a target QTL. Hence, daily replacement of nutrient solution in the hydroponic culture is an adequate modification for minimizing changes in nutrient concentrations. The growth conditions established in this study can simultaneously handle up to 1,000 seedlings in a nutrient solution volume of 80 L by one person. Another way to maintain nutrient concentrations in nutrient solution is to grow plants in a large volume of nutrient solution. Growth conditions described by Xu et al. 2004 used a protocol to grow about 5,000 plants in nutrient solution of 1,800 L, adding stock nutrient solution every week and replacing nutrient solution in hydroponic culture

Table 1 Co-localization of QTL for root length of seedlings grown in hydroponic conditions between previous studies and this study

Trait name	Chr[a]	Markers[b]	Region[c] (Mbp)	Additive effect	Positive allele	References
Seminal root length in 500 μM NH_4^+	1	R210	10.54	8.8	Koshihikari	This study
		−R2417	−33.18			
Seminal root length in 5 μM NH_4^+	1	C1370	31.21	9.1	Koshihikari	This study
		−C112	−44.75			
Seminal root length in 5 μM NH_4^+	2	C132	5.80	8.3	Koshihikari	This study
		−C499	−21.74			
Maximum root length day 14	2	RG171	18.27	14.9	Bala	Price and Tomas (1997)
Seminal root length in 5 μM NH_4^+	4	R2373	0	7.4	Koshihikari	This study
		C445	−36.06			
Seminal root length in 5 μM NH_4^+	6	R2549	25.79	12.8	Kasalath	This study
		−R1167	−32.12			
Seminal root length in 500 μM NH_4^+	6	R2549	25.79	4.1	Kasalath	This study
		−R1167	−32.12			
Seminal root length in 500 μM NH_4^+	8	S2104	0	6.4	Kasalath	This study
		−C390	−1.59			
Seminal root length in 500 μM NH_4^+	11	G320	6.67	5.6	Kasalath	This study
		−G1465	−26.51			
Maximum root length day 21	11	G44	10.00	15.6	Bala	Price and Tomas (1997)
Maximum root length day 28	11	RG2	20.61	41.7	Bala	Price and Tomas (1997)
Maximum root length day 40	11	G4001	25.13	5.0	Minghui 63	Xu et al. (2004)
		−RM254	−26.07			
Seminal root length in 500 μM NH_4^+	12	S10637A	2.19	5.1	Kasalath	This study
		−R1709	−23.70			
Root length day 14	12	RZ397	5.76	18.3	IR64	Nguyen et al. (2003)

[a] Chr indicates chromosome

[b] Markers indicate nearest marker or flanking markers of QTL

[c] Marker position or marker region refer to build 5 of the 'Nipponbare' genome of IRGSP. Related sequences of these markers were obtained from the GRAMENE Rice QTL database

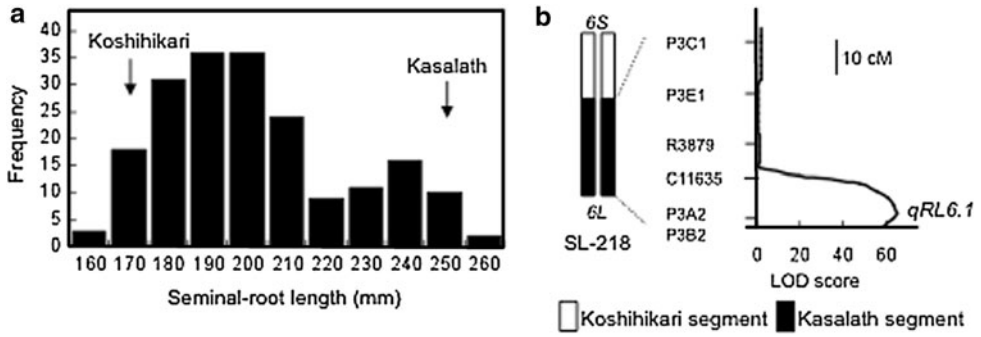

Fig. 4 Frequency distribution (**a**) and linkage analysis (**b**) for seminal root length of seedlings grown in 5 μM NH_4^+ using an F_2 population derived from a cross between SL-218 and 'Koshihikari'. A *graphical representation* of the SL-218 genotype is illustrated (**b**). The genetic map was reconstructed with six DNA markers, and the *vertical axis* indicates the genetic loci examined. The *horizontal axis* indicates the LOD score

every 2 weeks. Our growth conditions in a relatively small volume of nutrient solution with daily replacement have an advantage for analyzing the genetic control of root elongation in a small laboratory. A remaining modification in this study was that NH_4^+ was fed to plants as a sole source of nitrogen. Although combinations of NO_3^- and NH_4^+ as the nitrogen sources have been widely used for QTL mapping for RSA in hydroponic culture (Lian et al. 2005; Price et al. 1997; Shimizu et al. 2004; Xu et al. 2004), it is difficult to distinguish the NH_4^+ and NO_3^- effects.

Table 2 Putative QTL for seminal root length of plants grown in 5 μM NH_4^+ for F_2 plants derived from SL-218 and 'Koshihikari'

QTL	NH_4^+	Chromosome (μM)	Marker interval	R^2 (%)[a]	Additive effect[b]	Dominance effect[c]
qRL6.1	5	6	C11635–P3A2	76.4	12.7	0.4

[a] Phenotypic variation explained by *qRL6.1*

[b] The value was estimated as half of the difference between homozygotes of 'Koshihikari' and 'Kasalath' within the region between C11635 and P3A2

[c] The value was estimated as the difference between heterozygotes and the middle-point value for homozygotes of 'Koshihikari' and 'Kasalath' within the region between C11635 and P3A2

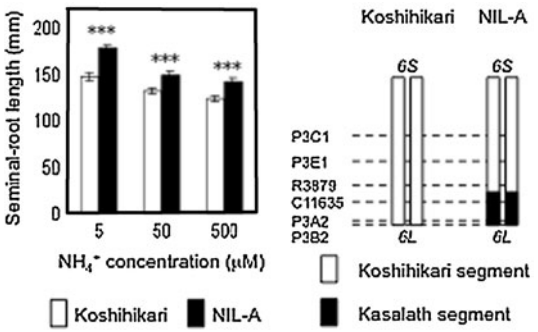

Fig. 5 Effects of *qRL6.1* on root elongation under a wide range of NH_4^+ concentrations. *Columns* indicate mean values of seminal root length of 'Koshihikari', genetic background, and NIL-A, NIL for *qRL6.1* with a 95% confidence interval ($n = 10$). *Asterisks* represents significant differences in seminal root length at the 0.01% level (paired Student's *t* test). A graphical representation of the NIL-A genotype is illustrated in the *right panel*

Rauh et al. (2002) showed that different loci govern root elongation of *Arabidopsis thaliana* plants grown in different nitrogen sources, i.e., NH_4^+ alone, NO_3^- alone or in combination. Genetic variations in root length in response to nitrogen source were also observed among 60 rice varieties, including 'Koshihikari' and 'Kasalath' (M. Obara et al., unpublished data). Hence, a supplement of NH_4^+ alone is adequate for mapping constitutive QTL controlling seminal root length in response to NH_4^+ concentration.

The 'Kasalath' allele on *qRL6.1* effectively promoted root elongation as a single locus when plants were grown in 5 μM NH_4^+. This positive growth effect was also observed with 50 and 500 μM NH_4^+. *qRL6.1* is a constitutive QTL controlling root length at all NH_4^+ concentrations. QTL are generally categorized into two types: constitutive QTL that are active across environmental conditions or adaptive QTL that respond to environmental conditions based on the nature of the QTL itself (Collins et al. 2008). In many cases, QTL for plant development are adaptive QTL to nitrogen concentration. For example, Lian et al. (2005) showed QTL controlling the weight of rice seedling shoots and roots grown in 0.24 mM NH_4NO_3. Adaptive QTL to nitrogen concentration were also found as yield-related traits of maize (Coque and Gallais 2006) and rice (Obara et al. 2004). Although a few constitutive QTL for root weight were observed across nitrogen concentrations, these QTL are apparently localized to the short-arm of chromosomes 5 and 12 (Lian et al. 2005). The 'Kasalath' allele associated with *qRL6.1* has the potential for improving the root system by enhancing root elongation under a wide range of NH_4^+ concentrations, because root elongation is generally depressed by increasing nitrogen concentrations. Introgression of *qRL6.1* that constitutively enhances root elongation leading to larger root systems will have a substantial advantage in enhancing biomass production or grain yield as a result of active acquisition of water and nutrients. These benefits will be particularly evident when plants are grown in managed paddy fields since significant changes in soil nitrogen content in these fields can occur as a result of supplementing nitrogen fertilizer throughout the crop's growth period.

Root elongation in rice seedlings is apparently regulated by several genes, since significant differences in seminal root length were observed between 'Koshihikari' and several CSSLs grown under both NH_4^+ concentrations (Fig. 2). Ebitani et al. (2005) showed the efficacy of detection and delimitation of QTL for heading date by substitution mapping with the same CSSLs. A total of eight QTL were mapped by substitution mapping (Fig. 3). These data indicate that at least four QTL within the region substituted by the 'Kasalath' segment contribute to increases in root elongation.

Although most QTL other than *qRL6.1* detected in this study were defined by a wide chromosomal region (Fig. 3; Table 1), the chromosomal localization of QTL found in this study and those reported previously can be roughly compared. Three out of eight QTL region in this study included QTL for root length of seedlings grown in hydroponic conditions in different mapping populations previously reported (Table 1). A QTL region detected on the long-arm of chromosome 2 in this study included a QTL for maximum root length of 14-day-old seedlings detected in a population developed from 'Bala' and 'Azucena' (Price and Tomas 1997). Another QTL region detected on the long-arm of chromosome 11 in this study included QTL for maximum root length of 14- or 28-day-old seedlings in a population developed from 'Bala' and 'Azucena' (Price and Tomas 1997). Furthermore, QTL for

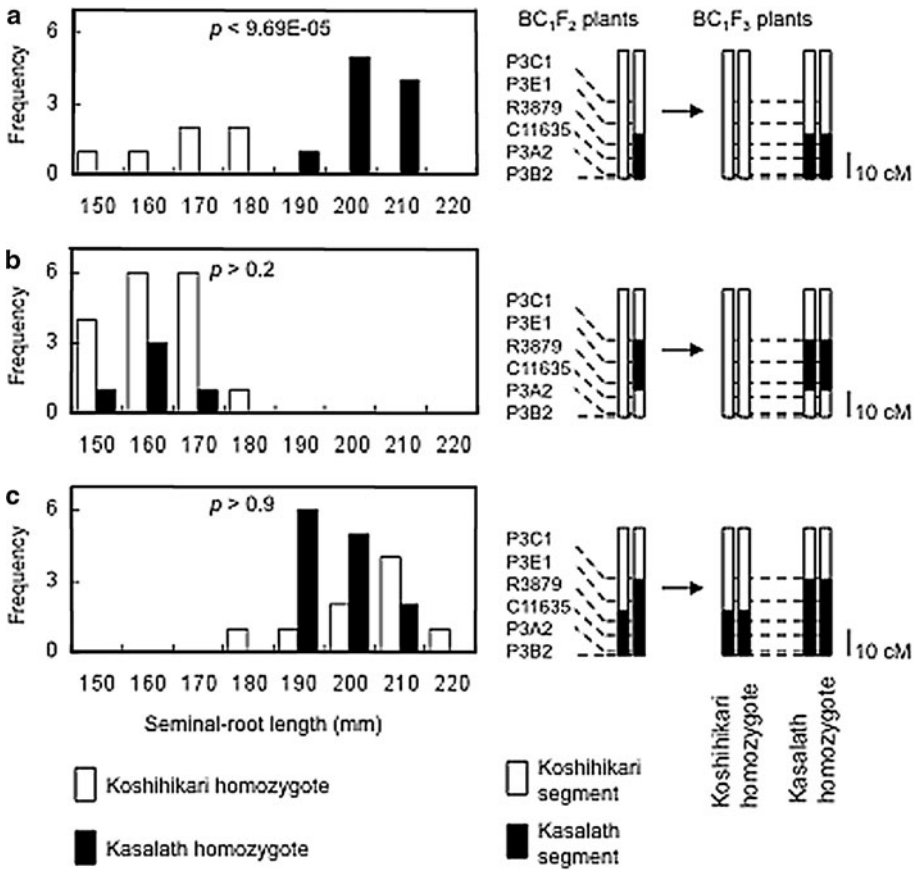

Fig. 6 Frequency distribution of seminal root length for 'Koshihikari'-fixed-genotype or 'Kasalath'-fixed-genotype F_3 plants grown in 5 μM NH_4^+. Genotyping analysis with DNA markers showed that the genotypes of parental F_2 plants at *qRL6.1* were heterozygous (**a**), 'Koshihikari'-fixed (**b**) or 'Kasalath'-fixed (**c**), respectively. The genotypes of each F_2 plant are illustrated in the *right panel*. The seminal root length of F_3 plants was compared between the homozygote of 'Koshihikari' and that of 'Kasalath' within a whole segregated chromosomal region. A total of 48 F_3 plants were used in each line. Probability is represented in *each panel* (paired Student's *t* test)

root length of 40-day-old seedlings was mapped on the long-arm of chromosome 11 in a population developed from 'Zhenshan 97' and 'Minghui 63' (Xu et al. 2004). The remaining QTL region detected on the long-arm of chromosome 12 in this study included a QTL for root length of 14-day-old seedlings detected in a population developed from 'IR64' and '*Oryza rufipogon*'(accession 106424) (Nguyen et al. 2003). Agreement with the QTL regions previously reported suggests that our hydroponic conditions were adequate to detect QTL. Although we could not conclude that the QTL detected in previous reports and this study were same locus/loci or different ones, fine-mapping analysis for the QTL on chromosomes 2, 11 or 12 will provide a precise position of these QTL as demonstrated by delimitation of *qRL6.1*.

Fine-mapping of the target QTL is necessary prior to introducing the QTL into a crop to avoid co-introgression of undesirable traits. To date, however, there are only a limited number of reports describing finely mapped QTL for root length of crops that were grown either in greenhouses or field conditions (Shimizu et al. 2008). Low mapping resolution for QTL associated with heading date was also observed in substitution mapping with the same CSSLs (Ebitani et al. 2005); however, when CSSL were used for QTL mapping, we could quickly produce advanced backcross progeny for verification, confirmation and fine-mapping of target QTL. In fact, we could successfully confirm and fine-map *qRL6.1* on chromosome 6. Recently, a new genetic map with 164 DNA markers was developed in a population developed from a cross between 'Bala' and 'Azucena' and the previous phenotypic data including root traits as maximum root length were reevaluated in a meta-QTL analysis (Khowaja et al. 2009). A total of 675 root QTL of 29 traits from a drought QTL database were summarized and localized in physical map based on the 'Nipponbare' genome (Courtois et al. 2009). A survey of the TropGENE database (2009) showed that two QTL for maximum root length of seedlings grown in soil culture were newly mapped in the region of *qRL6.1*. One is a QTL for maximum root length of plants grown in low nitrogen conditions (MacMillan et al. 2006), whereas the other is a QTL for maximum root length for plants grown in water-stressed conditions (Price et al. 2002). Additional surveying of the GRAMENE QTL Database (2010) in combination with the Rice Annotation Project Database (2010) showed that *qREP-6* maps in the vicinity of *qRL6.1*. *qREP-6* is a QTL for root length under phosphorus deficiency (Shimizu et al. 2008). The 'Kasalath' allele on *qREP-6* promoted root elongation under phosphorus deficiency in the 'Nipponbare' genetic background,

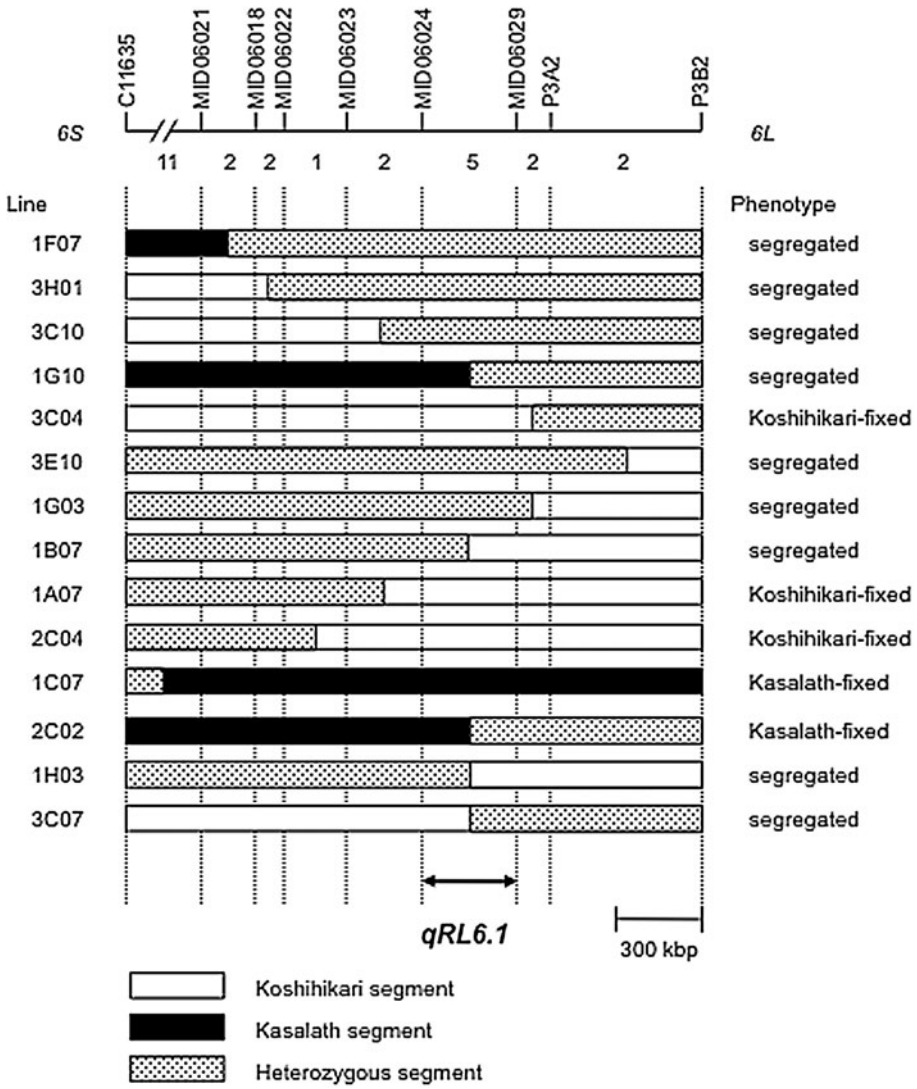

Fig. 7 Physical map and delimitation of *qRL6.1*. The *horizontal line* indicates a physical map with nine DNA markers based on the sequence position within the 'Nipponbare' genome. *Numbers* under the *horizontal line* indicate the number of plants in which recombination occurred between both sides of the DNA marker. *Horizontal columns* indicate the genotype of each F_2 lines. The *dotted vertical line* indicates the position of each DNA marker as described in the *upper panel*. *Arrows* indicate the region of *qRL6.1*

and the positive allele is similar to that for *qRL6.1*. Our fine-mapping analysis clearly showed that *qRL6.1* was located in a 336 kb-long region in the genome sequence and the position was from 30.82 to 31.16 Mbp on chromosome 6. Four loci (*qRL6.1*, a QTL for maximum root length of seedlings grown in soil culture with low nitrogen nutrient, a QTL for maximum root length of plants grown in water-stressed condition and *qREP-6*) are located in the same region of chromosome 6. It is very difficult to conclude whether the four QTL are same locus or different loci based on current information, because three QTL were mapped by QTL analysis, not by fine-mapping as was conducted for *qRL6.1*. Additional fine-mapping will be required to conclude an allelic relationship among the four loci.

Further high-resolution mapping analyses are also necessary for the molecular identification of the *qRL6.1* gene because investigation of the Rice Annotation Project Database (2010) revealed that 42 candidate genes are annotated within the *qRL6.1* region. Positional cloning is an efficient strategy for identifying the functional polymorphisms underlying QTL in rice and other crops (Yamamoto et al. 2009; Yano et al. 2000). We have just started to select lines in which recombination has occurred between the flanking markers of *qRL6.1*, MID06024 and MID06029, from approximately 4,000 segregating plants. High-resolution mapping analysis with these lines will lead to the molecular identification of the *qRL6.1* gene because our established growth conditions are superior for fine-mapping analysis of major QTL controlling seminal root length. We anticipate that *qRL6.1* will contribute to the improvement of rice RSA by molecular breeding in the near future.

In conclusion, *qRL6.1*, a major QTL for root elongation among QTL detected in this study, was finely mapped as a single Mendelian factor upon the successful establishment of hydroponic growth conditions. *qRL6.1* was found to be a constitutive QTL for root elongation to NH_4^+

concentration because seminal roots of NIL *qRL6.1* were significantly longer than those of 'Koshihikari' at all NH_4^+ concentrations tested. Our results indicate that the 'Kasalath' allele at *qRL6.1* has the potential for enhancing root system development by increasing root length. These achievements benefit the understanding of the genetic control of rice root growth and the goal of applying *qRL6.1* for high yield breeding with marker-assisted selection. Further analyses for validating effect of *qRL6.1* on root growth or yield and identifying the *qRL6.1* gene are ongoing.

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