博士論文

Identification of root traits for nitrogen-deficiency tolerance in rice through QTL analysis

（イネの低窒素耐性向上に役立つ根系形質の QTL 解析を用いた同定）

小川　諭志

OGAWA Satoshi

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ABSTRACT

Nitrogen (N) is an essential nutrient taken up in large amounts and usually is the most yield-limiting nutrient in rice production around the world (Samonte et al. 2006). However, estimates of the world nitrogen use efficiency (NUE) have been calculated to be as low as 33% (Raun and Johnson, 1999). Colombia, one of the major rice importing countries in Latin America, showed high rice production costs compare to US and other Latin American countries due to the high cost in N fertilizer use. The improvement of NUE has a significant potential for the rice producers in Colombia.

Root plays an important role in acquisition of nutrients. Improvement of root system architecture (RSA) is an important breeding target for producing higher yield through improvement of acquisition efficiency of nutrients such as N (De Dorlodot et al. 2007). However, RSA showed high degree of plasticity in response to changes of the nutrient environments (Ogawa et al. 2014a; Wissuwa et al. 2005) and these plasticity traits may assist plants to scavenge the nutrients in heterogeneous soils to increase water- and nutrient- acquisition efficiency. However, little is known about the interaction between RSA traits and agronomic performances under field environments and their genetic control. The objective of this study was to elucidate the root architectural plasticity to N level, and RSA ideotype in rice to improve agronomic performance under N-deficient conditions.

To clarify the interaction between RSA traits and agronomic performances, we conducted three different experiments at both greenhouse and field from 2012 to 2015, in CIAT. We used diverse accessions of both commercial cultivars and non-*sativa* species of rice. The first experiment was to evaluate seminal root elongation response to different N forms (NH_4^+, NO_3^- and NH_4NO_3) and concentrations (5, 50 and 500 μM) by using floating mesh method at eight days seedling stage. The result indicated that there is a genotypic difference in the response of seminal root elongation to the forms and concentrations of N even at seedling stage. I also found that root elongation in some commercial varieties such as Curinga was sensitive to N, especially NH_4^+. As NH_4^+ concentration increases, root elongation of Curinga was inhibited but some non-*sativa* species such as *O. rufipogon* was not. In the 2nd experiment, we examined the variation in root growth angle and plasticity among rice genotypes grown under hydroponics conditions at 40 days old with different NH_4^+ concentrations using basket method. We also observed that there is a genotypic variation of rooting pattern in response to NH_4^+. Especially, rooting pattern as ratio of deep rooting (RDR) in *O. glaberrima* was insensitive to NH_4^+ concentration, while that in Curinga was sensitive.

In the 3rd experiment, five contrasting genotypes with distinct rooting patterns (monomorphic-shallow, monomorphic-deep and dimorphic root system) were evaluated for the plant agronomic performance under paddy field conditions with different N applications, and the nitrogen-deficiency tolerance (NDT) traits were evaluated. Dimorphic root system varieties that have both shallow and deep root system showed less yield reduction when the fertilizer application was reduced compared to monomorphic- deep and shallow varieties. We concluded that dimorphic rooting system would be helpful to enhance NDT traits in yield under paddy filed conditions.

To gain a better understanding about the genetic basis of the relationships between RSA traits and agronomic performance, we evaluated a set of CSSLs derived from crosses between two genotypes of contrasting root plasticity, Curinga and *O. rufipogon* (accession IRGC105491) under three experimental settings similar to the above mentioned experiments.

QTL analysis was conducted with average data of RSA traits, agronomic traits and NDT traits using CSSL finder v. 0.84 computer program (Lorieux 2005). Following QTLs analysis of each experiment, we identified a total of 18 QTLs; including five QTLs for RSA traits on chromosomes 1 and 12, three QTLs for NDT on chromosomes 1, 7, 8, and 10 QTLs for agronomic traits on chromosomes 3, 4, 5, 7, 9, 10 and 12. Even if we should take the undesirable genetic linkage and pleiotropy into account, the identified QTLs could be used as target region for future breeding because of the possibility of simultaneous improvement in NDT traits.

Interestingly, we found that a QTL for deeper root number identified in the region of SNP markers between id1012330 and id1021697 on chromosome 1 under hydroponic conditions overlapped with a QTL for NDT trait of relative grain yield (RGY). These results suggest that there are some relationship and/or recombinant effect between deeper rooting trait and grain yield, although we cannot yet conclude that these QTLs are controlling those two traits. The QTL associated root system architecture could potentially be used in future breeding efforts to increase agronomic performance and to maintain grain yield under nitrogen-deficient conditions.

Genetic variation in RSA and its plasticity to nutrient conditions may be appropriate targets for marker-assisted selection to improve rice nutrient acquisition efficiency. However, RSA is a complex trait that combines root length and root growth angle (Abe and Morita 1994). Our challenge is to discover useful RSA traits that improve NAE and to identify relevant gene that control interesting RSA traits for future rice breeding. Future studies would be to pyramid useful RSA QTLs effectively in single genetic

background using advanced molecular tools and understanding interactions of Genotype x Genotype and Genotype x Environment for the development of rice varieties suitable for N deficit conditions.

ABBREVIATIONS

ANOVA; analysis of variance

CIAT; (International Center for Tropical Agriculture, or "*Centro Internacional de Agricultura Tropical"* in Spanish*)*

CSSL; chromosome segment substitution line

DAS; day after sowing

DAT; day after transplanting

FP; farmer's practice

N; nitrogen

NAE; nitrogen acquisition efficiency

NDT; nitrogen-deficiency tolerance

NIL; near isogenic line

NUE; nitrogen-use efficiency

O. barthii; *Oryza barthii*

O. glaberrima; *Oryza glaberrima*

O. rufipogon; *Oryza rufipogon*

O. sativa; *Oryza sativa*

PNN; partial nitrate nutrition

QTL; quantitative trait locus

RBM; relative biomass yield

RDR; ratio of deep rooting

RGY; relative grain yield

RIL; recombinant inbred line

RNC: relative nitrogen contents

RPV; rooting pattern value

RSA; root system architecture

SNP; single nucleotide polymorphism

SPAD; soil and plant analyzer development

SSR; simple sequence repeat

TRQ; tariff-rate quote

US; United States of America

CHAPTER 1 INTRODUCTION

Nitrogen; the most important nutrient for plant growth

Nitrogen (N) is the most important nutrient for plant growth, because it is the basic component of many organic molecules such as nucleic acids and proteins (Lea and Mifflin 2011). In rice, N promotes rapid growth and improves grain yield through tiller number increase, leaf area development, grain formation, grain filling, and protein synthesis. There are two inorganic N forms that is available to plants, i.e., NH_4^+ mainly in the soils of paddy fields and NO_3^- in those of well-drained fields. In soil, NH_4^+ form is produced from organic matter or N fertilizers. Bacteria present in the soil convert NH_4^+ to NO_3^- through NO_2^- (nitrification). Plants mainly use these two forms of inorganic N (NH_4^+ and NO_3^-) for their growth, however, the response to these two forms of N is different among plant species.

In the world, more than 100 million tons of N fertilizer per year was applied to the field to improve the agronomic productivity (FAO 2011). About 60% of global N fertilizer is used for producing the world's three major cereals including rice (Ladha et al. 2005). However, to avoid the risk of yield reduction, farmers have applied more N fertilizers for cultivation, and thus consumption of N fertilizer remarkably increased all over the world though consumption of phosphorus and potassium fertilizers reached plateau (Stuart et al. 2014). Price of N fertilizers such as urea, anhydrous ammonia and N solution also increased due to high demand in all over the world (FAO 2011).

Subbarao et al. (2013) calculated the direct annual economic loss from worldwide N-fertilizer application and estimated the cost of urea-N to be reaching US$ 0.45 per kg of N in 2008; which will result in nearly US$ 81 billion loss in the world, or US$ 17 billion for cereals crops only. Moreover, other external costs such as the contamination and damage to the environment are difficult to be quantified in economic terms and have not yet been adequately addressed (Ryden et al. 1984; Schlesinger 2009; Tilman et al. 2001; Viets 1975). The applied N is not effectively utilized by plants usually (Cassman et al. 2003). There are

several reports for the causes of N losses such as: leaching up to 36 - 45 kg/ha/year (Zhu et al. 2009), runoff around 13% of the total applied N (Chichester and Richardson 1992), losses through leaves up to 45 kg/ha/year (Stutte et al. 1979), volatilization up to 5% from available N per day (Hoeft, 2004), denitrification between 20% and 50% of total applied N (Garcia and Tiedje 1982). The world nitrogen-use efficiency (NUE) was calculated to be as low as 33 % for cereals (Raun and Johnson, 1999). Due to the low recovery of N fertilizer by crop plants, there is increasing interest in reducing fertilizer-N inputs by improving plant NUE. Thus the remaining N from fertilizers are lost to the atmosphere or leached into the groundwater and other freshwater bodies (Raun and Johnson 1999; Glass 2003), which is causing severe N pollution and becoming a risk for global ecosystems (Anbessa and Juskiw 2012). N_2O is one of the principal emitted greenhouse gas from N fertilizers, having 310 times higher global warming effect than CO_2.

Because of high amount of N loss, N deficiency is one of the most common problems in rice cultivation. It is common in all rice-growing fields where modern varieties with higher N requirement are grown without sufficient mineral N fertilizer. It often occurs at critical growth stages of the plant, such as tillering and panicle initiation, when the demand for N is high. N deficiency also occurs when a large amount of N fertilizers are applied but at the wrong timing or in the wrong way.

Table 1.1 Comparison of rice production cost between Colombia and US in 2010

	Colombia	US
Farm fee (US$ / ha)	328	423
Fertilizer cost (US$ / t)	449	238
Yield (t / ha)	5.3	8.16
Total production cost (US$ / ha)	2,359	2,153

Yield production cost (US$ / t)	444	265.6
Price for end user in Colombia (US$/ t)	1064.93	1099.8(with 80 % tariff)

Source: FEDEARROZ official report 2010 and FEDEAROZ website

In Latin America, which accounts for 20% of world's urea import (Maene, 2000), farmers are suffering from increased cost of fertilizers. The total fertilizer costs per tons of rice are higher in most Latin American countries than in US (FEDEARROZ 2010). The production costs in Brazil, Uruguay, Peru and Ecuador was 277, 316, 320 and 380 US$ per ton, respectively, and that in US was 265.6 US$ per ton in 2010. And thus, most of Latin American countries are experiencing higher rice production cost because of the higher fertilizer application cost and lower productivity. Especially, Colombia experienced the highest rice production cost in Latin America (444 US$ per ton) (FEDEARROZ 2010). According to the FEDEARROZ (Association of Rice Producers in Colombia) Official Report for 2010 in FEDEARROZ website, total rice production cost of Colombia was 67.2% higher than that of US (Table 1.1). Particularly, fertilizer cost was one of the most expensive components of production cost in Colombia, which was 88.7% higher than that in US. In addition, the US concluded the free trade agreement with Colombia in June 2007, establishing an initial 79,000 tons (milled basis) tariff-rate quota (TRQ) for all types and forms of US rice from 2012 (for detailed information see: The Colombia Rice Export Quota, Inc. (COL-RICE) web site: http://www.colom-peq.org/). The duty on imported rice was 80% before 2014, but within the TRQ, it will decrease around 6% every year and will be zero by 2030. The contingent import rice quality will be unlimited, that means the free trade for rice will start (Ministerio de Agricultura Desarrollo Rural, 2013). After 2018, imported US rice into Colombia is estimated to be cheaper than the domestically produced rice. After starting TRQ, Colombian rice farmers need to compete with cheaper US rice in the national market. Decreasing fertilizer cost is one of the approaches to win the price competition with imported rice. Thus, the improvement of nitrogen-use efficiency (NUE) has a significant impact on the economy and food security in Colombia.

Roots: the most important organ for nutrient acquisition

Roots are the most essential organ for the uptake of nutrients and water. For nutrient uptake, the individual nutrient ion must be in position adjacent to the root. The soluble fraction of nutrient such as N which are present in soil solution (water) and not held on the soil fractions flows to the root as water in soil is taken up by the roots (mass flow). Nutrient; such as phosphorus and potassium which are absorbed strongly to soil and only present in small quantities in the soil solution move to roots by diffusion. After reaching the surface of the roots, nutrient ions are transported to the center of the root, the stele, in order for the nutrients to reach the conducting tissues, xylem (Norman et al. 2013). The Casparian strip, a cell wall outside of the stele but within the root, prevents passive flow of water and nutrients and regulates the uptake of nutrients and water (Norman et al. 2013). And then water and nutrients are transported within the plant through xylem. Water potential plays a key role in a plants nutrient uptake. If the water potential is more negative within the plant than the surrounding soils, the nutrients will move from the higher solute concentration (soil) to lower solute concentration (plant). There are three ways with which plants uptake nutrients through the root: 1) simple diffusion, the passive movement of nonpolar molecule, such as O_2, CO_2, and NH_3 along the concentration gradient without the help of transport proteins, 2) facilitated diffusion, the rapid movement of solutes following a concentration gradient, facilitated by transport proteins, 3) active transport, the transport of molecules against the concentration gradient that requires an energy sources, such as ATP (Norman et al. 2013).

Besides the physiological root function, root morphology and root system architecture (RSA) are the important traits to uptake water and nutrition from the soil. However distribution of plant root systems are affected by soil physical and biochemical conditions (Takeuchi and Hasegawa 1959; Marschner 1986; Iijima et al. 1991). It was reported that vertical root distribution that is determined by a combination of the root growth angle and maximum root length is important for the water uptake (Yoshida and Hasegawa 1982, Uga et al. 2011) and nutrients (Lynch 2013) from deeper layers and, on the other hand, a shallow

root system would be at advantage for top soil foraging of phosphorus (Wissuwa et al. 2005) and mineralized N (Zhu et al. 2005). Most plants have acquired nutrient acquisition mechanisms through the evolution to overcome nutrient limitations and adapt to their native soils (Morgan and Connolly 2013). One of the universal adaptations to nutrient-deficiency environment is a plasticity of RSA to increase access to new nutrient sources. High degree of root growth plasticity was observed in response to changes in the supply of vital nutrients (Hodge 2009). In case of N deficient paddy conditions, root incorporates high root length densities, which reduce the distance NH_4^+ must diffuse in the rhizosphere to reach the root surface, and the proliferation of roots in NH_4^+-rich patches (White et al. 2013). Marzec et al. (2013) reported root hairs were produced longer and with higher density under N starvation conditions. Root plasticity is observed not only in nutrient-deficiency conditions but also in excessive conditions.

There are many reports about plasticity of RSA, and significant genetic variation in the morphology of root growth is also reported (Kato et al. 2006; Uga et al. 2009; Fig.1.1). Garnett et al. (2009) assumed that root morphology may have considerable impact on enhancing nutrition acquisition dependent on the target environment in question. I agree to his hypothesis that modified RSA can improve nutrient uptake, but only a few successes were reported to breed new rice genotype with root improvement such as deeper rooting (Uga et al. 2013; Wissuwa et al. 2005). If the genes which control root system architecture (RSA) to enhance nutrient acquisition are identified successfully, they would be useful for developing new rice varieties suitable for nutrient-deficient conditions.

Fig. 1.1 Natural variation in vertical root distribution of cultivated rice

White shaded area and outermost white dotted line indicate major and maximum root distributions, respectively (Personal communication from Dr. Uga, NIAS).

Breeding through root morphological improvement to enhance NUE

Root traits have been claimed to be critical for increasing yield under soil related stresses such as nutrient excess or deficiency (Lynch 2007, Serraj et al. 2004). The improvement of root system architectural traits might be a convenient strategy to increase productivity and NUE under low-input environments (Postma et al. 2013). The simplest way to increase nutrient uptake might be improved RSA such as lateral root production, root length density and root surface area (White et al. 2013). Thus, the improvement of root system architecture is an important breeding target for producing higher yields under N deficient conditions (de Dorlodot et al. 2007).

However, so far, limited reports are available to explain the relationship between root traits and grain yield under N limited treatments (Lynch, 2013; Arai-Sanoh et al. 2014). A part of the reason might have been that it was empirically assumed that the growth of roots is entirely governed by the physico-chemical properties of the soil, and much lesser degree by the genetics of the host plants (Kell, 2011). It was known that RSA traits were influenced by many factors such as soil texture, nutrient concentrations, soil micro- and macro-organisms and so on (e.g. Kirk and Du 1997; Shimizu et al. 2004). This is particularly true of N availability, which is the major growth-limiting nutrient in natural environments. These nutrients have been reported to alter post-embryonic root development and, therefore, RSA (López-Bucio et al. 2003).

For understanding the interactions of the complex traits such as RSA, QTL (quantitative trait locus) analysis serves as a powerful tool for identifying the genetic factors influencing quantitative traits and provides useful information. The achievements of QTL analysis for RSA traits improved the understanding of the genetic control of rice root growth. Doussan et al. (2003) and Kato et al. (2006) reported constitutive QTLs that were detected under several cultivation conditions. Most of identified QTLs were detected under the specific physico-chemical environments (Fitter and Stickland 1991; Cahill et al. 2010) including soil organisms (de Dorlodot et al. 2007; Lynch 2007). Some QTLs for RSA were detected by hormone and chemical interactions (Tanimoto 2005; Santner et al. 2009). According to Courtois et al. (2009), a total of 103 QTLs for root length have been reported as important root QTLs in rice and Ahamadi et al. (2014) reviewed QTLs for RSA including root morphology and function. A new major QTL controlling the ratio of deep rooting (RDR; means the proportion of total roots that elongated through the basket bottom) called *DRO1* (*DEEPER ROOT 1*) gene increases the frequency of high root growth angles (50 – 90° with respect to the horizontal, that is, deeper root) (Uga et al. 2011b). The opposite of *DRO1*, *qSOR* (*SOIL SURFACE ROOTING 1*) is related to the growth roots closer to soil surface, that is, shallow roots (Uga et al. 2011a). Except *DRO1*, there have been few reports of mapped

QTLs associated with root growth angle on chromosome 4 and 7 in rice (Uga et al. 2013; 2011). Obara et al. (2010; 2011) mentioned the potential QTLs (qRL1.1, qRL6.1) for enhancing root system development that can increase root length may be helpful for high yield breeding. Some nitrogen-deficiency tolerance (NDT) and NUE traits were also identified in rice by QTL analysis. Lian et al. (2005) identified 14 NDT traits in recombinant inbred lines (RILs) derived from the cross of Zhenshan97 / Minghui63. Between these parents, Wei et al. (2012) also detected eight QTLs for NDT trait and six QTLs for NUE. In addition, root length of plants grown in hydroponic culture has been widely used to detect QTL associated with improved root systems in both stressed and non-stressed rice fields (Champoux et al. 1995; Price and Tomas 1997; Shimizu et al. 2004).

Uga et al. (2013) developed near-isogenic line (NIL) in which root growth angle was improved due to a functional allele of *DRO1* introduced from the deep-rooting cultivar 'Kinandang Patong' has deeper roots in the background of shallow-rooting parent variety 'IR64', which has a non-functional allele of *DRO1*. This developed NIL has been shown to improve the ability to enhance N acquisition under lowland conditions with both N limited and normal N application (Arai-Sanoh et al. 2014). *DRO1* is the first reported gene associated with RSA that has been shown to improve the ability to improve water and nutrient acquisition. However, yet there have been few successful reports of mapped QTLs associated with both RSA and NDT in rice. RSA traits have large potential to enhance yield and stress avoidance. For the future breeding, it is interesting to draw attention to the potentially substantial benefits that are to be gained from growing crops with ideal root systems. Characterization and identification of ideal root system may help to develop new rice variety that can sustain yield performance under N limited condition thorough improvement of N-acquisition efficiency.

HYPOTHESIS

To increase rice productivity and nitrogen use efficiency, improved root should be able to uptake more N under nitrogen-deficient conditions and maintain grain yield under such conditions.

GENERAL OBJECTIVE

To develop rice genotypes with high nitrogen-deficiency tolerance which will be useful to reduce fertilizer application?

SPECIFIC OBJECTIVES

- Root traits characterization for improving nitrogen-deficiency tolerance
- QTLs identification for nitrogen-deficiency tolerance

RESEARCH DESIGN

The research goal is for reducing the amount of N fertilizer application by developing new rice varieties with improved RSA to enhance NAE and/or high nitrogen-deficiency tolerance (NDT). To realize this research goal, I analyzed both root growth dynamics and agronomic performance under different environments of N status in this study.

In an effort to understand root growth mechanism, I studied the new traits in RSA under different N forms and concentrations using diverse rice genotypes (CHAPTER 2). I chose genotypes with contrasting RSA based on two RSA evaluation methods under hydroponic conditions (CHAPTER 2). Chromosome segment substitution lines (CSSLs) between selected two genotypes were used in same experimental procedures as CHAPTER 2 to identify QTLs for RSA traits (CHAPTER 3). To identify ideal root system which enhances NDT, I conducted agronomic traits evaluation using rice varieties with representative RSAs under two paddy field conditions with different N applications in CHAPTER 4. I have chosen a trait called NDT (Wei et al. 2012), the ratio between the trait values under low N to those under farmer's practice (FP); or sufficient N conditions as a parameter to evaluate N effect for plant growth. In CHAPTER 5, for the better understanding of interactions between RSA and NDT, I conducted QTL analysis for agronomic and NDT traits and compared identified RSA QTLs regions. In CHAPTER 6, I reviewed all results in this study to elucidate the interactions among root architecture in hydroponic experiments (CHAPTER 2 and 3) and yield related nitrogen-deficiency tolerance traits under field conditions (CHAPTER 4 and 5) as general discussion. In CHAPTER 7, I summarized the research findings from the previous CHAPTERS and gave suggestions for the future direction of the research.

CHAPTER 2 ROOT ARCHITECTURAL RESPONSE TO NH_4^+

2.1 SECTION 1 SEMINAL ROOT ELONGATION PLASTICITY

2.1.1 INTRODUCTION

"Deeper rooting" that has been considered as an ideal RSA trait to absorb N leached to the deeper soil layers efficiently (Lynch, 2013). However, the deep rooting is a complex trait consisting of the root growth angle and the length in the seminal and crown roots (Araki et al. 2002). Maximum root length and root growth angle are the major factors contributing to control deep rooting. In addition, the root response to selective pressure; plasticity, was observed to help plants forage for nutrients in heterogeneous soils. In this CHAPTER, we focus on two important RSA traits i.e., seminal root length (SECTION 1) and root growth angle (SECTION 2), which may be useful to absorb N efficiency from N leached deep soil layers. In this SECTION, I hypothesized that longer root trait in seedling stage under different NH_4^+ concentrations has potential for enhancing N uptake following Obara et al. (2011) and examined the N-mediated seminal root elongation response under hydroponic controlled conditions at seedling stage.

2.1.2 MATERIALS AND METHODS

Study site

This research was conducted in the greenhouse facilities of the International Center for Tropical Agriculture (CIAT), Palmira in Colombia (3^o30'N, 76^o 21'W; 1000 mm annual rainfall, 965 m above sea level, and 26 ° C in annual average temperature).

Materials

The 15 rice genotypes were used in this experiment, including *indica*, *japonica*, tropical *japonica* and non-*sativa* species (Ogawa et al. 2014a). The genotypes used in this study were originating from Asia, Latin America and Africa (Table 2.1).

Table 2.1 Rice genotypes used in this study

Name	Accession ID	Origin	Group	Ecosystem
IR64	IRGC66970	Philippines	*indica*	Lowland
Koshihikari	JP80825	Japan	*temperate japonica*	Lowland
ANAR2006	BCF2335	Nicaragua	*indica*	Lowland
NERICA4	Unknown	Cote d'Ivoire	tropical *japonica x O. glaberrima*	Lowland
Curinga	BCF2309	Brazil	tropical *japonica*	Upland
Caiapo	BCF873	Brazil	tropical *japonica*	Upland
FEDEARROZ733	BCF2355	Colombia	*indica*	Lowland
Zhenshang97	BCF1988	China	*indica*	Lowland
O. barthii	IRGC101937	Senegal	non-*sativa*	Unknown
O. glaberrima (MG12)	IRGC103544	Mali	African domesticate	Unknown
O. rufipogon	IRGC105491	Malaysia	non-*sativa*	Unknown
FEDEARROZ174	BCF2146	Colombia	*indica*	Lowland
CT21375	BCF2571	Colombia	*indica*	Lowland
Kasalath	IRGC117617	India	*aus*	Lowland
O. glaberrima	TOG5681	Nigeria	African domesticate	Deep forest swamp

Experiments were conducted during the period of September to December, 2012 in controlled greenhouse conditions at CIAT. Before performing the actual experiment, preliminary studies were conducted to set all the experimental conditions and to verify the reproducibility of the results. All the experiments were conducted in hydroponic conditions up to eight days seedling stage from sowing using floating mesh method.

Eight days seminal root length phenotyping with floating mesh method

The seeds of all genotypes used in this study were pre-screened for their germination rate and seedling vigor to ensure their potential for seminal root evaluation. Well-filled seeds were selected by soaking in a sodium chloride (NaCl) solution with gentle shaking. The methods of seed germination and growing seedlings by floating mesh method were adopted from Obara et al. (2010). Seedlings were hydroponically grown in greenhouse conditions with the temperatures ranging from 25 to 30 °C, average relative humidity 50 % with natural sunlight. The composition of the basal nutrient solution was according to Subbarao et al. (2006) with minor modification of pH change from 5.5 to 6.5 (Table 2.2). Germinated seeds were sown on a stainless steel mesh (20 cm x 15 cm) with urethane sponge floating on the basal nutrient solution enriched with N form as NH_4^+ at three concentrations (5, 50 and 500 μM) in a large-scale tank (33 L of solution). The pH of the nutrient solution was monitored every day. If any change was detected, the whole solution was changed immediately. Each stainless steel mesh has 15 rows and twelve seeds per genotype were placed in each row arranged in 2 × 5 mm spacing between rows and seeds. The hydroponic nutrient solution was maintained lower than pH 6.5 throughout the experiment. Eight days after sowing, the seedlings were harvested for further phenotyping. Using the two most contrast genotypes (Curinga and *O. rufipogon*), I tried to evaluate seminal root length response to the other N forms (NO_3^- and NH_4NO_3) with two concentrations (5 and 500 μM).

Table 2.2 Component of CIAT hydroponic solution

Reagent	Molar mass	Concentration
Ammonium Sulfate; $(NH_4)_2SO_4$	132.1	5,50,500 µM
Calcium nitrate tetrahydrate; $Ca(NO_3)_2 \cdot 4H_2O$	236.1	5,50,500 µM
Ammonium nitrate (NH_4NO_3)	80.1	5,50,500 µM
Potassium Sulfate; K_2SO_4	174.3	270 µM
Sodium Phosphate; Na_2HPO_4	142	180 µM
Calcium Chloride Dehydrate; $CaCl_2$ $2H_2O$	147	360 µM
Magnesium Sulfate Heptahydrate; $MgSO_4$ $7H_2O$,	246.5	460 µM
Ethylendiamineteraacetic acid iron(III) Sodium Salt; Fe III EDTA	367.1	45 µM
Boric acid; H_3BO_3	61.83	18 µM
Manganese (II) Sulphate Monohydrate; $MnSO_4$ H_2O	169	4.6 µM
Zinc Sulfate Heptahydrate; $ZnSO_4$ $7H_2O$	287.5	1.5 µM
Cupric Sulfate; $CuSO_4$	249.7	1.5 µM
Sodium Molybdate; Na_2MoO_4	242	1.0 µM

Trait measurement and data analysis

At eight days after sowing, lengths of seminal root and shoot were measured with a ruler. The percent root length plasticity at higher NH_4^+ concentrations was calculated as [seminal root length at high NH_4^+ concentration / seminal root length at low NH_4^+ concentration] × 100. The experiments were repeated twice. Since I obtained a good correlation between the experiments ($P < 0.001$), average data over the two experiments were used for further statistical and QTLs analysis. All statistical analyses were performed

using the XLSTAT, an add-in for EXCEL. The significance of difference of the means between genotypes were determined by Student's t test.

2.1.3 RESULTS

Variation in seminal root elongation in response to NH_4^+ supply among rice genotypes

Significant difference in root length ($P < 0.01$) was found among 15 rice genotypes at different NH_4^+ concentrations (Fig. 2.1). Seminal root length in most genotypes was reduced sharply at higher NH_4^+ concentrations, except for *O. rufipogon, O. glaberrima* (MG12) and Zhenshang97, in which root length was not significantly affected. The percentage of root length plasticity varied significantly (Fig. 2.1). Between low (5 μM) and high (500 μM) NH_4^+ concentrations, Curinga, NERICA4 and FEDEARROZ733 showed highly NH_4^+ sensitive response, with root length being reduced by 49.7%, 47.7% and 52.3%, respectively. On the other hand, Caiapo and IR64 showed moderate sensitivity to NH_4^+, with 35.0% and 35.6% reductions, respectively. Koshihikari and ANAR2006 were least sensitive, with 21.4% and 21.7% reductions, respectively. These results indicate that *O. rufipogon* and *O. glaberrima* (MG12) are not sensitive, being constitutively capable of elongating seminal roots under 5, 50 and 500 μM NH_4^+ concentrations. This insensitivity contrasts with the response in *O. barthii* (Fig. 2.1).

Shoot height growth was less affected by NH_4^+ concentrations. Only *O. barthii* was inhibited sharply when the exogenous NH_4^+ concentration was increased (Fig. 2.1). On the other hand, increased shoot height in proportion to NH_4^+ concentrations was observed in Caiapo, ANAR2006 and CT21375. Shoot height of the other rice genotypes was unchanged across the different NH_4^+ concentrations.

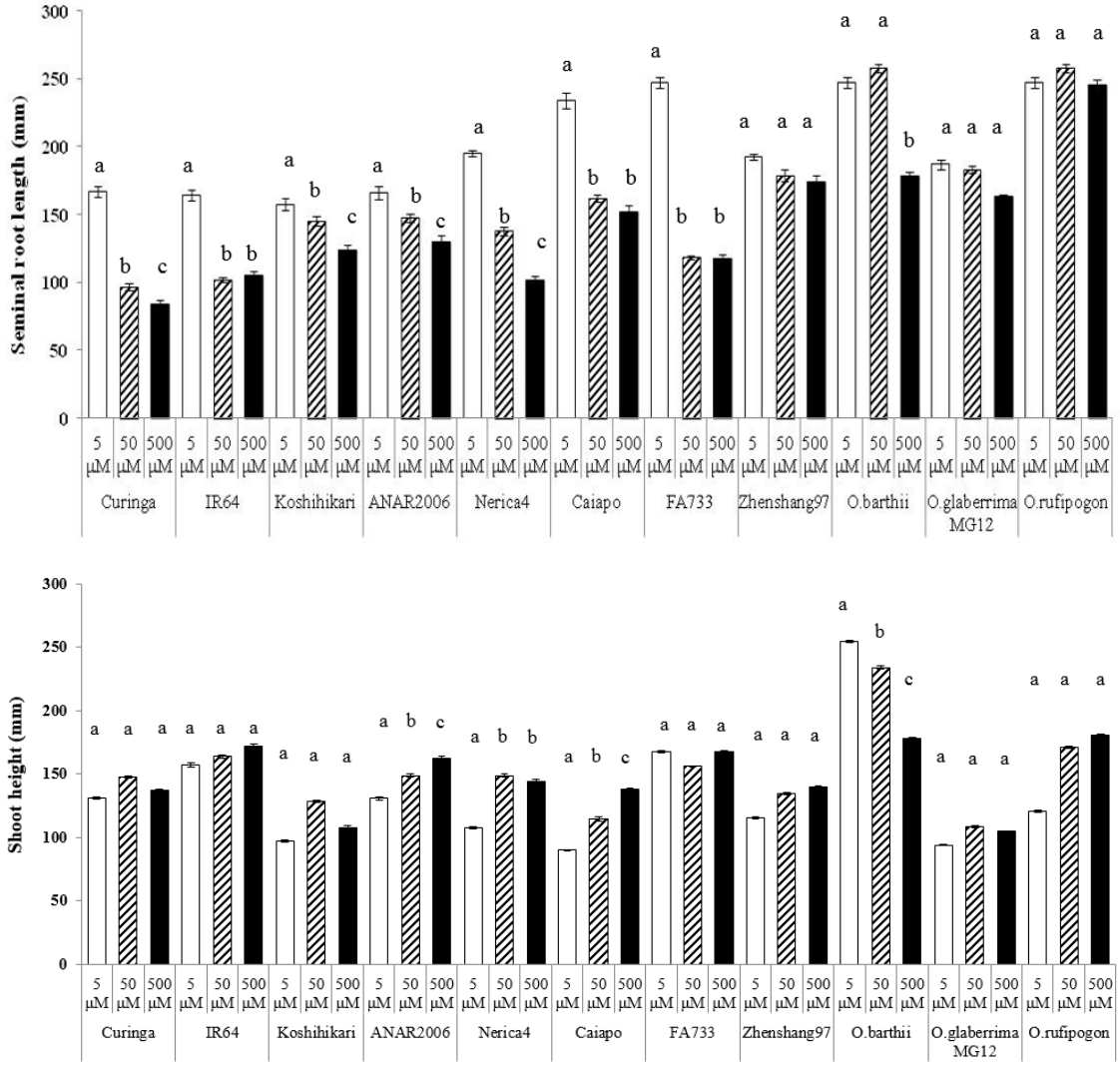


Fig. 2.1 Comparison of seminal root length and shoot height among tested rice genotypes grown in hydroponic culture under a wide range of NH_4^+ concentrations. Data are means of 10 replications ± SD. Means with the same letter within a genotype are not significantly different at $P < 0.05$ according to two-way ANOVA followed by Tukey's test.

Differential response of Curinga and *O. rufipogon* to different N forms and concentrations

There was a significant ($P < 0.001$) difference between Curinga and *O. rufipogon* in the response of seminal root elongation to N forms and concentrations. *O. rufipogon* elongated seminal roots irrespective of N forms and/or concentrations (Fig. 2.2). The percentage of root length plasticity upon exposure to

NH_4^+, NO_3^- or NH_4NO_3 was significantly higher in Curinga (41.2%, 17.5% and 44.5%, respectively) than in *O. rufipogon* (4.1%, 7.3% and 0.3%, respectively) (Fig. 2.2).

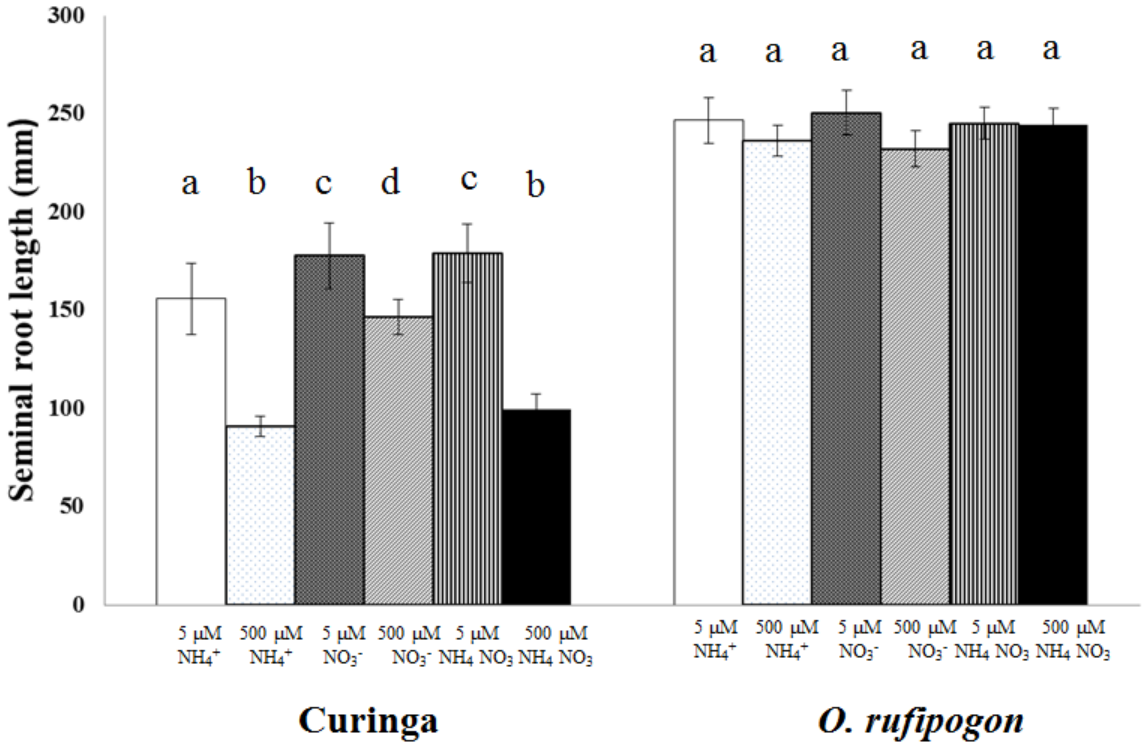


Fig. 2.2 of Comparison of seminal root length Curinga and *Oryza rufipogon* grown in hydroponic culture with three different forms and two concentrations of N. Data are means of 10 replications ± SD. Means with the same letter within genotypes are not significantly different at $P < 0.001$ according to two-way ANOVA followed by Tukey's test.

2.1.4 DISCUSSION

NH_4^+ sensitivity in relation to NAE

Rice is known as a unique plant species tolerant to NH_4^+ excess (Wang et al. 1993). Nevertheless, rice can be negatively affected by the elevated NH_4^+ levels (Balkos et al. 2010) commonly found in agricultural soils, with obvious symptom of stunted root growth (Gerendas et al. 1997; Britto and Kronzucker 2002; Balkos et al. 2010; Roosta and Schjoerring 2008). Chen et al. (2013) also reported the reduction in

vegetative biomass and yield due to excessive NH_4^+ levels. However, to enhance root elongation at the presence of exogenously applied inorganic N (Bloom et al. 2006), and therefore, we should understand root elongation response to NH_4^+ levels at the seedling stage which leads to faster biomass accumulation and higher NUE at later growth stages (Song et al. 2011).

In our study, *O. rufipogon*, *O. glaberrima* and Zhenshang97 consistently displayed enhanced root elongation across different NH_4^+ concentrations, in contrast to other rice genotypes which showed root elongation response to different NH_4^+ concentrations. We therefore classified *O. rufipogon*, *O. glaberrima* and Zhenshang97 as NH_4^+ insensitive genotypes, and characterized the other genotypes as medium or sensitive. Our results suggest that root elongation is governed by a combination of genetic and environmental factors, with some genotypes being more responsive to nutrient availability than others.

Changes in nutrient availability also cause changes in RSA that improve plant adaptation to environmental conditions and/or allow them to efficiently search for limiting nutrients in soil (Linkohr et al. 2002; Zhang et al. 2007; Da Silva and Delatorre 2009). Genetic variation in seminal root length among rice varieties in response to exogenous NH_4^+ concentrations may be attributed to variation in genes associated with N pathways (Obara et al. 2011).

Our results also suggest that *O. glaberrima* and *O. rufipogon* harbor alleles for root architectural traits that may be useful to breeders working with *O. sativa*. *O. glaberrima* has been previously used as a donor of many useful agronomical traits, such as resistance to drought or blast disease (Jones et al. 1997). Similarly, *O. rufipogon* has been utilized as a source of biotic and abiotic stress tolerance (Brar and Khush 1997) and as a source of yield-enhancing alleles for *O. sativa* rice cultivar (Imai et al. 2013; Fu et al. 2010). The high cross ability of *O. rufipogon* and *O. sativa* suggests that this progenitor species represents a valuable source of useful traits for rice improvement, including root plasticity and root architectural traits related to nutrient uptake.

Seminal root response to different N forms

Although NH_4^+ is generally considered to be the preferred form of N nutrition for rice plants (Wang et al. 1993), in recent years researchers have increasingly focused on NO_3^- nutrition which partially replacing NH_4^+ (partial nitrate nutrition [PNN]) of rice crops. It has recently been shown that lowland rice is exceptionally efficient in absorbing NO_3^-, which is formed by nitrification in the rhizosphere (Duan et al. 2007). In my study, I prepared hydroponic solution with three different forms of N; PNN (NH_4NO_3 [50:50]), NH_4^+ and NO_3^-. *O. rufipogon* seedlings constitutively elongated seminal roots irrespective of the forms of N applied (Fig. 2.4). These results confirmed that this genotype possesses a constitutive trait for seminal root elongation. Conversely, the response of Curinga leads to hypothesize that Curinga possesses different mechanisms for elongating its seminal roots in response to PNN and either NH_4^+ or NO_3^- (Fig. 2.4). When the NH_4^+ form alone is supplied at high concentration, many plant species are affected by ammonium toxicity which can be alleviated by co-provision of nitrate (Kronzucker et al. 1999; Roosta and Schjoerring 2008). The effect of this PNN on root growth is partly dependent on plant genotype (Song et al. 2011). The results of this study, therefore, suggest that N preference varies within genotypes.

NH_4NO_3 has been widely used as the N form supplied to plants when mapping QTLs for root system architecture (RSA) in hydroponic conditions (Lian et al. 2005; Price et al. 1997; Shimizu et al. 2004; Xu et al. 2004). Distinguishing the individual effects of NH_4^+ and NO_3^- is difficult, however, as root elongation response varies depending on the applied form of N. In our study, seminal root length among different forms of N (NH_4^+, NO_3^- or NH_4NO_3) was correlated (R = 0.89 to 0.93; $P < 0.01$). These results imply the existence of multiple N assimilation mechanisms for different forms of N in rice, similar to what has been reported that root length of *Arabidopsis thaliana* was positively correlated to one another among all of N treatments (NH_4^+, NO_3^- or NH_4NO_3) (Rauh et al. 2002).

2.2 SECTION 2 ROOT GROWTH ARCHITECTURE PLASTICITY

2.2.1 INTRODUCTION

As I mentioned in INTRODUCTION of CHAPTER 2 SECTION 1, deeper rooting has been considered as an ideal RSA trait to absorb N efficiently from N leached to deeper soil layers (Lynch 2013). In SECTION 1, I focused on maximum seminal root length, as one of the complex traits, deep rooting. In this SECTION 2, I focus on the other important trait relating to deep rooting, root growth angle. Root growth angle in rice has also been quantitatively characterized by Kato el al. (2006) and Uga et al. (2009). In their studies, a ratio called RDR, "ratio of deep rooting" was used to evaluate the roots growth angle (Oyanagi et al. 1993). This ratio indicates the percentage of vertically growing roots (deep root) that were distinguished by the border line for the root growth angle at 50° on the basket. However, so far limited reports are available to understand the response of RSA traits in rice under different N treatments, because each root growth shows plasticity for the survival of plants under continuously changing heterogeneous environmental conditions. Therefore, in this SECTION, I focused on the response of RSA; especially root growth angle under hydroponic conditions with different N supply in more details.

2.2.2 MATERIALS AND METHODS

This research was conducted in the greenhouse facilities of CIAT as was mentioned 2.1.2.

The nine genotypes from diverse rice genotypes were studied in this experiment including *indica*, *japonica*, tropical *japonica* and non-*sativa* species selected as the result of SECTION 1. The genotypes used in this study were originated from Asia, Latin America and Africa (Table 2.3).

Table 2.3 Rice genotypes evaluated in this study

Name	Accession ID	Origin	Group	Ecosystem
IR64	IRGC66970	Philippines	*Indica*	Lowland
NERICA4	Unknown	Cote d'Ivoire	tropical *japonica x O. glaberrima*	Lowland
Curinga	BCF2309	Brazil	tropical *japonica*	Upland
Caiapo	BCF873	Brazil	tropical *japonica*	Upland
FEDEARROZ174	BCF2146	Colombia	*Indica*	Lowland
O. barthii	IRGC101937	Senegal	non-*sativa*	Unknown
O. glaberrima (MG12)	IRGC103544	Mali	African domesticate	Unknown
O. glaberrima	TOG5681	Nigeria	African domesticate	Deep forest swamp
O. rufipogon	IRGC105491	Malaysia	non-*sativa*	Unknown

Variation in RSA among rice genotypes under different NH_4^+ conditions

The basal nutrient solution used in this study was the same as described by Subbarao et al. (2006) with modification of pH from 5.5 to 6.5 (Table 2.2). Three concentrations of NH_4^+ supplied in the form of $(NH_4)_2SO_4$ were used: 5, 50 and 500 μM as control, low and high treatments, respectively (Obara et al. 2011). The experiments were conducted in randomized complete block design with three replications.

I used the root basket method developed by Uga et al. (2009) with minor modifications to evaluate selected genotypes listed in Table 2.3. A stainless steel mesh basket (6 cm in top diameter, 1.5 cm in bottom diameter, 6 cm in height and 0.5 mm in mesh size; Rejilla para lavaplatos, Bemor International Ltd., Colombia) was used together with the polyvinyl chloride tube (8 cm in diameter and 12 cm in height). The polyvinyl chloride tube to supports basket was not filled with soil as was in the original

method. Instead, the opaque polyvinyl chloride tube was used so that the root growth can be monitored easily. The basket was filled with river sand instead of soil in the original method to support plant base. To confirm the reliability of the modified method, I have selected some common rice genotypes already evaluated by Uga et al. (2011) such as IR64, Kinandang Patong and *DRO1*-NIL, and confirmed that the results of root growth were basically the same in both methods.

Well-filled seeds were incubated at 30 ℃ for 2 days with wet paper towels in an incubator for germination, and then each pre-germinated seed was carefully placed on the sand in the basket at the center and then the baskets were placed on the polyvinyl chloride tubes arranged in plastic tanks containing water. Seven days after sowing (DAS), only uniform seedlings in the basket were transferred to the plastic tanks with 33 L of hydroponic solution containing different NH_4^+ concentrations. The distance between the tubes was 2 cm. The solutions were replaced every five days.

At 40 DAS, maximum root length, dry root biomass, deep root number, shallow root number, shoot height, dry shoot biomass, tiller number, SPAD reading at the middle of top leaf using a SPAD-502 Chlorophyll Meter (Konica Minolta Inc., Tokyo, Japan) were measured. Deep root number and shallow root number were identified root growth angles 50 – 90º with respect to horizontal and rest, respectively. Vertically growing roots and more horizontally growing roots were distinguished by the border line for the root growth angle at 50° on the basket. Vertically growing deep roots are the ones penetrate the mesh within the border of basket, and on the contrary, more horizontally growing shallow roots are the ones that penetrate the mesh outside of the border. These numbers of the deep and shallow roots were used to calculate the ratio of deeper root (RDR), one of the indicators to evaluate the root growth angle according to the protocol described by Oyanagi et al. (1993) and Uga et al. (2009). RDR was calculated by dividing the number of roots that penetrated through the bottom of the basket mesh (deeper roots) with the total root number that penetrated the whole mesh using following formula.

RDR = Deep root number / Total root number

Low RDR indicates shallow (< 50º) and high RDR indicates deep root system (≥ 50º). The experiment was repeated two times. While repeating the experiment, in addition to the above said root and shoot traits, changes of maximum root length, number of deep and shallow roots were recorded every two days until the end of experiment.

In order to classify the monomorphic and dimorphic root system, we derived the rooting pattern value (RPV) using the following formula.

RPV = | Deep root number - Shallow root number |

I classified RPV of less than 10 as dimorphic root system and more than 10 as monomorphic-deep or shallow rooting pattern. The RDR of these rooting pattern empirically corresponded to 40-60, higher than 60, and lower than 40, respectively.

In addition, root-shoot ratio was calculated by dividing dry root biomass with dry shoot biomass.

Analysis of variance (ANOVA) based on randomized complete block design was carried out for all plant characteristics. All statistical analyses were performed using GLM procedures of ANOVA (SAS Institute Inc. 2004, SAS/STAT, 9.1).

2.1.3 RESULTS

Root system architecture responses to NH_4^+ concentrations

Significant variation (ANOVA, $P < 0.01$) of selected parameters among rice genotypes was obtained under different concentrations of NH_4^+ (Tables 2.4; 2.5). All genotypes had different maximum root length between 50 and 500 µM NH_4^+ concentrations except IR64 (Table 2.4). Root biomass was found to be the most sensitive trait and it increased with increasing concentration of NH_4^+, but the percent increment varied among the genotypes (Table 2.6). The total root number of Curinga, Caiapo and *O.*

glaberrima (MG12) increased proportionally to NH_4^+ concentrations, but surprisingly opposite trend was observed in *O. rufipogon* where the root number decreased with increasing NH_4^+ concentrations. Among the tested genotypes, only Curinga showed different RDR between 50 and 500 µM NH_4^+ concentrations (Table 2.4). In addition, there was no significant difference in the number of deep roots between 50 and 500µM NH_4^+ concentrations (17.67 ± 0.88, 16.67 ± 2.40, respectively). However, the number of shallow roots increased sharply (from 5.3 ± 0.88 to 17.00 ± 0.58) with increasing concentrations of NH_4^+. Thus it was elucidated that the development of shallow roots in Curinga is highly influenced by the concentration of NH_4^+. Except Curinga and *O. rufipogon*, all other genotypes did not show any significant change in the number of deep and shallow roots under different NH_4^+ concentrations. Surprisingly, *O. rufipogon* showed 50% reduction in the numbers of deep and shallow roots when the NH_4^+ concentrations increased to 500 µM. The RPV varied significantly among the genotypes studied. IR64, NERICA4, Caiapo, FEDEARROZ174, *O. glaberrima* (MG12 and TOG5681) and *O. rufipogon* showed no change in their rooting pattern between 50 and 500µM NH_4^+ concentrations (Table 2.6). However, Curinga and *O. barthii* had changed their rooting pattern from monomorphic to dimorphic (Table 2.6). Based on the normal range of NH_4^+ concentration (20 – 200 µM) available in the field conditions (Owen and Jones 2001), I classified the rice genotypes as monomorphic-shallow root system (IR64), monomorphic-deep root system (Curinga, NERICA4, *O. barthii*) and dimorphic root system (Caiapo, FEDEARROZ174, *O. glaberrima* (TOG5681), *O. glaberrima* (MG12) and *O. rufipogon*) from RDR and RPV at 50 µM NH_4^+ concentration (Fig. 2.4).

The effect of NH_4^+ on shoot growth

In contrast to root growth, shoot traits such as plant height, shoot biomass, tiller number and SPAD value were found to be increased with NH_4^+ concentrations (Table 2.5). In particular, shoot biomass increased sharply when the NH_4^+ concentration increased, but the percent increment varied among the genotypes

studied. Percentage increase in shoot biomass between 50 and 500 μM was observed to be higher than that in root biomass.

Table 2.4 Root growth variation response to different NH_4^+ concentrations

	Maxuimum Root length (cm)			Dry Root biomass (mg)			Total Root number			RDR		
Genotype	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM
IR64	24.33±0.88 a	21.67±1.20 a	18.00±1.46 a	123.0±4.1 a	244.3±4.1 b	600.0±10.6 c	14.00±1.00 a	39.33±2.02 b	39.67±3.84 b	47.9±5.7 a	23.5±3.6 b	23.8±2.8 b
Nerica4	37.33±3.18 a	43.00±7.37 a	21.00±2.52 b	90.3±2.8 a	186.3±4.1 b	342.3±9.9 c	6.00±0.00 a	21.00±3.21 a	22.67±4.81 a	94.4±5.5 a	76.1±2.9 ab	66.1±1.9 b
Curinga	29.00±1.15 a	28.00±2.08 a	19.33±1.20 b	99.0±7.2 a	228.0±10.0 b	426.0±7.8 c	9.33±1.45 a	23.00±0.57 b	33.67±2.02 c	97.2±2.7 a	76.6±3.7 b	49.0±2.0 c
Caiapo	33.33±1.67 a	38.33±2.19 a	26.33±2.67 b	93.7±4.4 a	181.3±3.8 b	251.0±10.6 c	8.33±1.45 a	22.00±3.60 ab	33.33±3.18 ac	72.8±6.0 a	54.1±3.4 a	59.6±2.4 a
Fedearroz174	26.33±2.90 a	22.00±1.15 ab	15.33±0.33 b	97.6±2.3 a	364.3±6.4 b	540.7±8.9 c	9.67±1.76 a	36.33±2.02 b	29.33±2.85 b	80.1±2.8 a	57.4±1.8 b	61.8±1.5 b
O.barthii	49.67±3.48 a	45.67±1.85 ab	26.00±1.73 c	37.0±2.8 a	80.3±3.5 b	324.6±13.5 c	12.30±0.67 a	38.67±4.06 b	45.00±4.04 b	81.6±10.0 a	68.2±2.6 a	53.2±3.3 a
O. glaberrima (MG12)	34.00±3.05 a	44.00±1.15 b	26.33±1.86 c	53.0±2.3 a	166.0±3.8 b	319.3±7.6 c	6.33±0.88 a	23.33±2.60 b	32.33±0.88 c	86.1±7.3 a	60.7±4.0 ab	50.4±2.6 b
O. glaberrima (TOG5681)	41.00±1.52 a	47.33±2.90 a	28.33±1.76 b	105.3±5.5 a	188.7±5.5 b	362.3±6.4 c	6.00±0.00 a	26.00±1.73 b	31.00±0.58 b	67.5±9.6 a	57.8±4.0 a	61.2±3.9 a
O. rufipogon	40.33±2.96 a	45.67±0.33 ab	32.67±1.67 ac	104.3±1.4 a	401.7±7.4 b	515.0±4.7 c	16.33±1.20 a	50.67±2.91 b	26.33±0.33 c	67.5±4.3 a	53.4±4.4 ab	46.8±2.7 b

Data are mean ± SE of three replications; diferent letters indicate significant difference among NH_4^+ concentrations in each rice ecotype (Tukey test, $P < 0.05$)

Table 2.5 Shoot growth variation response to different NH_4^+ concentrations

	Plant height (cm)			Dry Shoot biomass (mg)			Tiller number			SPAD		
Genotype	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM	5 μM	50 μM	500 μM
IR64	28.50±1.80 a	38.17±3.20 a	41.33±4.80 a	238.7±5.6 a	406.7±1.2 b	1699.7±6.4 c	1.00±0.00 a	3.00±0.00 b	8.00±0.00 c	19.37±1.39 a	23.36±1.23 ab	35.10±1.60 c
Nerica4	21.17±1.92 a	42.00±1.53 b	47.67±1.20 b	246.3±6.4 a	391.7±8.2 ab	1188.3±57.5 c	1.00±0.00 a	2.67±0.33 b	3.67±0.33 b	21.90±2.87 a	33.10±2.00 ab	41.40±2.48 b
Curinga	27.67±1.01 a	39.17±2.53 a	45.83±8.92 a	219.3±1.4 a	421.0±4.6 b	1364.0±4.8 c	1.00±0.00 a	2.33±0.33 b	5.33±1.20 b	15.40±3.47 a	26.20±0.57 ab	39.03±1.19 c
Caiapo	29.33±1.20 a	45.67±1.45 b	54.00±5.29 b	213.7±6.1 a	270.3±7.5 ab	1149.7±28.9 c	1.00±0.00 a	2.67±0.33 b	4.00±0.00 c	18.50±0.90 a	28.57±2.07 b	37.53±2.54 b
Fedearroz174	26.67±1.92 a	34.17±2.49 ab	41.33±0.88 b	119.0±2.6 a	490.0±5.7 b	1450.3±12.1 c	1.00±0.00 a	2.67±0.33 a	7.67±0.88 b	13.83±2.39 a	24.83±1.72 b	36.40±1.44 c
O.barthii	27.50±0.76 a	42.17±3.21 b	53.33±3.18 b	91.0±5.2 a	246.7±9.8 ab	1140.0±91.6 c	1.00±0.00 a	3.67±0.33 a	11.33±0.88 b	11.63±2.04 a	22.47±0.66 b	33.63±0.52 c
O. glaberrima (MG12)	25.33±2.48 a	43.33±3.33 ab	45.83±1.92 b	137.3±7.9 a	381.6±8.2 b	1143.3±35.2 c	1.00±0.00 a	2.00±0.57 ab	4.67±0.88 b	14.50±0.17 a	26.27±0.93 b	34.70±0.23 c
O. glaberrima (TOG5681)	28.33±0.44 a	50.50±4.80 b	58.83±3.32 b	235.3±6.4 a	407.7±4.8 b	1478.7±48.4 c	1.00±0.00 a	2.33±0.33 a	5.33±0.33 b	14.40±1.35 a	27.43±0.96 b	37.37±0.24 c
O. rufipogon	39.17±1.92 a	50.33±1.45 ab	64.67±3.28 c	229.0±2.6 a	499.0±7.2 b	1700.0±14.9 c	1.00±0.00 a	3.67±0.33 ab	6.00±1.15 c	16.83±1.49 a	25.07±0.54 b	33.77±0.55 c

Data are mean ± SE of three replications; diferent letters indicate significant difference among NH_4^+ concentrations in each rice ecotype (Tukey test, $P < 0.05$)

Table 2.6 Root-shoot-ratio and RPV variation response to different NH_4^+ concentrations

	Root-shoot-ratio			RPV			Rooting pattern		
Variety	5 µM	50 µM	500 µM	5 µM	50 µM	500 µM	5 µM	50 µM	500 µM
IR64	0.52±0.013 a	0.60±0.034 a	0.35±0.017 a	0.67±0.41 a	20.67±0.09 b	21.00±0.07 b	Dimorphic	M. Shallow	M. Shallow
NERICA4	0.37±0.028 a	0.48±0.020 a	0.29±0.058 b	5.33±0.33 a	11.00±0.14 b	12.67±0.39 b	Dimorphic	M. deep	M. deep
Curinga	0.45±0.051 a	0.54±0.022 a	0.31±0.016 a	8.67±0.29 a	12.33±0.16 b	0.33±0.06 c	Dimorphic	M. deep	Dimorphic
Caiapo	0.44±0.014 a	0.67±0.020 b	0.22±0.027 c	3.67±0.42 a	1.33±0.15 a	6.67±0.07 b	Dimorphic	Dimorphic	Dimorphic
FEDEARROZ174	0.82±0.011 a	0.74±0.011 a	0.37±0.014 b	5.67±0.29 a	5.67±0.05 a	7.60±0.05 a	Dimorphic	Dimorphic	Dimorphic
O.barthii	0.41±0.019 a	0.33±0.028 a	0.28±0.068 a	7.67±0.78 a	14.00±0.07 b	3.00±0.07 c	Dimorphic	M. deep	Dimorphic
O. glaberrima (MG12)	0.39±0.034 a	0.44±0.022 a	0.28±0.046 a	4.33±1.15 a	5.33±0.17 a	0.33±0.08 b	Dimorphic	Dimorphic	Dimorphic
O. glaberrima (TOG5681)	0.45±0.012 a	0.46±0.011 a	0.25±0.076 b	4.00±1.6 a	4.00±0.15 a	7.00±0.13 b	Dimorphic	Dimorphic	Dimorphic
O. rufipogon	0.46±0.018 a	0.81±0.010 b	0.30±0.032 c	5.67±0.26 a	4.00±0.09 a	1.67±0.10 b	Dimorphic	Dimorphic	Dimorphic

Data are mean±SE of three replications; diferent letters indicate significant difference among NH_4^+ concentrations in each rice ecotype (Tukey test, $P < 0.05$). M.shallow and M. deep indicate monomorphic shallow and monomorphic deep, respectively.

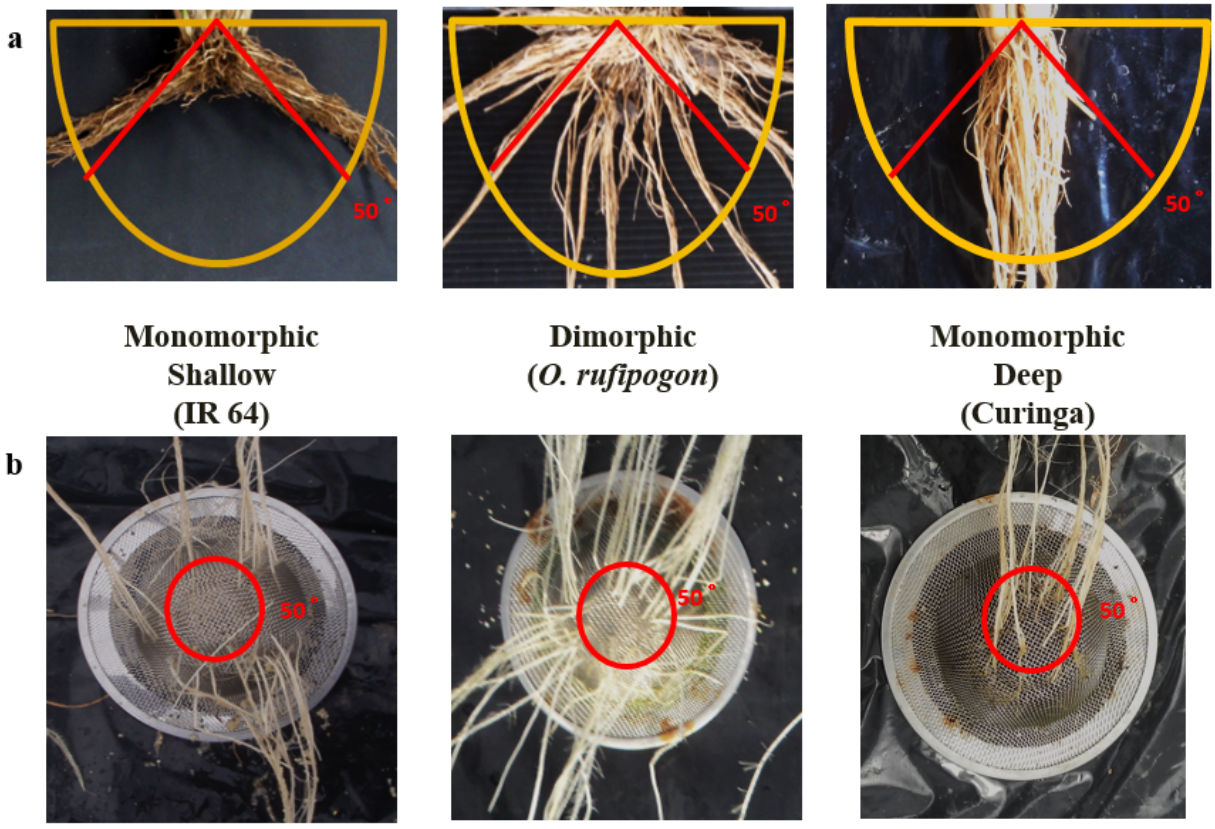


Fig. 2.3 Monomorphic and dimorphic rooting pattern of selected rice genotypes

(a) Rooting pattern classification at maturity stage in the field trial using basket method. Yellow line around the roots indicates basket area where deep and shallow roots penetrate and red lines indicates 50 degree angle (b) 40 days hydroponic study using basket method. Red circles show the boundary where the deep and shallow roots penetrate the basket.

N responsive root growth dynamics

The dynamics of maximum root length, total root number and RDR under different NH_4^+ concentrations over the entire growth period were found consistent between the treatments. I showed an example of root growth dynamics pattern of important representative genotypes like IR64 (monomorphic-shallow), Curinga (monomorphic-deep) and *O. rufipogon* (dimorphic) in Fig. 2.4. In all rice genotypes, deep roots appeared earlier than shallow roots; therefore, they showed higher RDR in early growth stages. The maximum root length, root number and RDR responded differently over the period of time under different NH_4^+ concentrations. Maximum root length started to be different among NH_4^+ treatments from 7 DAS, but the number of total roots showed differences among NH_4^+ treatments only after 14 DAS (Fig. 2.4). The growth trend of the maximum root length and total root number was quite similar to all genotypes studied. In RDR, before 27 DAS, we did not notice any difference among NH_4^+ treatments but at 40 DAS some genotypes showed different RDR responses to NH_4^+ concentrations (Fig. 2.4).Based on these results, we concluded that maximum root length and total root number are earlier responsive root traits to NH_4^+ concentrations than root growth angle.

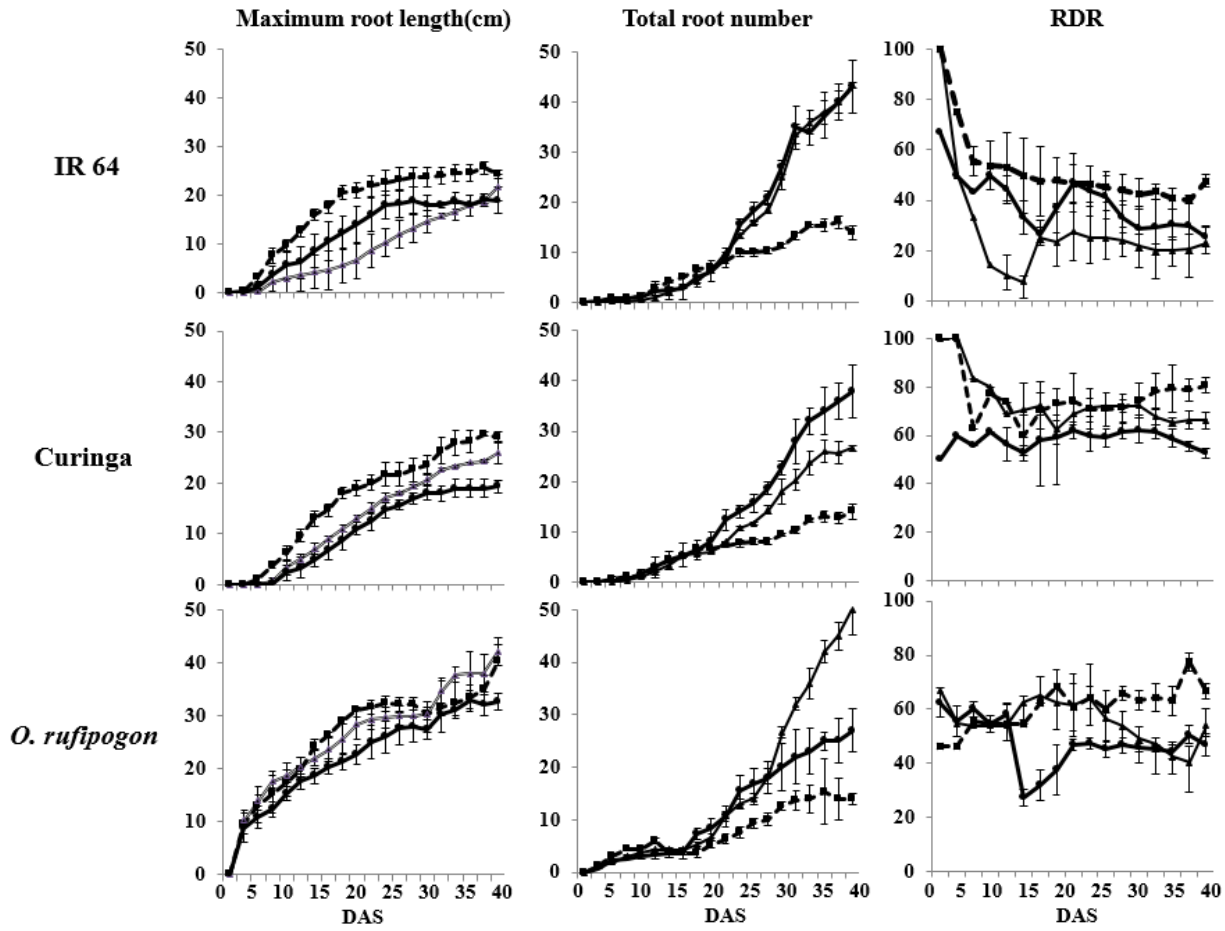


Fig. 2.4 Time courses of root growth; maximum root length, total root number and RDR in different RSA rice genotypes

Plot shows mean ± SE (n=3), Each line with square, triangle and circle showed mean of RDR in 5, 50 and 500 μM NH_4^+ concentrations

2.2.4 DISCUSSION

N-responsive root architectural changes in rice

RSA is a trait that exhibits significant plasticity because of its sensitivity to soil environmental factors. However, to a significant degree it is hereditarily controlled as suggested by surveys of its natural genetic variation in rice (Uga et al. 2013; Lynch 2011; Kato et al. 2006). A key controller of RSA has been QTL through the natural variation approach in the di-cotyledon model, *Arabidopsis* (Kellermeier et al. 2013).

This provides a proof of principle that allelic variation for RSA traits exists, is genetically tractable, and might be exploited for crop breeding (Pacheco and Hardtke 2012).

Here, we investigated root architectural changes in rice response to external NH_4^+ supply. I quantified root traits of seedlings grown on control (5 μM), low (50 μM) and high (500 μM) NH_4^+ concentrations and found significant contributions of genotype and genotype-treatment interactions. Analysis of individual genotype based on root phenotypic data revealed a gradient of sensitivity towards different NH_4^+ concentrations. Our previous study (CHAPTER 2 SECTION 1, 8 days) on the response of seminal root length to NH_4^+ indicated the presence of natural variation among rice genotypes. *O. rufipogon* and *O. glaberrima* (MG12) did not show any plasticity to in the seminal root length in response to various NH_4^+ concentrations (Ogawa et al. 2014a). Conversely, in the present study (40 days) these non-*sativa* species showed root plasticity in the traits such as maximum root length and total root number (Table 2.4). Interestingly, this trend was reversed in case of IR64. This result indicates that RSA plasticity to NH_4^+ levels is stage- and genotype- dependent and it is developmentally regulated as Grossman and Rice (2012) reported for the root plasticity in barley. The RSA response to nutrient level and form, and its relationship with the level of domestication are the research topics of the future.

In the previous studies using seminal root length (Obara et al. 2010, 2011), it was suggested that NH_4^+ insensitive seminal roots may be useful for NAE under N deficit conditions. However, plasticity to nutrient concentrations is also one of the useful traits for NAE (Gruber et al. 2013). Root plasticity is a trait that can invest more resources to root systems, when grown in low-nutrient soils plants allocate more root to seek higher-nutrient locations (Grossman and Rice 2012). In our study, *O. rufipogon* showed highest root number under 50 μM NH_4^+ concentrations than in other concentrations (Table 2.4). This unique plasticity might be useful to improve NAE. Results of this study are important to understand the mechanism of root plasticity under low nutrient conditions. Since dry matter fractionation in cereal crops

is conservative across the levels of domestication (Wacker et al. 2002), there may be potential to incorporate traits for constitutive RSA trait and greater root plasticity from non-*sativa* rice into genotypes that can translate such plasticity into increased yield in nutrient deficient soils.

2.3 CONCLUSIONS

In this study, we have demonstrated that different N forms and concentrations have remarkable and even contrasting effects on root growth such as seminal root length and root growth angle in rice. Root system architectural traits were investigated at eight days using mesh float method and at 40 days using root basket method, and significant variation in NH_4^+ responsive root architectural changes were observed among studied rice genotypes. Most of genotypes showed high plasticity to different N forms and concentrations. However, we also found interesting trait, i.e., insensitive response to N forms and concentrations. Seminal roots of some non-*sativa* species such as *O. glaberrima* and *O. rufipogon* were not inhibited in their elongation in higher NH_4^+ concentration. Similar to seminal root length response root growth angle as RDR of *O. barthii* and *O. glaberrima* were not affected by the NH_4^+ concentration. It is to be studied in the future whether these plasticities in root length and root architecture may be useful to increase nutrient uptake in rice breeding program. To the best of our knowledge, this is the first report revealing the influence of different N forms and concentrations on seminal root elongation and root angle in rice.

CHAPTER 3 QTL ANALYSIS FOR ROOT SYSTEM ARCHITECTURE RESPONSE TO NH_4^+

3.1 SECTION 1 SEMINAL ROOT ELONGATION QTLS

3.1.1 INTRODUCTION

QTL analysis has become a powerful tool for identifying the genetic factors influencing quantitative traits, and it provides useful information for understanding the processes of the complex traits such as root system architecture (RSA). Several QTLs for root traits have been reported in rice under various conditions (Uga et al. 2011; Redoña and Mackill 1996; Price and Tomos 1997), they did not, however, evaluate the root growth under different N forms and concentrations. In rice, Obara et al. (2010) have described adaptive and constitutive QTLs associated with root elongation in response to NH_4^+ concentrations, and recently identified major constitutive QTLs for seminal root elongation in response to NH_4^+ (Obara et al. 2011). Information is lacking, however, for the root response to other N forms such as NO_3^- and NH_4NO_3. Because rice can take up both NH_4^+ and NO_3^- forms N simultaneously, it is essential to investigate the root trait response to different inorganic N forms. In this CHAPTER, genetically controlled components of RSA will be quantitatively characterized by QTL analysis. Detected QTLs will be useful for future root breeding program.

3.1.2 MATERIALS AND METHODS

Material development for QTL analysis

In this research, we used 48 CSSLs (BC_3F_3) developed by CIAT Rice Genomic Laboratory in collaboration with Cornell University in 2008 (Fig. 3.1). These lines derived from a cross between Curinga and *O. rufipogon*, where Curinga (CT11251-7-2-M-M-BR1) is a major Brazilian commercial upland variety with drought tolerance ability (Sakai et al. 2010) and *O. rufipogon* (IRGC105491) originating from south Asia (Yeo et al. 1994). It has utilized as the source for QTL to increase yield (Thomson et al. 2003). These parents and CSSLs were selected based on the result of our preliminary study (See CHAPTER 2 SECTION 1). The roots of Curinga showed sensitive response to the exogenous

NH_4^+ concentrations, while *O. rufipogon* did not. In CSSLs developing process, *O. rufipogon* was crossed with Curinga to produce F_1 plants. The F_1 plants were backcrossed three times with Curinga and selfed to obtain BC_3F_3 population. In each backcross generation, plants heterozygous for the target region were selected by using SNPs markers for further backcrossing or self-pollination (Arbelaez et al. 2015). Additionally, I surveyed genotypes of whole chromosomes using 238 SNPs markers. Selected BC_3F_2 plants were backcrossed with Curinga and self-pollinated. Developed BC_4F_3 plants were selected by marker assisted selection using 238 SNPs markers. Position of the markers closest to QTLs in CSSLs population derived from a cross between Curinga and *O. rufipogon* was shown Fig 3.2.

Response of seminal root elongation in parental and CSSLs to various forms and concentrations of N

In order to study the N response of seminal root length to N and to detect the QTLs contributing that trait, 48 CSSLs derived from Curinga × *O. rufipogon* along with their parents were tested with three different N forms; $(NH_4)_2SO_4$ as NH_4^+ form**,** Ca $(NO_3)_2$ as NO_3^- form and NH_4NO_3 as mixed at low (5 μM) and sufficient (500 μM) concentrations. The seedling growth conditions, seed arrangement and experimental design, harvest method are the same as explained in experiment in CHAPTER 2 SECTION1. Experiments were independently conducted with each N forms (NH_4^+, NO_3^- or NH_4NO_3) with two temporal replications.

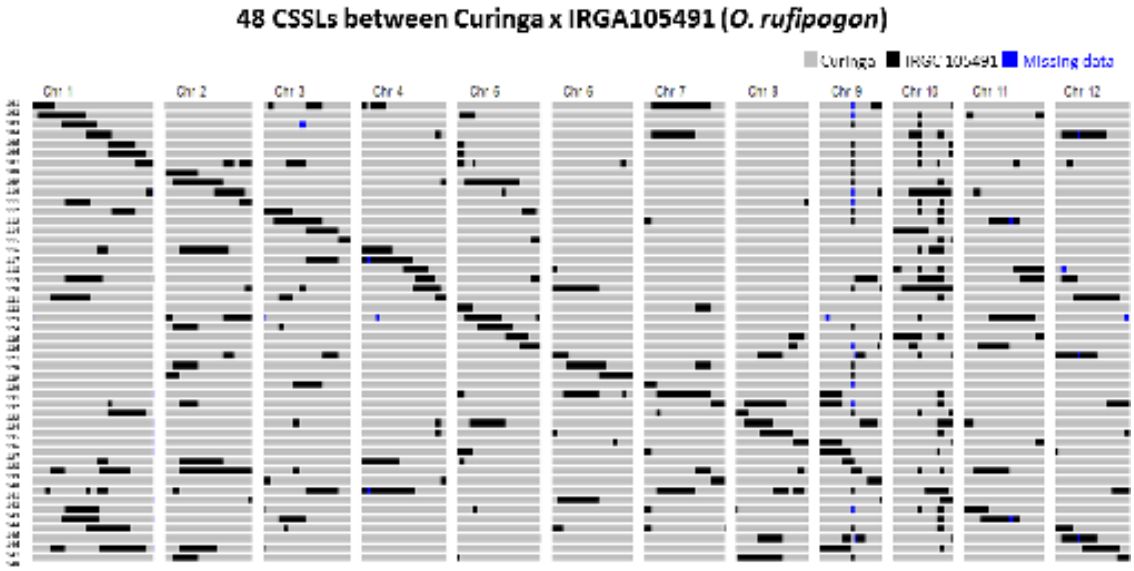


Fig. 3.1 Graphical representation of genotype of 48 CSSLs. The 12 rice chromosomes are arranged horizontally. They are covered by 238 SNPs markers. The genotypes are displayed vertically.

Fig. 3.2 Position of the markers for this study in CSSLs derived from a cross between Curinga and *O. rufipogon* Chromosome numbers are indicated above and the marker names are indicated to right of each linkage map with physical map position (Mb).

QTL analysis

QTL for each experiment were detected based on student *t*-test of the difference between the mean value for each CSSL and 'Curinga', the recurrent parent of the CSSL. QTL detections were performed with two temporal replications. A significant threshold of $P < 0.001$ was used for QTL detection in this study to avoid false-positives. Linkage rough maps with 238 DNA markers were constructed from genotype data with CSSL finder v. 0.84 computer program (Lorieux 2005). Averaged values of both shoot and root traits of floating mesh method as I mentioned in CHAPTER 2 SECTION 1) were used. In addition to shoot and root traits, the percentage of root length at low N concentrations (5 μM) to high concentration (500 μM) in each N form was used as the indicator of root plasticity to N level.

3.1.3 RESULTS

Variation in seminal root elongation among CSSLs

A total of 48 CSSLs were used to locate QTLs (chromosomal regions) controlling seminal root length in response to three different N forms (NH_4^+, NO_3^- and NH_4NO_3) and two different concentrations (5 and 500 μM). Curinga had shorter seminal roots (155.9 ± 18.1 mm) than *O. rufipogon* (246.6 ± 11.7 mm) when seedlings were grown under 5 μM NH_4^+. Seminal root lengths of the 48 CSSLs ranged from 133.8 to 214.4 mm. Significant differences ($P < 0.01$) in seminal root length were observed between 18 CSSLs and Curinga (Fig. 3.3a). Seminal root lengths of 14 lines were longer than Curinga, whereas it was shorter than Curinga in four lines (Fig. 3.3a). When plants were grown in 500 μM NH_4^+, the seminal root length of Curinga was 90.9 ± 4.9 mm and that of *O. rufipogon* was 236.5 ± 7.8 mm. Seminal root lengths of the 48 CSSLs ranged from 62.3 to 157.5 mm (Fig. 3.3b). Significant differences ($P < 0.01$) in seminal root length were detected between 35 CSSLs and Curinga (Fig. 3.3b). Seminal roots of 23 lines were longer than Curinga, while they were shorter in 12 lines (Fig. 3.3b). Under 5 μM NO_3^- conditions, the seminal root length of Curinga was 177.6 ± 16.9 mm and that of *O. rufipogon* was 250.5 ± 11.2 mm. Seminal root lengths of the 48 CSSLs ranged from 100.3 to 199.2 mm. Significant differences ($P < 0.01$) in seminal

root length were observed between 29 CSSLs and Curinga (Fig. 3.3c). Seminal root lengths of two lines were longer than Curinga, and those of 27 were shorter (Fig. 3.3c). When plants were grown in 500 μM NO_3^-, the seminal root length of Curinga was 146.5 ± 9.0 mm and that of *O. rufipogon* was 232.5 ± 9.3 mm. Under these conditions, seminal root lengths of the 48 CSSLs ranged from 95.4 to 182.3 mm (Fig. 3.3d). Significant differences ($P < 0.01$) in seminal root length were observed between 32 CSSLs and Curinga (Fig. 3.3d). Seminal roots of eight lines were longer than Curinga, whereas 24 lines had shorter seminal roots (Fig. 3.3d). When seedlings were grown with 5 μM NH_4NO_3, the seminal root length of Curinga was 179.0 ± 14.6 mm and that of *O. rufipogon* was 245.1 ± 8.0 mm. Seminal root lengths of the 48 CSSLs ranged from 133.6 to 204.5 mm. Significant differences ($P < 0.01$) in seminal root length were noted between 26 CSSLs and Curinga (Fig. 3.3e). Seminal root length was longer than Curinga in two lines, and comparatively shorter in 24 lines (Fig. 3.3e). When plants were grown in 500 μM NH_4NO_3, the seminal root length of Curinga was 99.3 ± 8.1 mm and that of *O. rufipogon* was 244.4 ± 8.3 mm. Seminal root lengths of the 48 CSSLs ranged from 76.0 to 158.0 mm (Fig. 3.3f). Significant differences ($P < 0.01$) in seminal root length were observed between 22 CSSLs and Curinga (Fig. 3.3f). Seminal roots were longer than Curinga in 11 lines, and shorter in 11 other lines (Fig. 3.3f). Moreover, Curinga and its derived CSSLs showed significantly less root length plasticity to treatment with NH_4NO_3 compared with NH_4^+ (Fig. 3.3).

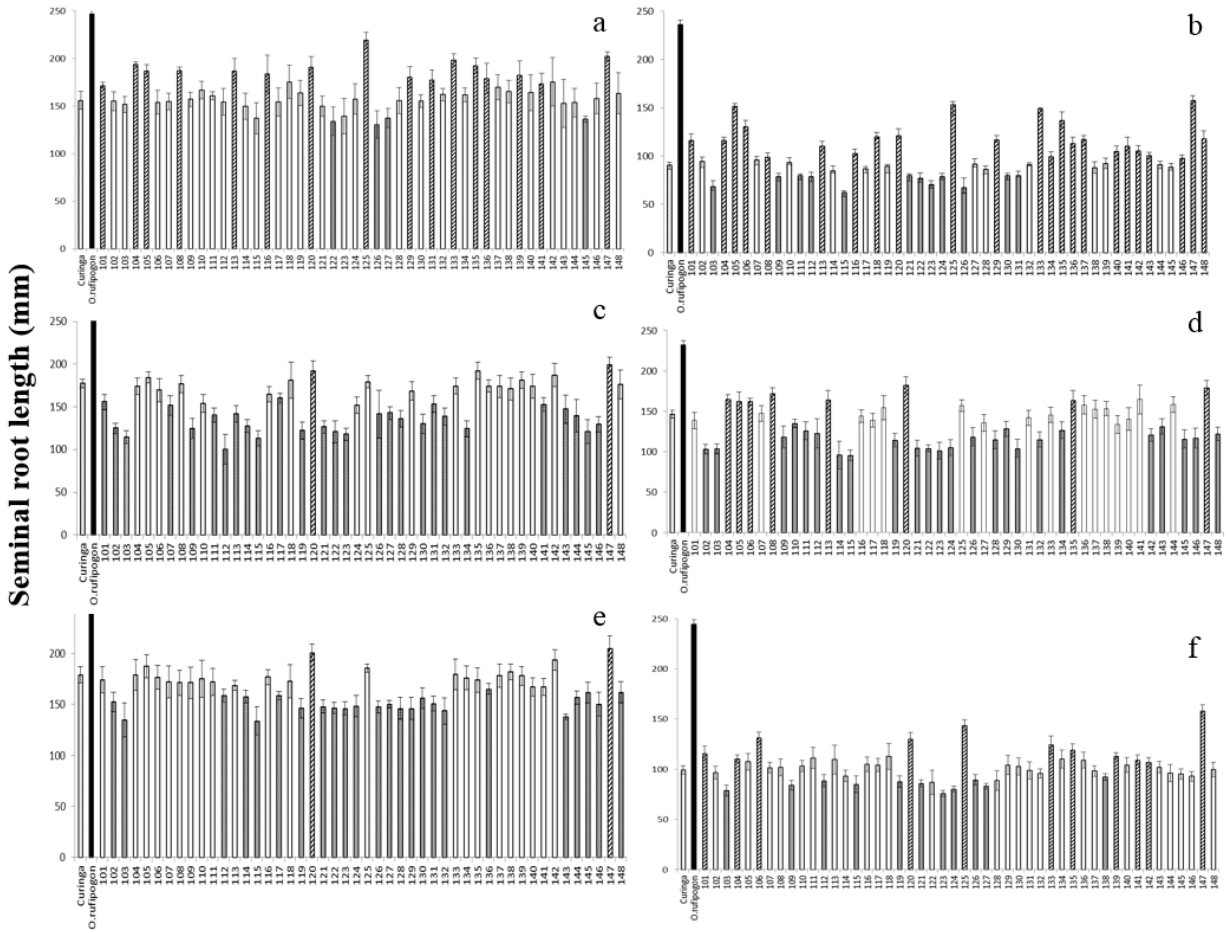


Fig. 3.3 Comparison of seminal root length of parental lines and CSSLs grown in hydroponic culture with different forms of N at two concentrations. Data are means of 10 replications ± SD. Letters a–f indicate different concentrations and forms of N: a 5 μM NH_4^+, b 500 μM NH_4^+, c 5 μM NO_3^-, d 500 μM NO_3^-, e 5 μM NH_4NO_3 and f 500 μM NH_4NO_3. Dotted horizontal bars indicate mean values of Curinga seminal root lengths. Hatched bars show negative significant differences compared with Curinga at $P < 0.001$, and shaded bars show positive significant differences at $P < 0.001$. Open bars indicate CSSLs that are not significantly different from Curinga

Correlation analysis between shoot and root traits among CSSLs

Pearson's correlation coefficient analyses between shoot height and seminal root length using 48 CSSLs (Table 3.1) were conducted to know effect of N forms. Shoot height and seminal root length were significantly and positively correlated in all N forms with Pearson's coefficients ranging from 0.52 to 0.68 ($P < 0.001$) except in NH_4NO_3 at 5 μM ($R = 0.45$). Highly significant positive correlations for seminal root length among N forms and concentrations were also obtained ($R = 0.50$ to 0.67; $P < 0.001$). However, percentage of root length plasticity under sole NO_3^- conditions did not show any correlation with NH_4^+ and NH_4NO_3 conditions ($R = -0.07$, -0.15, respectively; $P > 0.05$). In addition, shoot height was significantly correlated among different N forms and concentrations ($R = 0.52$ to 0.68; $P < 0.001$) except 5 μM NO_3^- and 500 μM NH_4NO_3 (Table 3.1).

Table 3.1 Correlation coefficient (*R*) between shoot height and seminal root length under different N treatments

N-concentration	5 μM	500 μM	5 μM	500 μM	5 μM	500 μM
N form	NH_4^+	NH_4^+	NO_3^-	NO_3^-	NH_4NO_3	NH_4NO_3
R	0.56**	0.68**	0.54**	0.66**	0.45*	0.52**

** $P < 0.001$; * $P < 0.01$

QTL analysis for seminal root elongation

Using CSSL Finder (Lorieux 2005), two co-located QTLs associated with seminal root length were detected when plants were exposed to 500 μM concentrations of NH_4^+ and NH_4NO_3. However, any QTL for seminal root length was not detected when plants were exposed to 5 μM concentrations. These QTLs were located on chromosome 1 between SNP markers id1014841 (26.55 Mb) and id1023347 (38.79 Mb) (Fig. 3.4; Table 3.2). Four CSSLs (105, 106, 133 and 147) each carry an introgression in this region, and all had significantly longer seminal roots than Curinga ($P < 0.001$, student's *t*-test) when grown under

high concentrations of NH_4^+ and NH_4NO_3. An NH_4^+-insensitive seminal root QTL was also identified in the same region (Fig. 3.4; Table 3.2).

Fig. 3.4 Putative QTL region for NH_4^+ insensitive seminal root length on rice chromosome 1. Solid grey bars on right indicate % of root length plasticity for each line. Lines with the least root length plasticity located within the black frame. The most probable location of the root elongation QTL is indicated by the black frame which defines a common introgressed region between the lines having insensitive roots.

Table 3.2 QTLs for root length identified in this study in seedlings grown under hydroponic conditions compared with QTL regions from previous studies

Trait	Condition	Chr.	Marker	Region (Mb)	Positive allele	Reference
Seminal root elongation	500 μM NH_4^+	1	id1014841-id1023347	26.55-38.79	*O.rufipogon*	This study
Seminal root elongation	500 μM NH_4NO_3	1	id1014841-id1023347	26.55-38.79	*O.rufipogon*	This study
Insensitive seminal root	% of reduction between high and low NH_4^+	1	id1014841-id1023347	26.55-38.79	*O.rufipogon*	This study
Seminal root length	500 μM NH_4^+	1	R210 -R2417	10.54 - 33.18	Koshihikari	Obara et al. 2010
Seminal root length	5 μM NH_4^+	1	C1370 -C112	31.21 -44.75	Koshihikari	Obara et al. 2010
Seminal root length	500 μM NH_4^+	1	RM6648-RM5407	34.1 - 38.1	IRGC104038	Obara et al. 2011
Seminal root length	500 μM NH_4^+	1	RM1361-RM5362	40.8 - 41.09	IRGC104038	Obara et al. 2011
Seminal root length	5 μM NH_4^+	1	RM1361-RM5362	40.8 -41.09	IRGC104038	Obara et al. 2011
Seminal root length	500 μM NH_4^+	6	R2549-R1167	25.79 -32.11	Kasalath	Obara et al. 2010
Seminal root length	5 μM NH_4^+	6	R2549-R1167	25.79 - 32.12	Kasalath	Obara et al. 2010

3.1.4 DISCUSSION

SNP marker regions for seminal root elongation

In this study, I successfully identified a QTL on chromosome 1, delineated by SNP id1014841 and SNP id1023347, associated with seminal root elongation under sufficient concentrations (500 μM) of NH_4^+ and NH_4NO_3. It means this locus has the function that the root elongation was not inhibited under high NH_4^+ concentration. It is interesting to note that this particular QTL was expressed under two different N forms (NH_4^+ and NH_4NO_3), but not under NO_3^-. This result indicates that, in rice, different genetic factors are likely involved in the control of the root elongation process in response to different forms of N. This finding is consistent with the result of Rauh et al. (2002), who found QTLs to control root elongation in *Arabidopsis thaliana* in response to different N forms.

When I compared the QTLs discovered in this study with previously reported root QTLs in rice using the Gramene Annotated Nipponbare Sequence 2009 map (www.gramene.org), several overlapping loci were found. The major QTL region on chromosome 1 reported here (SNP id1023347) co-locates with a QTL for root length reported by Obara et al. (2010 and 2011), which was identified in rice seedlings grown under sufficient NH_4^+ conditions (500 μM) using two different mapping populations derived from Koshihikari × Kasalath and Taichung 65 × *O. glaberrima*. In our study, an introgression from *O. rufipogon* (IRGC 105491) in the genetic background of the tropical *japonica* variety Curinga was associated with greater seminal root growth in response to both NH_4^+ and NH_4NO_3.

Several important candidate genes, including *OsAAT* 1, *OsAAT* 2 and *OsAMT* 2 involved in N metabolism, were located in our QTL region (Song et al. 1996; De la Torre et al. 2006; Suenaga et al. 2003). These genes are known to be involved in the production of a key enzyme necessary for amino acid synthesis and enzymes serving as functional NH_4^+ transporters. In addition to N metabolism genes, several yield QTLs, e.g., *spp1.1, gpp1.1* and *yld1.1* from *O. rufipogon* (Fu et al. 2010), were also co-located in the same region. Recently, Zhao et al. (2013) reported that aluminum tolerance in rice is

synergistic with NH_4^+ preference, based on a root elongation study at the seedling stage. This relationship was evident in our QTL analysis. The genomic region (SNP marker interval id1014841–id1023347) regulating seminal root length on chromosome 1 was also found to be co-located with the most important QTL region associated with aluminum tolerance in other rice populations (Wu et al. 2000; Nguyen et al. 2002; Famoso et al. 2010). Four CSSLs (105, 106, 133 and 147) carrying an introgression across this genomic region also showed a significantly lower percentage of root growth plasticity under Al / NH_4^+ hydroponic conditions than did Curinga (R=0.91, $P < 0.001$). These results collectively reveal that this genomic region is important for understanding Al / NH_4^+ synergism in rice, and may be of potential use to plant breeders interested in enhancing seminal root growth in response to different forms of N.

3.2 SECTION 2 ROOT GROWTH ANGLE QTLS

3.2.1 INTRODUCTION

Root distribution has also been quantitatively characterized by using several traits, including root length, volume, and density in the soil at different depths, and these characteristics differed among rice cultivars (Nemoto et al. 1998, Hirayama et al. 2007, Kato et al. 2006). Root growth angle is one of the factors to determine root growth distribution without biomass change. As yet, there have been few studies regarding the determination of QTL that is related to root growth angle. A new major QTL controlling the ratio of deep rooting (RDR; means the proportion of total roots that elongated through the basket bottom in detail information see CHAPTER 2) called *DRO1* (*DEEPER ROOT 1*) gene causes the increased frequency of high root growth angles (50 – 90° with respect to the horizontal, that is, deeper root) (Uga et al. 2011). The opposite of *DRO1*, *qSOR* (*SOIL SURFACE ROOTING 1*) is related to growth of soil surface roots, that is, shallow roots (Uga et al. 2012). In this SECTION, I focused on detecting QTLs controlling RSA traits such as root length, root number and root growth angle at 40 days after sowing using the basket method under hydroponic conditions.

3.2.2 MATERIALS AND METHODS

RSA of parental and CSSLs response to concentrations of NH_4^+

In order to detect the QTLs for RSA contributing the NH_4^+ response, shoot and root traits were measured by using root basket method as I mentioned in CHAPTER 2 SECTION 2 under hydroponic conditions with three different NH_4^+ concentrations (5, 50 and 500 μM). In addition to CHAPTER 2 SECTION 2, I analyzed deep root number and shallow root numbers with average of four plants of both experiments with two replications. QTL analysis was conducted by CSSL finder v. 0.84 computer program (Lorieux 2005) with average values of two replications with using 48 CSSLs and these parents; Curinga and *O. rufipogon* (CHAPTER 3 SECTION 1).

3.2.3 RESULTS

RSA variation estimated for each CSSL and parents

Significant variation (ANOVA, $P<0.001$) among the CSSLs was observed among phenotyped RSA (Table 3.3). Between two parents, all RSA traits except of deeper root number showed significant differences (Table 3.3). Deeper root number is a unique trait that did not show significant difference between parents, but ranged from 11.50 to 24.25 (mean 16.11) and showed significant variation (ANOVA, $P<0.001$) among the CSSLs (Table 3.3). Root biomass was found to be the most variable trait across the CSSLs (Table 3.3).

Table 3.3 Performance of RSA traits of parental lines and CSSL population of Curinga / *O. rufipogon* tested under hydroponic conditions with 500 μM NH_4^+

Traits	Parents		CSSLs		ANOVA(*P*-value)
	Curinga	*O. rufipogon*	Mean	Range	Genotype
Deeper root number	17.00	18.00ns	16.11	11.50-24.25	< 0.001
Shallow root number	8.25	15.25***	16.96	6.25-26.50	< 0.001
Total root number	25.25	33.25**	33.07	20.50-47.25	< 0.001
Ratio of deeper root	67.56	54.09***	48.94	36.11-69.71	< 0.001
Rooting pattern value	8.75	2.75***	5.60	1.50-13.25	< 0.001
Maximum root length (mm)	177.50	327.75***	187.17	151.25-235.50	< 0.001
Root biomass (mg)	449.75	1069.75***	713.24	245.75-1333.00	< 0.001

***, ** and ns indicated significant difference for the same trait between two parents at $P<0.001$, 0.01 and >0.05 (n=8).

Correlation among root and shoot traits under different NH_4^+ regimes

The results obtained in Pearson's correlation coefficient of root and shoot traits are presented in Table 3.4. In 5 μM NH_4^+ concentrations, I did not find any correlation between roots and shoot traits. In both 50 and 500 μM concentrations, maximum root length with plant height ($R=0.824$; $P<0.001$, $R=0.914$; $P<0.001$), root biomass with shoot biomass ($R=0.868$; $P<0.001$, $R=0.874$; $P<0.001$), root number with tiller number ($R=0.803$; $P<0.001$, $R=0.752$; $P<0.01$) had high significant correlations (Table 3.4). In 50 μM, root number was also correlated with SPAD ($R=-0.692$; $P<0.05$), but not in 500 μM. Surprisingly, both RDR and RPV did not show any correlation between themselves and with any of the root or shoot traits studied. Time course of maximum root length and total root number were highly correlated ($R=0.871$ to 0.997; $P<0.001$, $R=0.781$ to 0.996; $P<0.001$) among the all genotypes, but no such correlation was observed with respect to RDR ($R=-0.701$ to 0.933).

Table 3.4 Correlation coefficients among root and shoot traits under hydroponic conditions

					5 μM				
	MRL	RB	RN	RDR	RPV	PH	SB	TN	SPAD
MRL	1								
RB	-0.670	1							
RN	-0.164	0.319	1						
RDR	0.24	-0.526	-0.557	1					
RPV	0.377	-0.446	-0.052	0.773	1				
PH	0.105	0.292	0.712	-0.492	-0.013	1			
SB	-0.328	0.791	0.094	-0.306	-0.375	0.186	1		
TN	-0.450	0.376	0.403	-0.218	-0.058	0.069	0.273	1	
SPAD	-0.356	0.540	-0.015	-0.149	-0.478	-0.137	0.760	0.098	1
					50 μM				
	MRL	RB	RN	RDR	RPV	PH	SB	TN	SPAD
MRL	1								
RB	-0.382	1							
RN	-0.141	0.544	1						
RDR	0.348	-0.228	-0.515	1					
RPV	-0.447	-0.236	0.252	-0.292	1				
PH	***0.824	-0.066	0.025	-0.012	-0.528	1			
SB	-0.328	***0.868	0.346	-0.094	-0.102	-0.096	1		
TN	0.141	0.189	***0.803	-0.205	0.249	0.143	-0.102	1	
SPAD	0.299	-0.13	*-0.692	0.429	-0.331	0.216	-0.011	-0.44	1
					500 μM				
	MRL	RB	RN	RDR	RPV	PH	SB	TN	SPAD
MRL	1								
RB	-0.415	1							
RN	-0.083	-0.02	1						
RDR	0.053	-0.537	-0.501	1					
RPV	-0.487	0.422	0.053	-0.358	1				
PH	***0.914	-0.279	-0.176	0.172	-0.41	1			
SB	-0.027	***0.874	-0.108	-0.542	0.358	0.136	1		
TN	-0.146	0.333	**0.752	-0.309	0.07	-0.096	0.15	1	
SPAD	-0.387	-0.268	-0.539	0.478	0.21	-0.239	-0.283	-0.636	1

MRL: Maximum root length; RB: Root biomass; RN: Root number; RDR: Ratio of deeper root; RPV: root pattern value; PH: Plant height; SB: Shoot biomass; TN: Number of tillers; SPAD: SPAD value leaf chlorophyll concentration. *** $P < 0.001$, ** $P < 0.01$, * $P<0.05$

Correlation among root traits

The results obtained in Pearson's correlation coefficient of each RSA traits are presented in Table 3.5. Deep root number showed the correlation with total root number, ratio of deeper root and root biomass (P<0.001). Shallow root number also showed the correlation with total root number, ratio of deeper root but not root biomass. Root biomass correlated with total root number.

Table 3.5 Phenotypic correlations among root traits observed in the CSSLs of Curinga / *O. rufipogon* in hydroponic conditions with 500 μM

	Deep #	**Shallow #**	**Total #**	**RDR**	**RPV**	**MRL**	**Root Biomass**
Deeper root number	1						
Shallow root number	0.11	1					
Total root number	**0.70*** **	**0.79*** **	1				
Ratio of deeper root	**0.58*** **	**-0.64*** **	-0.10	1			
Rooting pattern value	-0.06	0.08	0.02	-0.14	1		
Maximum root length	0.14	-0.03	0.07	0.19	-0.24	1	
Root biomass	**0.53*** **	0.43	**0.64*** **	0.03	-0.18	0.09	1

Deep #; deeper root number, Shallow #; shallow root number, Total #; total root number, RDR; ratio of deeper root, RPV; rooting pattern value, MRL; maximum root length. *** indicated a significant at P<0.001

QTL analysis for root growth angle

Using CSSL Finder (Lorieux 2005), two QTLs associated with root growth angle were detected: one is the deeper root number, and the other is the shallow root number (Fig. 3.5). Deeper root number QTL was located on chromosome 1 between SNP markers id1012330 (23.45 Mb) and id1021697 (36.46 Mb) (Fig. 3.5; Table 3.4). The QTL for shallow root number was located on chromosome 12 between SNP markers id1012330 (23.45 Mb) and id1021697 (36.46 Mb) (Fig. 3.5; Table 3.6).

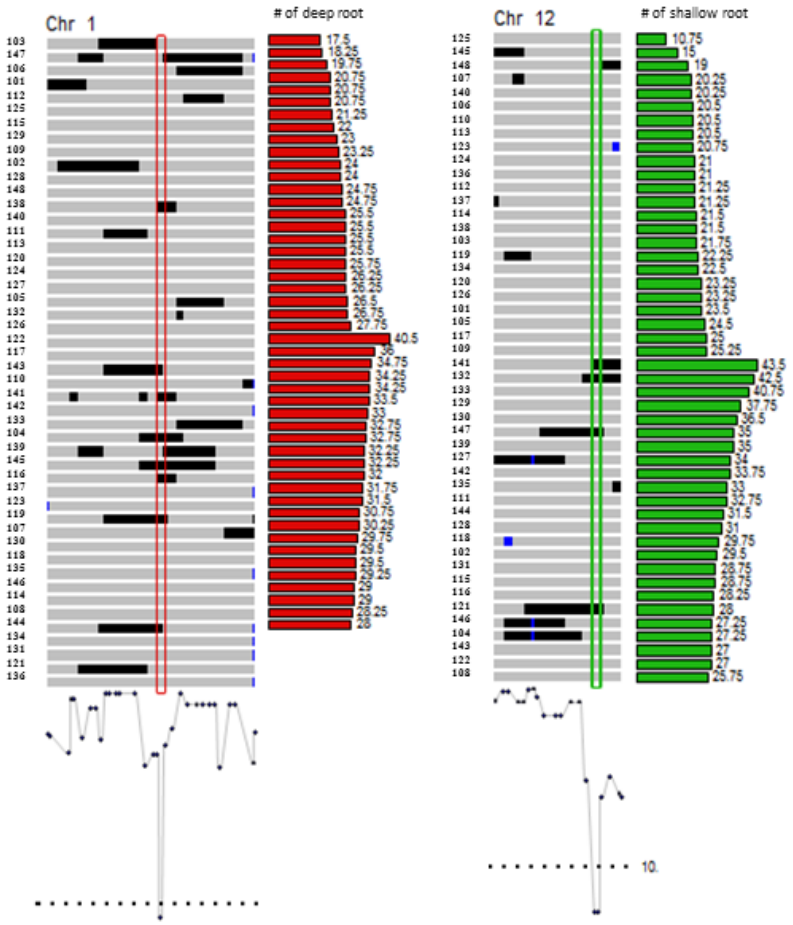


Fig. 3.5 Putative QTLs region for root growth angle on rice chromosome 1 and 12. Solid blue bars on right indicate number of deep or shallow roots for each line. Lines with the number of each deep and shallow root located within the blue frame. The most probable location of the deep or shallow root number QTL is indicated by the blue frame which defines a common introgressed region between the lines having higher number of deep or shallow roots.

3.2.4 DISCUSSION

N-responsive RSA plasticity for improved NAE

An important factor that determines the distribution of the RSA in the soil is the root growth angle (Forde and Lorenso 2001). Trachsel et al. (2013) reported that deeper rooting plasticity with root growth angle was observed in response to low N application conditions in maize. His result indicates that modulation of deeper rooting by root growth angle is one of the candidate traits to improve N acquisition. In my case, RSA was not influenced by NH_4^+ concentrations in most of genotypes, but Curinga showed high plasticity (Table 2.4). I hypothesized both constitutive RSA and RSA plasticity are the key traits to adapt to poor nutrient soils. Rooting pattern plasticity is very important to seek the nutrition in heterogeneous soils. In rice, the deep root system is generally correlated with tall plant stature (Yoshida et al. 1982) and root-shoot-ratio is generally decreased under high N supply. It has long been known that roots will proliferate in response to localized patches of high N (Drew and Saker 1975; Laine et al. 1995). This would appear to be an evolutionary adaptation so that root allocation is not wasted in areas with low N (Garnett et al. 2009). In our study, the maximum root length is one of the components of deep root system that showed high correlation with plant height under both low and high NH_4^+, but did not show any correlation with other traits in hydroponic study (Table 3.5). It is interesting to note that RDR and RPV did not show any correlation with either root or shoot traits indicating that rice can adapt to the change in root growth angle in response to different NH_4^+ concentrations to maintain yield component (Table 3.5).

QTL analysis for root growth angle

Under hydroponic conditions in this study, a total of two QTLs; deeper root number and shallow root number were detected on chromosome 1 and 12, respectively (Table 3.6). It is of interest to compare the markers associated with our QTLs with QTLs that have been reported earlier. Recently, a major QTL controlling the ratio of deeper rooting called *DRO1, DRO2* was reported to increase the frequency of deeper roots on chromosome 9 and 4, respectively (Uga et al. 2011, Uga et al. 2013), in contrast to QTL

called *qSOR* (Soil surface rooting 1) which regulates the growth of shallow roots on chromosome 7 reported by Uga et al. (2012). Surprisingly, in this study I could not find any co-location to previously reported regions, instead I found new QTLs to regulate root growth angle (Fig 3.5; Table 3.6).

Table 3.6 QTLs for root angle identified in this study under hydroponic conditions compared with QTL regions from previous studies

Trait	Condition	Chr.	Marker	Region (Mb)	Positive allele	Reference
Deeper root number	500 μM NH_4^+	1	id1012330-id1021697	23.45-36.46	*O.rufipogon*	This study
Shallow root number	500 μM NH_4^+	12	id12007161-id12008796	19.93-24.85	*O.rufipogon*	This study
Ratio of deeper root	363 μM NH_4^+	9	RM24393-RM7424	16.67 -17.29	Kinandang Patong	Uga et al. 2010
Ratio of deeper root	363 μM NH_4^+	4	RM6089	29.59	Kinandang Patong	Uga et al. 2013
Soil-surface rooting	363 μM NH_4^+	7	RM21941-RM21976	24.78-25.59	Gemdjah Beton	Uga et al. 2011
Ratio of deeper root	363 μM NH_4^+	7	RM5508	23.00-23.95	Kinandang Patong	Uga et al. 2015

3.3 GENERAL DISCUSSION

QTL analysis for RSA traits at different growth stages

For the two hydroponic experiments in this CHAPTER, a total of five QTLs; seminal root elongation, deeper root number and shallow root number were detected on chromosome 1 and 12, respectively (Table 3.2 and 3.6). Interestingly, QTL regions for seminal root elongation and deeper root number were overlapped each other. This overlapped region was also reported as QTLs for constitutive seminal root elongation (Obara et al. 2011), N metabolism relative QTLs (Song et al. 1996; De la Torre et al. 2006; Suenaga et al. 2003) and yield relative QTLs (Fu et al. 2010). Thus, I assumed that understanding this QTLs region of the genetic control of RSA has immense potential to enhance agronomic performance in rice.

3.4 CONCLUSIONS

Our results of QTL analysis indicate that the *O. rufipogon* allele on chromosome 1 has the potential to enhance early root growth and root system architecture development under 500 μM NH_4^+ hydroponic

conditions. Interestingly, both deeper root number QTL and seminal root length QTL regions were overlapped. This knowledge contributes to our understanding of the genetic control of seminal root growth, root growth angle, and also addresses the goal of defining QTL regions associated with water and nutrient acquisition efficiency for future rice breeding programs. Further studies are underway to confirm the impact of this QTL on overall agronomic performance under different N environments.

CHAPTER 4 ASSOCIATION BETWEEN ROOT SYSTEM ARCHITECTURE TRAITS AND NITROGEN-DEFICIECY TOLERANCE IN THE FIELD

4.1 INTRODUCTION

Improved root system architecture (RSA) can enhance agronomic performance of plants by increasing water- and nutrient-acquisition efficiencies. However, little is known about the interaction between RSA and agronomic performances under field conditions. To gain a better understanding on the genetic basis of these relationships, in this CHAPTER I tried to determine the significance and magnitude of variations of agronomic traits related to yield performance for representative genotypes with contrasting RSA (CHAPTER 2). To validate the importance of agronomic performance, I chose an index called nitrogen-deficiency tolerance (NDT), the ratio of a trait under low N conditions to that under normal N fertilized conditions or farmer's practice (FP) in Colombia (Wei et al. 2012). The NDT of different traits were proposed to be used as the selection criteria to improve plants' NDT (Wei et al. 2012). Grain yield response to N is commonly affected by the environment, genotype-by-environment interaction, and the type of the N fertilizers, application method and timing of application (Peng et al. 2006).

The objective of this study was to determine the magnitude of variation in NDT traits among rice genotypes with different RSA, with which different responses of seminal root elongation and early root growth to N concentrations were already tested (CHAPTER 2, Ogawa et al. 2014ab). I intended to verify if there were positive correlations between NDT traits and RSA traits.

4.2 MATERIALS AND METHODS

Field phenotyping of NDT among common varieties

Based on the current hydroponic study (CHAPTER 2) and our previous study on these genotypes (Ogawa et al. 2014a; Uga et al. 2009), five representative genotypes with contrasting RSA were selected. Rooting pattern of selected genotypes was as follows: IR64, monomorphic-shallow; Curinga and NERICA4, monomorphic-deep; FEDEARROZ174 and *O. rufipogon,* dimorphic with both shallow and deep root systems with stable rooting pattern value (RPV) regardless of the NH_4^+ concentration in the hydroponic solution (see CHAPTER 2, Fig. 2.4).

Two field trials were conducted with randomization, one in the dry season (August-December) of 2012, and another in the rainy season (February-June) in 2013, both at CIAT, Colombia. Before starting each

experiment, maize was planted for two consecutive cycles to make the field homogeneously deficient in N. Soil samples were taken before transplanting, flowering and after harvest at 30 points in each field at 0-15 cm depth by using metal tube with 8 cm diameter and mixed (Table 4.1). Organic matter content (Walkley and Black method), ammonium (1M KCl method) and nitrate (1 M KCL method) N, and total N (dry combustion method) was analyzed according to Salinas and Garcia (1979) (Table 4.1). The experiments were laid out in a split-plot design with three N treatments as the whole-plot factor and genotypes as the split-plot factor, replicated thrice. The N treatments were: 1) Native, with 0 kg ha^{-1} N application, 2) Farmers' practice (FP) in Colombia (Berrio et al. 2002), with total N application rate of 180 kg ha^{-1}, which were applied in the form of urea in three equal splits: 60 kg ha^{-1} N as basal at two days after transplanting (DAT), 60 kg ha^{-1} N at 10 DAT, and 60 kg ha^{-1} N at 30 DAT. 3) 50 % FP with total N application rate of 90 kg ha^{-1}, applied in three equal splits at the same timing as of FP treatment. The other nutrients (KH_2PO_4; 70 kg, KCl; 60 kg, $ZnSO_4$: 25 kg, $FeSO_4$: 80 kg, B: 0.4 kg and 60 kg of micronutrient ha^{-1}) were applied at the same dose in all the three treatments as per the standard commercial rate of Colombia at two DAT. The seeds were sown on germination tray and 21 days-old seedlings were transplanted with the spacing of 20 x 25 cm in a block of 1 × 1.4 m in size (40 plants per hill). Integrated agronomic practices were adopted to control pests and weed to avoid yield loss throughout the crop duration. At flowering, leaf chlorophyll content (SPAD value) was measured at the middle position of the flag leaf. At harvest, seven plants from each replication were sampled for trait measurements. Samples were dried in screen house for 12 days to determine dry matter weight with electronic balance (Sartorius, M-power, 3100g d=0.01, Germany). The parameters for NDT such as relative single plant grain yield (RGY) and relative single plant biomass yield (RBM) and of each variety at both native and 50 % of FP were calculated using the following formula reported by Wei et al. (2012).

$$RGY_{NDT0} = \text{Individual grain yield}_{native} / \text{Individual grain yield}_{FP}$$

RGY_{NDT50} = Individual grain yield $_{50\ \%\ FP}$ / Individual grain yield $_{FP}$

RBM_{NDT0} = Individual biomass yield $_{native}$ / Individual biomass yield $_{FP}$

RBM_{NDT50} = Individual biomass yield $_{50\ \%\ FP}$ / Individual biomass yield $_{FP}$

During experiment, I also monitored the N content in the soil as NH_4^+ and NO_3^- during the cultivation to understand N dynamics in filed soil condition before the experiment.

Data analysis

For the analysis of agronomic traits, seven individual plants with three replications were used. All statistical analyses were performed using the XLSTAT, an add-on for Microsoft Excel. Differences in mean values between genotypes were evaluated using Bonferroni's multiple comparisons or Tukey's test. Pearson's correlation coefficient analysis was conducted for correlation analysis.

Table 4.1 Soil N properties before the experimental field trials in 2012 and 2013

		N treatments		
Soil chemical property	**Year**	**Native**	**50 % FP**	**100 % FP**
Organic matter (g/kg)	2012	12.87 ± 0.23	12.83 ± 0.26	13.07 ± 0.66
	2013	12.76 ± 0.31	13.73 ± 0.78	13.13 ± 0.29
NH_4^+ (mg/kg)	2012	2.93 ± 0.10	2.95 ± 0.14	4.01 ± 0.16
	2013	7.79 ± 0.70	8.47 ± 0.24	11.29 ± 0.39
NO_3^- (mg/kg)	2012	0.21 ± 0.00	0.42 ± 0.20	0.41 ± 0.02
	2013	0.62 ± 0.07	0.40 ± 0.13	0.21 ± 0.04
EC (ds/m)	2012	0.32 ± 0.03	0.29 ± 0.01	0.35 ± 0.04
	2013	0.33 ± 0.02	0.42 ± 0.01	0.37 ± 0.02
Soil texture (%)	**Clay**	**Silt**	**Sand**	**Texture**
	15.7	65.4	18.9	Silty loam

Data are mean ± SE of three replications.

4.3 RESULTS

Field evaluation for NDT

Significant variations in agronomic traits were obtained between the years, genotypes and N treatments, and their interactions (Table 4.2). The agronomic traits such as plant height, number of productive tillers, shoot biomass and grain weight increased in response to N application in the both years (Table 4.2). In 2013, NERICA4, FEDEARROZ174 and *O. rufipogon* had no significant reduction in grain weight (single plant grain yield) between 50 % FP and FP treatments. *O. rufipogon* is a unique rice genotype that showed no reduction in shoot biomass between native and 50 % FP; and 50 % FP and FP treatments in 2012 and 2013, respectively. One thousand grain weight of all the genotypes was not influenced by N treatment except for FEDEARROZ174. Earlier flowering was recorded in native N treatment as compared to other N treatments in general. Conversely, delayed flowering at native N treatment was observed in IR64 (Table 4.2).

Table 4.2 Field traits of selected lines under three different N applications over two seasons

	Treatment	FD	PH	SB	TN	PN	PL	GW	1000GW	SPAD
Dry season (Aug. - Dec., 2012)										
IR64	0	71.5 a	82.14 a	17.44 a	9.86 a	9.36 a	21.02 a	21.21 a	25.69 a	33.1 a
	50	69.0 b	96.57 b	38.79 b	15.43 b	14.93 b	23.44 b	40.12 b	26.84 a	36.1 b
	100	68.5 b	101.00 b	55.43 c	21.29 c	20.07 c	24.44 b	52.48 c	27.32 a	39.7 c
NERICA4	0	55.0 a	96.20 a	8.67 a	4.11 a	4.07 a	22.64 a	14.37 a	28.53 a	41.76 a
	50	57.0 b	109.66 b	17.40 b	7.13 b	7.04 b	23.02 a	26.78 b	27.97 a	45.12 ab
	100	58.0 c	117.69 c	22.23 c	8.41 c	8.03 c	25.76 b	33.74 c	27.97 a	52.21 b
Curinga	0	59.5 a	93.33 a	11.97 a	7.33 a	7.00 a	22.50 a	15.8 a	26.06 a	44.0 a
	50	61.2 a	101.00 b	16.27 b	9.00 b	9.00 b	22.83 b	23.7 b	26.30 a	44.8 ab
	100	60.0 a	110.00 c	23.71 c	12.00 c	11.67 c	22.67 c	34.4 c	27.03 a	46.3 b
FEDEARROZ174	0	79.0 a	76.48 a	14.65 a	10.23 a	10.00 a	23.29 a	19.99 a	25.20 a	41.46 a
	50	79.0 a	90.38 b	19.45 b	11.88 b	11.41 b	24.93 b	27.13 b	26.60 ab	44.37 ab
	100	81.0 b	95.59 c	26.43 c	15.62 c	15.28 c	25.30 b	37.37 c	27.87 b	48.51 b
O. rufipogon	0	59.7 a	123.33 a	22.12 a	13.33 a	13.00 a	24.33 a	23.38 a	27.09 a	28.1 a
	50	65.0 b	144.00 b	24.64 a	16.67 b	16.33 b	23.76 a	28.97 b	27.12 a	31.2 ab
	100	64.7 b	148.67 b	37.24 b	18.67 c	19.33 c	26.77 b	37.20 c	26.91 a	34.2 b
	Genotype	**	**	**	**	**	**	**	**	**
	Treatment	**	**	**	**	**	**	**	**	**
	G x T	*NS*	**	**	*	*	*	**	**	**
Rainy season (Feb. - Jun., 2013)										
IR64	0	71.0 a	82.10 a	17.05 a	10.00 a	9.85 a	21.50 a	23.99 a	26.97 a	30.67 a
	50	69.0 b	87.33 b	23.31 b	11.33 b	11.28 b	23.06 b	31.34 b	27.94 b	36.09 b
	100	68.0 c	88.88 b	40.69 c	16.71 c	16.53 c	24.29 c	51.54 c	28.11 b	39.67 c
NERICA4	0	54.0 a	105.81 a	13.81 a	6.33 a	6.33 a	23.98 a	22.26 a	28.20 a	43.97 a
	50	56.7 a	122.15 b	28.32 b	9.40 b	9.35 b	26.33 b	39.64 b	28.01 a	56.78 b
	100	60.3 a	135.38 c	34.11 c	11.43 c	11.14 c	27.02 b	45.50 b	27.90 a	55.19 b
Curinga	0	59.3 a	95.10 a	16.64 a	8.33 a	8.24 a	22.81 a	19.59 a	26.10 a	40.12 a
	50	62.3 a	113.7 b	34.50 b	14.14 b	13.81 b	22.78 a	40.74 b	26.94 a	45.45 b
	100	60.3 a	118.5 c	40.88 c	15.24 b	14.90 b	24.34 b	47.07 c	27.79 b	48.60 b
FEDEARROZ174	0	72.0 a	89.52 a	20.55 a	9.90 a	9.67 a	23.80 a	24.69 a	27.13 a	27.97 a
	50	65.0 b	101.14 b	39.52 b	18.42 b	18.52 b	25.02 b	45.80 b	26.83 b	32.82 b
	100	64.7 b	113.85 c	45.67 c	22.20 b	20.80 b	25.82 c	47.68 b	27.63 c	38.06 c
O. rufipogon	0	59.7 a	123.95 a	25.60 a	13.33 a	13.29 a	21.03 a	23.92 a	27.70 a	28.09 a
	50	65.0 b	152.20 b	50.06 b	22.52 b	23.43 b	23.25 b	43.91 b	27.72 a	31.18 b
	100	64.7 b	159.60 c	56.92 b	23.90 b	23.90 b	23.76 b	45.07 b	27.61 a	34.07 c
ANOVA	*Genotype*	**	**	**	**	**	**	**	**	**
	Treatment	**	**	**	**	**	**	**	**	**
	G x T	*NS*	**	**	**	**	*	**	**	**
	Year	**	**	**	**	**	**	**	**	**

FD: Flowering date (DAT); PH: Plant height (cm); SB:Shoot biomass (g); TN: Number of tillers (n); PN: Panicle number (n); PL: Panicle length (cm); GW: Grain weight (g); 1000GW: 1000 grains weight (g); SPAD: relative leaf chlorophyll concentration (SPAD units)
Different letter indicated significant differences for the each genotype at P <0.01 with Bonferroni correction
*, ** indicate significance at 0.05 and 0.01 levels, respectively. NS indicates no significance

The two traits related to NDT, i.e., relative grain yield (RGY) and relative biomass yield (RBM) also showed wide variations among the genotypes studied (Table 4.3). In both years, dimorphic root genotypes (FEDEARROZ174 and *O. rufipogon*) showed higher RGY_{NDT0} and RBM_{NDT0} than monomorphic root genotypes (IR64, NERICA4 and Curinga). In 2013, dimorphic root genotypes showed better performance in RBM_{NDT50} and RGY_{NDT50} than monomorphic root genotypes. Interestingly, the rooting pattern value (RPV) at 50 μM NH_4^+ concentration in hydroponic experiment (CHAPTER 2) showed significant negative correlations (P<0.05) with RBM_{NDT0}, RGY_{NDT0}, RBM_{NDT50} and RGY_{NDT50} in 2012 (Table 4.3) and also, with RBM_{NDT50} and RGY_{NDT0} in 2013 (Table 4.3). This indicates that the dimorphic root system

(RPV less than 10) has a significant positive role on NDT. However, RDR showed positive correlation with RBM_{NDT50}, RGY_{NDT50} only in 2012 (Table 4.3). To visualize above results, Figure 4.1 indicates the representative root systems of both dimorphic and monomorphic, in relation to the grain yield under the nitrogen sufficient (FP) and nitrogen deficient (Native) conditions. Dimorphic root varieties, FEDEARROZ174 and *O. rufipogon* showed less yield reduction between native and FP conditions as compared to both monomorphic shallow and deep varieties; IR64 and NERICA4, Curinga, respectively (Fig. 4.1).

Table 4.3 Parameters for nitrogen-deficiency tolerance for shoot biomass and grain weight in field experiments for two seasons, and their correlations with the root architectural traits measured in hydroponic experiments

		2012		2013	
		RBM	RGY	RBM	RGY
NDT_0	IR64	31.5 ± 2.4 a	40.4 ± 4.4 a	41.9 ± 1.7 a	46.5 ± 1.7 a
	NERICA4	39.0 ± 2.4 a	42.6 ± 6.0 a	40.5 ± 1.7 a	44.4 ± 1.9 a
	Curinga	50.5 ± 2.7 b	45.9 ± 2.7 a	40.7 ± 3.4 a	41.6 ± 4.7 a
	FEDEARROZ174	55.4 ± 1.3 bc	53.5 ± 2.0 b	45.0 ± 1.6 b	51.8 ± 2.5 b
	O. rufipogon	59.4 ± 3.0 c	62.9 ± 5.5 b	45.0 ± 8.9 b	53.1 ± 2.3 b
	RDR	*R=0.79*	*R=0.55*	*R=0.44*	*R=0.41*
	RPV	*R=-0.88* *	*R=-0.87* *	*R=-0.82*	*R=-0.87* *
NDT_{50}	IR64	70.0 ± 3.7 a	76.5 ± 4.4 a	57.3 ± 3.8 a	60.8 ± 4.2 a
	NERICA4	78.3 ± 8.1 a	79.4 ± 2.4 a	83.0 ± 3.4 b	87.1 ± 3.6 b
	Curinga	68.6 ± 3.1 a	68.9 ± 1.9 b	88.0 ± 4.2 b	86.6 ± 7.9 b
	FEDEARROZ174	73.6 ± 2.1 a	72.6 ± 7.1 a	86.5 ± 1.0 b	96.1 ± 4.4 c
	O. rufipogon	66.2 ± 1.8 a	77.9 ± 5.5 a	87.9 ± 14.7 b	97.4 ± 5.3 c
	RDR	*R=0.88* *	*R=0.97* *	*R=0.60*	*R=0.59*
	RPV	*R=-0.88* *	*R=-0.93* *	*R=-0.88* *	*R=-0.83*

Data are mean ± SE of three replications;

Different letter indicated significant differences among genotypes at $P < 0.05$ with Bonferroni's correction

* indicates significant difference with $P < 0.05$

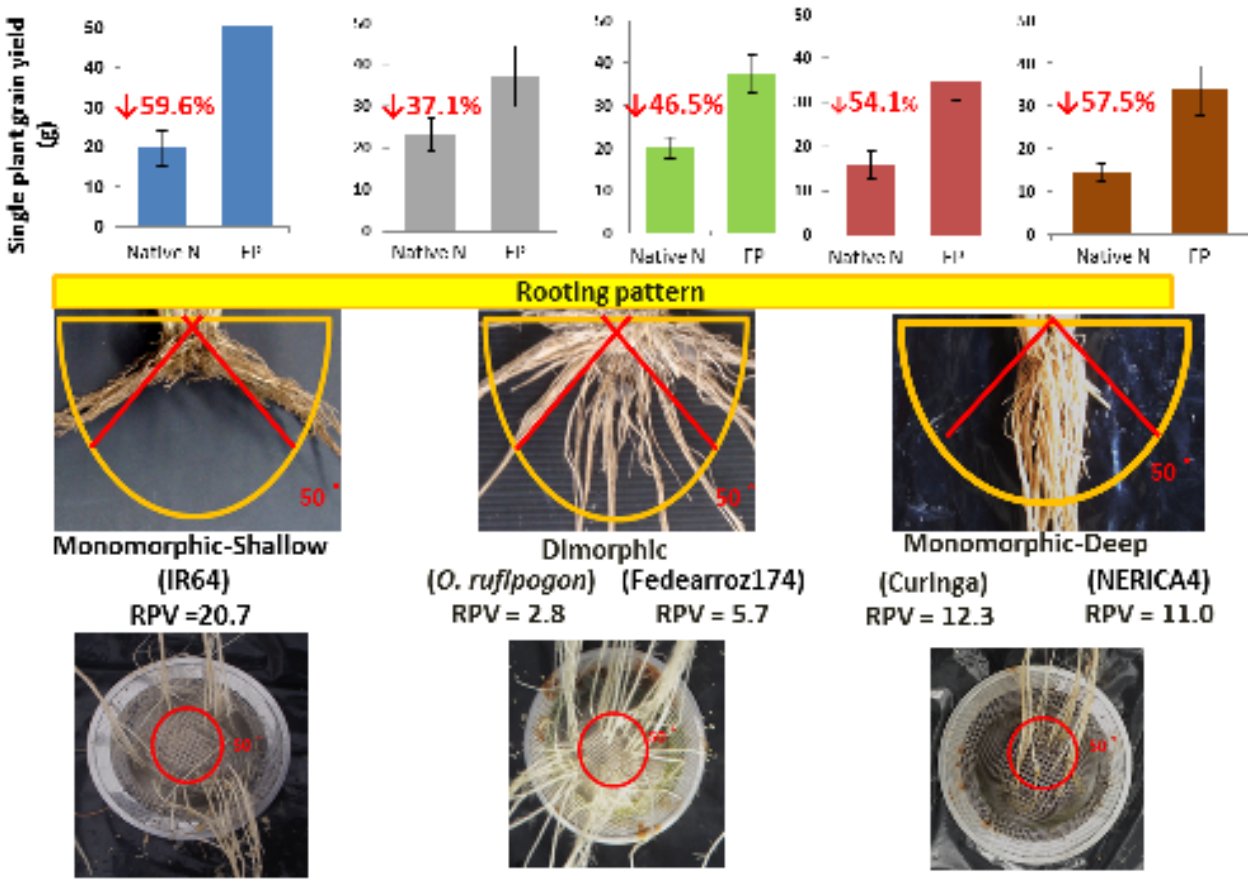


Fig. 4.1 Rooting pattern versus individual plant yield between native and FP conditions (2012).

4.4 DISCUSSION

Field NDT traits among varieties

I obtained different performance in overall agronomic traits between the seasons although I repeated the same N treatments and experimental conditions (Table. 4.3). The field data clearly showed that the grain yield in rainy season; Feb. – Jun., 2013 was better than in dry season; Aug. - Dec., 2012. Both precipitation and radiation were higher in 2013 than in 2012, which are assumed to be the main reason of the yield difference between two years. Particularly the radiation during peak flowering time was distinctively lower in 2012 (IDEAM; http://www.ideam.gov.co/). Although there was a significant

difference in overall agronomic performance among the trials (seasons), the trends of agronomic performance among tested genotypes showed similar response to N treatments (Tables 4.2; 4.3).

NDT traits have been considered as indirect selection criteria for the improvement of nitrogen-use efficiency (NUE) (Chen et al. 2008, Feng et al. 2010; Lian et al. 2005; Namai et al. 2009). In this study, I elucidated the relationship between RSA traits from hydroponics experiment and NDT traits under field conditions as Price and Tomas (1997) and Shimizu et al. (2004) reported. In maize, hydroponics experiment has been demonstrated to be useful for detecting QTL regions associated with root traits at an early growth stage, and also for influencing grain yield in the field (Tuberosa et al. 2002). The dimorphic root genotypes such as FEDEARROZ174 and *O. rufipogon* showed higher relative grain yield (RGY), one of the most important NDT trait compared to monomorphic root genotypes (either deep or shallow) over the two seasons, but I could not find any correlation with other major agronomic traits (Table 4.3). In addition, there was a significant correlation between RPV under 50 μM NH_4^+ in greenhouse and NDT traits under field conditions.

Our result suggests that dimorphic root system such as intermediate RDR (around 50) and RPV nearer to zero might be useful for improving NAE under N limited conditions. Since inorganic N transformation and leaching to deeper layer (Lynch 2013), the proportion of roots in the deeper soil layer increases in paddy fields (Morita and Yamazaki 1993), in contrary, N mineralization (Murphy et al. 1998) and P availability are generally highest at the top soil layer (Zhu et al. 2005). In beans under combined stress conditions, a large dimorphic root system permitted vigorous rooting both in the surface and deep soil horizons. This dimorphic root genotype had also the most vigorous shoot because increased diversion of biomass to the root system was not at the expense of aboveground growth and may also be important for the acquisition of soluble nutrients, especially NO_3^- N (Liao et al. 2006; Palta et al. 2007). Thus, the dimorphic root system is likely to be an important contributor to the higher efficiencies of nutrient acquisition in rice.

The association between dimorphic root system and NDT traits can be further confirmed by using homozygous genetic material such as near-isogenic lines (NILs). To further clarify the interaction between dimorphic root system and NDT traits, detailed analyses of interested CSSLs with Curinga and *O. rufipogon* would be the next step. To the best of our knowledge, this is the first report showing the variation of RDR, RPV and root growth pattern response to different NH_4^+ concentrations in paddy rice. This will benefit the understanding of the genetic control of RSA response to NH_4^+ concentrations in lowland conditions, and this trait can be a target for nutrient acquisition efficiency in paddy rice.

4.5 CONCLUSIONS

Five contrasting genotypes with distinct rooting patterns (monomorphic-deep, monomorphic-shallow and dimorphic, selected based on the RDR and RPV from the hydroponic study (CHAPTER 2) were used in this CHAPTER. These distinct genotypes were evaluated in the field to identify the role of root architecture on plant performance under different N applications. Our field results revealed that the dimorphic rooting genotypes enhance the grain yield and shoot biomass under N deficient conditions compared to the monomorphic root genotypes. The yield reduction in native N conditions compared to that in FP was smaller in the dimorphic rooting genotypes than in the shallow or deep monomorphic root genotypes.

I suggest that dimorphic rooting pattern would be helpful to enhance the nitrogen-acquisition efficiency (NAE) of rice in paddy filed conditions. Our next challenge is to understand relationship between RSA and NDT without genotype x environment effect, 1) by using developed lines such as NIL in a single background and 2) by investigating N absorption mechanism from shallow root. These studies will shed light on the fundamental understanding for NUE enhancement. To gain a better understanding about the genetic basis of relationships between RSA and agronomic performance, I evaluated a set of CSSLs derived from crosses between two contrast root plasticity genotype; a tropical *japonica* rice cultivar

‘Curinga’ and ‘*Oryza rufipogon’* accession IRGC105491 (Fig. 3.1) under paddy field with native and FP N treatments above mentioned in the next CHAPTER.

CHAPTER 5 QTL ANALYSIS AND THE CORRELATION WITH ROOT SYSTEM ARCHITECTURE TRAITS USING CHROMOSOME SEGMENTS SUBSTITUTION LINES

5.1 INTRODUCTION

In 2010, Gewin (2010) unearthed some promising subterranean strategies in root modification to increase yield and agronomic performance that are called "underground revolution". Improved RSA can increase water and nutrient acquisition efficiency (Chapman et al. 2012). In CHAPTER 4, I determined the significance and magnitude of variations of NDT traits for yield performance using representative genotypes with contrasting RSA. To gain a better understanding about the genetic basis of relationships between RSA and NDT, I conducted field experiments for two seasons with different N applications using a set of CSSLs between Curinga and *O. rufipogon* that showed different N responses to seminal root elongation and early root growth under hydroponic conditions (CHAPTER 2 and 3; Ogawa et al. 2014ab) for QTL analysis.

QTL analysis has been adopted in studying NDT traits in rice (Wei et al. 2012). Wei et al. (2012) identified eight QTLs for NDT traits using recombinant inbred lines (RILs) derived from the cross of Zhenshan97 / Minghui63. Lian et al. (2005) also identified 14 NDT traits using same RILs. Several QTLs for N-uptake that have positive effects co-localize with QTLs for RSA traits, suggesting that increasing NUE can be achieved by breeding for a RSA traits (Coque et al. 2008) which consequently improves overall grain yield (De Dorlodot et al. 2007). In addition the NDT QTLs, qRL6.1 and qRL1.1 for root length associated with increased root length have the potential to enhance N-acquisition (Obara et al. 2010; Obara et al. 2011; Chin et al. 2010).

Lynch et al. (2013) proposed a steep, cheap and deep root system as the ideotype to enhance N-acquisition in maize. Here he was referring to the root growth characteristics of steep growth angle, low density of lateral roots per length of axial root (cheap), and greater lateral root length of crown roots (deep) to reduce inter-root competition, improve the metabolic efficiency of soil exploration, and

accelerate the elongation of axial roots. That kind of root system is helpful to improve optimal acquisition of water and N. Result of CHAPTER 4 suggested that dimorphic root system increases grain yields under N-deficit conditions. Even though it was indirectly estimated that RSA may have considerable impact on NDT, it is known that such effect is highly dependent on a specific environment (Garnett et al. 2009), therefore few successes of breeding new crop variety by improving RSA traits have been reported.

The objective of this study was to identify QTLs controlling NDT traits among a set of CSSLs between Curinga and *O. rufipogon,* for which wide difference in RSA was already identified in the previous CHAPTER (CHAPTER 3 and Ogawa et al. 2014ab). Those QTLs would be useful for breeding new cultivars adaptable to low N conditions. I also intended to elucidate the relationship between RSA QTLs that were detected in CHAPTER 3 and NDT traits QTLs found in this CHAPTER.

5.2 MATERIALS AND METHODS

Field phenotyping for NDT using CSSLs

A field experiment was conducted in both wet (February to June) and dry (August to December) seasons in 2014 by using N-omission lowland field plot facilities at CIAT with 48 CSSLs derived from a cross between the Curinga and *O. rufipogon* that were described in CHAPTER 3. Before starting each experiment, maize was planted for two consecutive cycles to make the field homogeneously deficient in N. Soil samples were taken before transplanting, flowering and after harvest at 30 points in each field at 0-15 cm depth by using metal tube with 8 cm diameter and mixed (Table 5.1). Organic matter content (Walkley and Black method), ammonium (1M KCl method) and nitrate (1 M KCL method) N, and total N (dry combustion method) was analyzed according to Salinas and Garcia (1979) (Table 5.1). The experiments were laid out in a split-plot design with two N treatments as first factor and genotypes as second factor, replicated twice with randomization. The N treatments involved were: “Native” with 0 kg N ha^{-1} application and “Farmers’ Practice (FP)” in Colombia (Berrio et al. 2002) where 180 kg N ha^{-1} was

applied. Rice cultivation and trait measurement were conducted as already described in CHAPTER 4. In addition to CHAPTER 4, parameters for NDT such as relative N contents (RNC) of each line at both native and FP were calculated using the following formula reported by Wei et al. (2012). N contents in plant tissue (flag leaf) were measured by Kjeldahl method at CIAT soil laboratory.

Relative N contents = N contents $_{native}$ / N contents $_{FP}$.

In addition to NDT traits parameters, Agronomic NUE (ANUE) was calculated as the difference of yield performance between native and FP treatments divided by fertilized N amount (180 kg Ha^{-1}) according to Craswell and Godwin (1984).

ANUE = (Grain Yield $_{FP}$ - Grain Yield $_{native}$) / Amount of fertilized N.

We also used the average value of root traits data that was determined in CHAPTER 3 for elucidating the relationship between RSA and NDT.

Table 5.1 Soil N properties before the experimental field trials in 2014

		N treatments	
Soil chemical property	**Year**	**Native**	**100 % FP**
Organic matter (g/kg)	Feb.-Jun.	-	-
	Aug.-Dec.	15.92 ± 0.76	13.75 ± 0.23
NH_4^+ (mg/kg)	Feb.-Jun.	8.82 ± 1.46	11.63 ± 1.28
	Aug.-Dec.	13.50 ± 1.15	11.88 ± 0.96
NO_3^- (mg/kg)	Feb.-Jun.	0.88 ± 0.12	0.46 ± 0.26
	Aug.-Dec.	6.10 ± 0.10	6.02 ± 1.45
Total N (mg/kg)	Feb.-Jun.	912.7 ± 94.1	995.7 ± 78.5
	Aug.-Dec.	1399.5 ± 47.0	1194.8 ± 42.23

Data are mean ± SE of three replications.

QTL analysis

For QTL analysis, average data of 21 individual plants was used, i.e., SPAD value, plant height, leaf N contents at flowing period, single plant grain yield, single plant shoot biomass and 1000 grain weight at harvesting time. Each trait under both native N and FP conditions were used in each year because there was a significant seasonal effect. In addition to agronomic traits, NDT traits calculated as value under native N conditions divided by that under FP conditions were used together with ANUE.

QTL analysis was conducted by CSSL finder v. 0.84 computer program (Lorieux 2005). See detail information at CHAPTER 3 SECCION 1. A set of 238 SNP markers were used to identify regions associated with each trait using QTL analysis. The location of each SNP markers closet to QTLs analysis is shown in Fig.3.2.

Analysis of correlation between nitrogen-deficiency tolerance (NDT) traits and root system architecture (RSA) using CSSLs

The results obtained in this CHAPTER were used as the NDT traits in the field, and the results in the solution culture experiment using basket method (CHAPTER 3 SECTION 2) was used as the traits for RSA, and the correlation analysis were performed between these parameters using XLSTAT, add-on for Microsoft Excel.

5.3 RESULTS

Agronomic performance of each CSSL and parents under different N treatments

Significant variations (ANOVA, $P<0.001$) were observed in agronomical traits between the variables year, genotype, N treatment and their interactions (Table 5.2). Performance of each CSSL and parents for four traits (single plant grain yield, single plant biomass yield, flag leaf N concentration and SPAD value) were investigated under native and FP treatments. The frequency distributions of these four traits in the CSSLs were shown in Fig. 5.1. All four traits segregated continuously and almost fitted normal

distribution under two N treatments (Fig. 5.1). Significant differences between two parents were observed. For NDT traits (Table 5.2), as compared with Curinga, *O. rufipogon* had higher values for RGY and relative biomass yield (RBM) but relative N concentration (RNC) showed different result between the tested seasons (Table 5.2). However, Agronomic Nitrogen Use Efficiency did not show any significant difference between the two parents.

Table 5.2 Performance of NDT and NUE traits of parental lines and CSSLs of Curinga / *O. rufipogon* tested over two seasons

Traits	Parents		CSSLs		ANOVA(*P*-value)		
	Curinga	*O. rufipogon*	Mean	Range	Genotype	Treatment	G x T
			Feb.-Jun.				
Relative grain yield	60.39	76.47	65.84	44.47-95.92	<0.001	<0.001	<0.001
Relative biomass yield	70.26	90.33	70.63	46.75-97.89	<0.001	<0.001	<0.001
Relative N concentration	61.75	79.09	66.22	48.34-94.38	<0.001	<0.001	0.011
Agronomic Nitrogen Use Efficiency	14.76	10.65	12.67	1.22-23.20	<0.001	<0.001	<0.001
			Aug.-Dec.				
Relative grain yield	56.12	68.3	64.14	42.75-95.18	<0.001	<0.001	<0.001
Relative biomass yield	54.04	58.09	65.54	49.22-86.51	<0.001	<0.001	<0.001
Relative N concentration	91.86	73.6	84.09	61.86-97.96	<0.001	<0.001	0.992
Agronomic Nitrogen Use Efficiency	12.32	12.87	10.65	1.14-26.39	<0.001	<0.001	<0.001

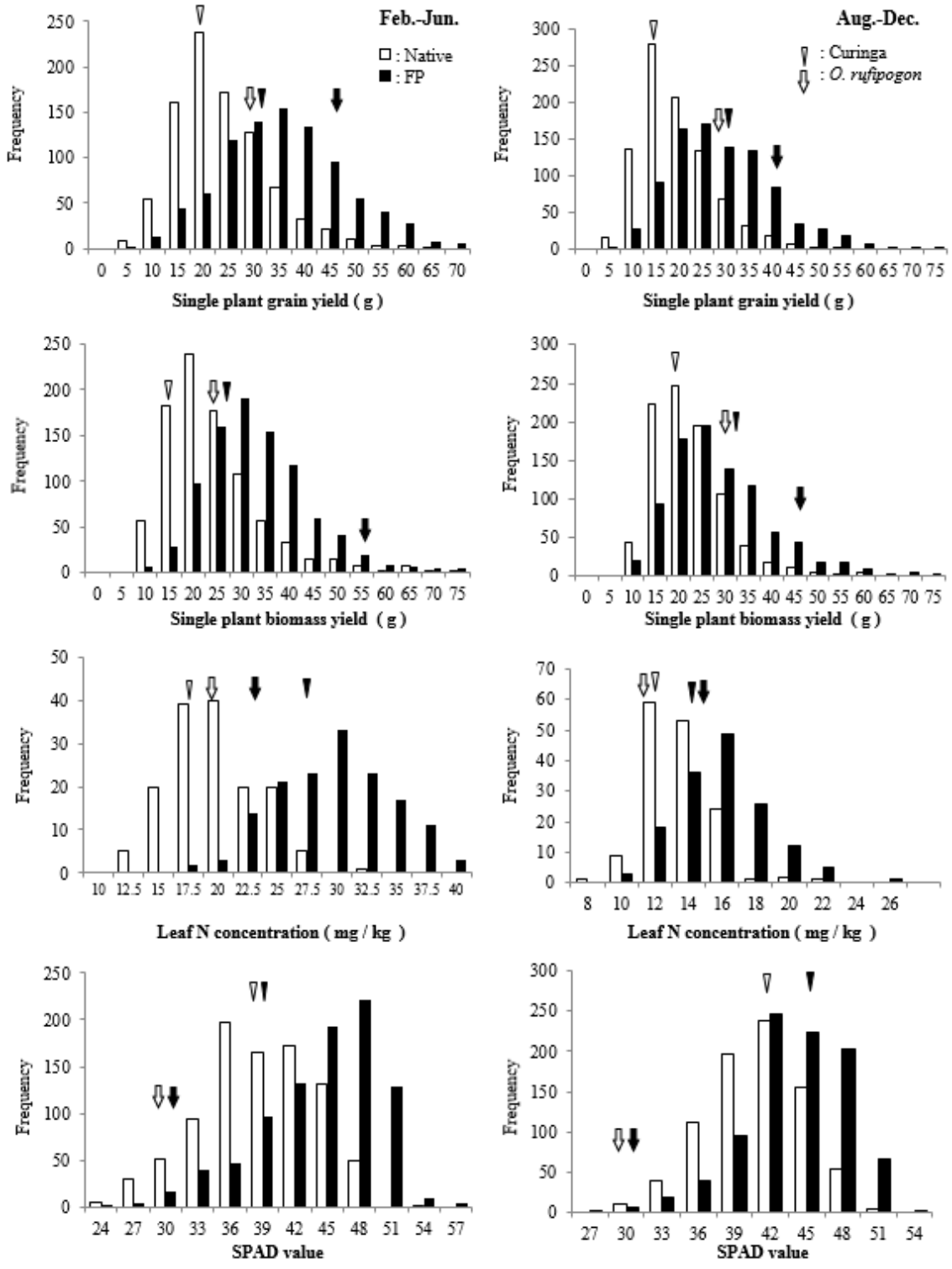


Fig. 5.1 Frequency distribution for single plant grain yield, single plant biomass yield, N concentrations (flag leaf), SPAD value (flag leaf) of the CSSLs measured under native and FP in two seasons in 2014

QTL analysis

A total of 11 putative QTLs for N deficient tolerance and morpho-physiological traits were identified on chromosomes 1, 3, 4, 5, 7, 8, 9, 10 and 12 (Table 5.3).

Table 5.3 Identified putative QTLs in this study compared with QTLs regions from previous studies

Trait	Treatment	Chr.	Marker	Position(Mb)	Positive allele	Trial/Reference
In this study						
Relative grain yield	NDT trait between Native and FP	1	id1010490-id103568	19.68 -27.24	*O.rufipogon*	Feb.-Jun.
Single plant grain yield	FP	3	id3002476-id3004123	4.32-7.68	*O.rufipogon*	Both trials
Shoot biomass	Native	4	id4005120-id4007907	17.68-24.36	*O.rufipogon*	Feb.-Jun.
1000 grain weight	Native and FP	5	Id5006603-id5012179	16.45-25.79	*O.rufipogon*	Both trials
SPAD value	FP	7	id7000142-id7000609	0.74-4.66	*O.rufipogon*	Both trials
Nitrogen content in leaf	FP	7	id7000142-id7000609	0.74-4.66	*O.rufipogon*	Feb.-Jun.
Relative N content	NDT trait between Native and FP	7	id7000142-id7000609	0.74-4.66	*O.rufipogon*	Feb.-Jun.
Relative SPAD value	NDT trait between Native and FP	8	id8000171	0.53	*O.rufipogon*	Feb.-Jun.
Tiller number	Native	9	id9000233-id9000580	0.88-10.75	*O.rufipogon*	Feb.-Jun.
Early flowering	Native and FP	10	id1005370-id1006910	18.66-22.34	*O.rufipogon*	Aug.-Dec.
Tiller number	FP	12	id12003803-id12005677	9.54-16.74	*O.rufipogon*	Aug.-Dec.
In previous studies						
Grain weight	Upland well water condition in Colombia	1	RM5-RM1232	23.97 - 27.63	*O.rufipogon*	Moncada et al. 2001
Grain per panicle	Lowland FC field in China	3	RM22-RM231	1.57 – 7.4	*O.rufipogon*	Fu et al. 2010
Spikelet per panicle	Lowland FC field in China	3	RM22-RM231	1.57 – 7.4	*O.rufipogon*	Fu et al. 2010
Tiller number	Lowland FC field in China	4	RM255-PSM115	30.77–34.58	*O.rufipogon*	Zhou et al. 2013
1000 grain weight	Lowland FC field in China	5	S15902070-S17002291	5.90 – 17.00	Lemont	Wang et al. 2014
1000 grain weight	Lowland FC field in China	5	S20781937-S23672252	20.78– 23.67	Tequing	Wang et al. 2014
NADH-GOGAT protein	Greenhouse	7	C261	0.79	Kasalath	Obara et al. 2001
Soluble protein	Greenhouse	8	R1963	28.17	Kasalath	Obara et al. 2001
Tiller number	Lowland FC field in China	9	RM6570-RM215	18.57-21.19	*O.rufipogon*	Zhou et al. 2013
Heading date	Greenhouse	10	RM171-RM228	18.80-21.98	*O.rufipogon*	Thanh et al. 2010
Tiller number	Artificial field	12	G2140-R3375	5.60-18.86	Kasalath	Wissuwa et al. 1998

For NDT traits, one QTL for RGY on chromosome 1 was detected in Feb.-Jun. trial. A RNC QTL on chromosome 7 was detected in Feb.-Jun. On the same region of the chromosome, QTLs for low SPAD value and low N concentration were detected under FP treatment in Feb.-Jun. trial. For relative SPAD value, one QTL on chromosome 8 was detected in Feb.-Jun. trial.

For agronomic traits, a QTL for tiller number on chromosome 9 and biomass yield QTL on chromosome 4 were also detected under native treatment in Feb.-Jun. trial. Single plant grain yield QTL on chromosome 3 was detected under FP treatment in both trials. Tiller number QTL on chromosome 12 was detected under FP treatment in Aug.-Dec. trial. QTL for thousand grain weight were detected constitutively on chromosome 5 under both native and FP treatments in both two trials. Early flowering QTLs also were detected on chromosome 10 under both native and FP treatments but only in Aug.-Dec. trial.

Correlation among NDT traits, and that between NDT and RSA traits

The results obtained in Pearson's correlation coefficient among NDT traits are presented in Table 5.4. RGY and RBM showed positive significant correlation in both seasons as previously reported elsewhere (Wei et al. 2012). The correlation between RGY and ANUE, and RBM and ANUE were significantly negative in both seasons, constitutively. However, RBM and RNC showed the positive correlation only in Feb.-Jun. season, but not in Aug.-Dec. season.

Table 5.4 Correlations among NDT traits observed in the CSSLs of Curinga / *O. rufipogon*. Below and above the diagonal is the correlation in Feb.-Jun. and Aug.-Dec., respectively.

	RGY	**RBM**	**RNC**	**ANUE**
RGY		**0.55*** **	-0.04	**-0.78*** **
RBM	**0.56*** **		0.01	**-0.52*** **
RNC	0.27	**0.46*** **		0.04
ANUE	**-0.93*** **	**-0.57*** **	-0.26	

RGY; Relative grain yield, RBM; Relative biomass yield, RNC; Relative N content, ANUE; Agronomic nitrogen-use efficiency. *** indicated a significant at $P < 0.001$.

The correlations between NDT and root traits are shown in Table 5.5. The RCN was significantly correlated with RPV in season 1 (Feb. – Jun.) and The RGY was also negatively correlated with RPV in season 2 (Aug. – Dec.). Low RPV; that means the dimorphic rooting system, showed higher relative grain yield. These results suggested that dimorphic root system is the one of the important root system architectures to improve nitrogen-deficient tolerance. Ratio of Deeper Roots (RDR) and ANUE had significantly negative correlation in both seasons. This suggests that lower RDR lines (shallow root system) are good to improve agronomic performance when sufficient N is applied in the field. In the trial Aug.-Dec., Rooting Pattern Value (RPV) showed positive correlation with ANUE. These results indicated that higher RPV lines (dimorphic root system) are important to improve NUE.

Table 5.5 Phenotypic correlations between root traits and NDT traits observed in the CSSLs of Curinga / *O. rufipogon*

	Deep #	**Shallow #**	**Total #**	**RDR**	**RPV**	**MRL**	**Root Biomass**
			Season 1 (Feb.-Jun.)				
RGY	0.00	-0.12	-0.09	0.18	0.04	0.04	**-0.42***
RBM	0.09	-0.13	-0.03	0.16	-0.08	-0.17	-0.16
RNC	0.12	-0.16	-0.04	0.15	**-0.38***	-0.04	-0.20
			Season 2 (Aug.-Dec.)				
RGY	0.06	-0.12	-0.05	0.24	**-0.35***	0.06	-0.03
RBM	**0.35***	-0.22	0.06	**0.48****	-0.25	0.19	0.03
RNC	-0.07	0.07	0.01	-0.10	-0.04	-0.06	0.14

RGY; Relative grain yield, RBM; Relative biomass yield, RNC; Relative N content, Deep #; deeper root number, Shallow #; shallow root number, Total #; total root number, RDR; ratio of deeper root, RPV; rooting pattern value, MRL; maximum root length

***, ** and * indicated a significant at $P<0.001$, 0.01 and 0.05.

5.4 DISCUSSION

Field NDT traits among CSSLs between Curinga and *O. rufipogon*

We repeated the field experiments for evaluating agronomic and NDT traits for two seasons, and we observed variation in overall agronomic performances due to difference in the environmental factors, particularly solar radiation, temperature and precipitation (IDEAM; http://www.ideam.gov.co/, ANEXO.1; Fig. 5.1). Agronomic performance of Feb.-Jun. in 2014 was similar to that of 2013 (Feb.-Jun.), because both were in the rainy season. In contrast, that of Aug.-Dec. in 2014 was similar to that of 2012 (Aug.-Dec.), because the growing season of the year was completely same. Although there was a significant difference in overall agronomic performance between two seasons, the trends of the responses of agronomic performance among CSSLs and their parents to N treatments were similar (Table 5.2 and Fig. 5.1). It seems that the strong trait-controlling QTLs were present throughout the two testing seasons, although some Genotype x Environment interaction existed.

The relationship between NDT and RSA was already tested and discussed in CHAPTER 4 using 5 genotypes having contrasting RSA, and I tentatively concluded that dimorphic root system has advantage over monomorphic shallow or deep root systems to attain higher NDT. To further test this hypothesis, I used plant materials with narrower variation than five root contrast varieties (CHAPTER 4) in root morphology but simpler genetic basis, that is, CSSLs between Curinga (monomorphic deep root system) and *O. rufipogon* (dimorphic root system). Using the same genetic background, it was assumed to be easier to identify effect of RSA on NDT in details. I used both the results of the root growth analysis in CHAPTER 3 and that of the field evaluation under native and FP N conditions in this CHAPTER to obtain the correlation results (Table 5.5). However, RSA is a complex trait consisting of root biomass, total root number, root length and root growth angle in both crown and secondary roots (Araki et al. 2002). Moreover, NUE and NDT traits are also complex consisting of various physiological processes such as: photosynthesis and respiration, N and carbon metabolism and plant hormone metabolism (Novoa

and Loomis 1981). Therefore, correlation between NDT traits and RSA was not clear in tested two seasons (Table 5.5). The results of the present experiment suggested that including lower RDR trait (shallow root system) into Curinga (monomorphic-deep rooting variety) genome background can improve grain yield once I apply sufficient N fertilizer under paddy field conditions. In other words, shallow root system can be helpful to maintain the yield even under the low N conditions (high NDT). Shallow root system has been known to preferentially take up nutrients such as mineralized N and phosphorus from the topsoil (Lynch 2011, Uga et al. 2013). Morita and Yamazaki (1993) reported fresh weight of superficial roots is positively correlated with grain yields in paddy fields. In contrary, deep root system was reported as an important RSA to take up leached inorganic N from deep soil layer (Thorup-Kristensen 2006, Lynch, 2013). Still, it must be remembered that lower RDR lines used in this experiment has almost dimorphic root systems contrasting to Curinga which has monomorphic-deep root system. The contribution of the root dimorphism system to enhance to NDT should be studied further.

QTL analysis for NDT and agronomical traits under different N treatments

From the results of QTL analysis for NDT traits in this study, some QTLs were identified in both seasons (Table 5.3). Some of them were not identified in the previous report, probably due to the genotype by environment interaction. As shown by ANOVA (Table 5.2), environmental conditions of two seasons such as temperature and radiation had large effects on yield responses to N. Between the two experiments used for different seasons, concentration of soil available N (NH_4^+) during the two trials was highly different, probably due to the ongoing mineralization and leaching due to the precipitation as reported previously (Wei et al. 2012, Ogawa et al. 2014a). Especially, the NH_4^+ level of the second trial were lower than in the first trial (Table 5.1).

A total of 8 QTLs for agronomical traits were detected on chromosomes 3, 4, 5, 7, 9, 10 and 12 (Table 5.3). A total of 3 QTLs for NDT traits were also detected on chromosomes 1, 7 and 8 (Table 5.3). Some

of these QTLs matched the QTLs that related to the similar traits in the previous reports. When I compared the QTLs discovered in this study with previously reported QTLs in rice using the Gramene Annotated Nipponbare Sequence 2009 map (www.gramene.org), several co-located loci were found (Table 5.3).

The genomic region flanked by SNP marker id1010490-id103568 on chromosome 1 was detected to have QTLs for relative grain yield in Feb.-Jun. season. Moncada et al. (2001) found that overlapped region was associated with grain weight under well-watered upland conditions. Fu et al. (2010) also identified QTLs in this region for thousand grain weight. Furthermore, in the middle of our QTL region, NRT2.1, a nitrate transporter to improve N assimilation was found (Araki and Hasegawa 2006). Katayama et al. (2009) reported that NRT 2.1 was the candidate genes to improve NUE and NRT2.1-overexpression lines enhanced vegetative growth. Overexpression of another nitrate transporter in rice (OsNRT2.3b) significantly increased yield and total N uptake (Xu et al. 2012). In addition, Feng et al. (2010) reported not only nitrate deficient conditions but also ammonia deficient conditions enhanced OsNRT2.1 gene expression.

A QTL for single plant grain yield was located in the region of id3002476-id3004123 on chromosome 3. In same region, Fu et al. (2010) detected QTLs for grain per panicle and spikelet per panicle under lowland FP in China. The marker interval id4005120 – id 4007907 on chromosome 4 for biomass yield and id9000233 – id9000580 for tiller number under native N treatment were reported as tiller number QTLs under lowland FP field in China by Zhou et al. (2013). The marker interval id700142-id700609 that was detected by SPAD value, N concentration, and relative N concentration was reported as NADH-GOGAT protein by Obara et al. (2001). In addition, Obara et al. (2001) also reported the tip of the short arm of chromosome 8 that include peak of id800171, which l also reported in this research as a QTL of relative SPAD value and a QTL of soluble protein. It is known that N content in plants is predominantly affected by the Rubisco content; one of the soluble protein, which strongly affects photosynthesis. About 50% of the

total soluble protein and 25% of the total N are in Rubisco protein in rice leaves (Makino 2003). QTL for 1000 grain weight between the marker id5006603-id5012179 was also reported by Wang et al. (2014). Interestingly, only this 1000 grain weight QTL was identified consistently over the different N treatments (native and FP) and two seasons. This region from *O. rufipogon* could be a good candidate for introducing stable yield traits into rice varieties. Our QTLs analysis has also confirmed the previous results (Wei et al. 2012) of NDT and NUE trait relationship in rice and identified NDT QTLs could be used as targets for developing rice cultivars adapted to N stress environment. These loci should be further investigated as candidates for utilization in marker assisted breeding programs to improve NUE in rice.

QTLs for RSA to enhance NDT, "underground revolution" associated root architectural QTLs

N is a limiting nutrient in plant growth that is usually taken up from soil by root system (Epstein and Bloom, 2005). To breed crops for naturally fluctuating N environments, mechanisms that mediate traits conditioned on the environment may be an important targets of crop improvement (Gifford et al. 2013). Although root system architecture showed different plasticity between hydroponic and soil conditions, previous studies have shown that the genetic variation for root traits of seedlings and young plants grown in hydroponic culture at an early growth stage is associated with variation in root traits at a later growth stages under field conditions (Tuberosa et al. 2002, Shimizu et al. 2004). Furthermore, RSA of plants grown in hydroponic culture has been widely used to detect QTL associated with improved root systems in both stressed and non-stressed rice fields (Uga et al. 2013, Obara et al. 2010). Although many of the QTLs identified in the present study were different across the seasons, they may be used after careful validation, in breeding programs demanding specific adaptability (Wei et al. 2012). A large number of QTLs or genes promising for improving rice performance in water and nutrient uptake are now available, there is a few success reports that the improved root architecture enhanced grain yield in rice breeding program (Reviewed in Ahmadi et al. 2014). Finally, I found that the genomic region controlling deeper root number under hydroponic conditions simulating paddy field was overlapped with the genomic region of NDT trait under field conditions, which indicates the importance of deeper root number for increasing grain yield under N stress conditions. Lynch et al. (2013) mentioned a 'steep, cheap and deep' root ideotype for optimal acquisition of water and N by maize. In rice, Arai-Sanoh et al. (2014) reported deeper root traits introgressed by *DRO1* gene can help to absorb more N under both native and N applied field conditions.

5.5 CONCLUSIONS

In a perspective of reducing inputs in rice production, there is a huge need for breeding new N efficient rice. The objective would be to introduce QTLs involved in N uptake and NUE under low N fertilization conditions in the newly rice breeding. Most of the QTLs identified in this study were different across season, suggesting that the use of these QTLs would be difficult in breeding for general stability. However, once validated, these QTLs can be used in breeding for specific adaptability. Considering the relatively small population size and the fact that separating QTL-by-season interaction was not possible, the results must be regarded as preliminary and further validation is required. Further studies are also underway to confirm the impact of QTLs for RSA to improve not only N-acquisition efficiency but also another nutrient- and water- acquisition efficiency that will be useful to enhance yield performance in future rice breeding program. Fine mapping is also needed for gaining more information about the regions simultaneously controlling NDT traits.

QTLs for root architecture and NDT traits were mapped using 238 SNP markers loci. A total of 13 QTLs for root system architectural, NDT and morpho-physiological traits were identified on chromosomes 1, 3, 4, 5, 7, 8, 9, 10 and 12. Interestingly, a QTL for deeper root number was identified at the region of SNP markers between id1012330 and id1021697 on chromosome 1 under hydroponic conditions overlapped with a QTL for NDT trait of relative grain yield. The overlapped region of QTL for root features with those for grain yield suggests the possible role of the former in determining the latter (Tuberosa et al. 2002). However, higher genetic resolution is required to ascertain accurately the role of linkage in the cosegregation of QTL effects for traits that are plausibly related on a functional basis [e.g. root architecture, plant water status, osmotic potential, concentration of abscise acid and reactive oxygen species, yield components and yield] (De Dorlodot et al. 2007). In addition, plant root systems show highly plasticity to environmental stimuli. Recent analyses of field-grown crops highlighted the importance of RSA in nutrient acquisition (Lynch 2013). This indicated that it is feasible in practice to

exploit genotypes or mutations giving rise to optimal RSA for crop design in the future, especially with respect to plant breeding for infertile soils (Kong et al. 2014). The QTL associated root architecture could potentially be used in future rice-breeding efforts to increase agronomic performance under N deficient conditions.

CHAPTER 6: GENERAL DISCUSSION

In this study, I conducted experiments to identify useful root traits to improve nitrogen-deficiency tolerance (NDT) traits for the future rice breeding program. As general discussion, I reviewed all detected results in this study to elucidate the interactions among root system architecture (RSA) traits in hydroponic experiments (CHAPTER 2 and 3) and yield related NDT traits under field conditions (CHAPTER 4 and 5).

Table 6.1 Correlation co-efficient between RSA traits under hydroponic conditions and NDT traits as relative grain yield under field conditions.

	Seminal root length (CHAPTER 2 & 3, SECTION 1)	NH_4^+ sensitivity (CHAPTER 2 & 3, SECTION 1)	Rooting Pattern Value (CHAPTER 2 & 3, SECTION 2)	Deeper root number (CHAPTER 2 & 3, SECTION 2)
5 genotypes Experiment 1 (CHAPTER 4)	0.52	-0.55	-0.87*	0.63
5 genotypes Experiment 2 (CHAPTER 4)	0.69	-0.65	-0.87*	0.95*
CSSL Experiment 1 (CHAPTER 5)	0.53*	-0.42*	0.04	0.00
CSSL Experiment 2 (CHAPTER 5)	0.31*	0.02	-0.35*	0.06

* indicated a significant at $P < 0.05$.

Low Rooting Pattern Value means dimorphic root system

Here, the correlation analysis between RSA traits under hydroponic conditions and NDT trait as relative grain yield (RGY) under field conditions are summarized. Although growth in general is different between hydroponic and filed/soil conditions, as I mentioned in the previous CHAPTERs, root characterization in hydroponic culture has been widely used to detect QTL associated with improved root systems in both stressed and non-stressed rice fields (Uga et al. 2013; Shimizu et al. 2004; Price and Tomas 1997). In the experiments in which five representative genotypes were used, the RGY was significantly correlated with RPV. And in the experiments using CSSLs, RGY was significantly correlated with seminal root length and RPV. In both two experiments, low RPV, which corresponds to

the dimorphic rooting system, showed higher RGY. As was mentioned in CHAPTER 4, dimorphic root system with low RPV (close to zero) might be useful for improving NAE under N limited conditions because of inorganic N transformation and leaching to deeper layer (Lynch 2013) and highest N mineralization (Murphy et al. 1998) at the top soil layer. There is limited evidence to conclude the effect of dimorphic root traits on the improvement of NDT traits. However, this dimorphic root trait may be useful in improving rice productivity not only in developing countries where the plants are suffered from limited N fertilizer applications, but also in developed countries where the cost of N fertilizer and environmental impact should be minimized. Table 6.2 Candidate genes for estimated region that control RSA and NDT

Candidate gene name	**Gene function**	**The Rice Annotation Project Database gene position**	**Reference**
PIN	Auxin efflux carrier component	Os01g0455500	Carraro et al. 2012
IAA	Amino acid hydrolase homolog precursor (involved in auxin homeostasis)	Os01g0510600	Ding et al. 2008
Tat protein	Twin-arginine translocation pathway signal domain containing protein	Os01g0456400	Fukao et al. 2011
ARFs	Auxin responsive factor 3	Os01g0480600	Wang et al. 2009
IAA8	Auxin-responsive protein	Os01g0484500	Groover et al. 2003
IAA8	Auxin-responsive protein	Os01g0488500	Groover et al. 2003
XPL1	Phosphoethanolamine N-methyltransferase	Os01g0500300	Luo et al. 2012
OsGLT1	NADH-glutamate synthase	Os01g0681900	Goto et al. 1998
OsAAT2 1 D14673	Aspartate aminotransferase	Os01g0760600	Song et al. 1996
OsAMT2;2 1	Ammonium transporter	Os01g0831300	Suenaga et al. 2003
OsAMT2;3	Ammonium transporter	Os01g0831900	Suenaga et al. 2003
OsAMT3;1	Ammonium transporter	Os01g0870300	Suenaga et al. 2003

OsAAT 1	Aspartate aminotransferase	Os01g0871300	de la Torre et al. 2006

Regarding the QTL analysis, it was found that, the QTL for deeper root number, identified in the region of SNP markers between 23.45 Mb and 36.46 Mb on chromosome 1, was overlapped with a QTL for NDT trait of RGY. QTL for deeper root number was also overlapped with seminal root length QTLs. Although there was no correlation between number of deep root and RGY among CSSLs, deeper root trait may have potential to maintain grain yield under N-deficient conditions. For the overlapped region, the auxin related genes to control root elongation and growth angle, and the ammonia transportation related genes to improve nitrogen-deficiency tolerance were already reported in the literature (Table 6.2). At this moment, I cannot conclude whether the improvement of NDT traits and the traits related to the RSA traits are carried out by the same one gene or by the gene interactions. To clarify this interaction between NDT and RSA, the interesting CSSLs were backcrossed to develop further generation material such as near isogenic lines for gene identification.

CHAPTER 7: CONCLUSIONS

Rice is one of the most important staple foods not only in Asia but also in other regions such as Latin America, because nearly half the world's population depend on rice for their diet (FAO 2011). Nitrogen (N) is an essential nutrient taken up in large amounts and usually is the most yield-limiting nutrient in rice production around the world (Samonte et al. 2006). However, estimates of the world nitrogen use efficiency (NUE) have been calculated to be as low as 33% (Raun and Johnson, 1999). Colombia, one of the major rice importing countries in Latin America, showed high rice production costs compare to US and other Latin American countries due to the high cost in N fertilizer use. Thus, improvement of NUE have a significant potential for the rice producers in Colombia.

Root plays an important role in acquisition of nutrients. Improvement of root system architecture (RSA) is an important breeding target for producing higher yield through improvement of acquisition efficiency of nutrients However, RSA showed high degree of plasticity in response to changes of the nutrient environment (Ogawa et al. 2014a; Wissuwa et al. 2005). Thus, root plasticity traits may assist plants to scavenge the nutrients in heterogeneous soils to increase water- and nutrient- acquisition efficiency. However, little is known about the interaction between RSA trait and agronomic performances under field environments and their genetic control. The objective of this study was to elucidate the root architectural plasticity to N level, and RSA ideotype in rice to improve agronomic performance under N-deficient conditions.

To clarify the interaction between RSA and agronomic performances, we conducted three different experiments at both greenhouse and field from 2012 to 2015, at CIAT. We used diverse accessions of both commercial cultivars and non-*sativa* species of rice (Table 2.1). The first experiment was to evaluate seminal root elongation response to different N forms (NH_4^+, NO_3^- and NH_4NO_3) and concentrations by using floating mesh method at eight days seedling stage. The result indicated that there is a genotypic

difference in the response of seminal root elongation to the forms and concentrations of N even at seedling stage (Fig. 2.1; 2.2). I also found that root elongation in some commercial varieties such as Curinga was sensitive to N, especially NH_4^+. As NH_4^+ concentration increases, root elongation of Curinga was inhibited but some non-*sativa* species such as *O. rufipogon* was not (Fig. 2.2). In the 2nd experiment, we examined the variation in root growth angle and plasticity among rice genotypes grown under hydroponics conditions at 40 days old with different NH_4^+ concentrations using basket method. We also observed that there is a genotypic variation of rooting pattern in response to NH_4^+ (Table 2.4; 2.5). Especially, rooting pattern as ratio of deep rooting (RDR) in *O. glaberrima* was insensitive to NH_4^+ concentration, while that in Curinga was (Table 2.4).

In the 3rd experiment, five contrasting genotypes with distinct rooting patterns (mono and dimorphic root system) selected in CHAPTER 2 SECTION 2 were evaluated for the plant agronomic performance under paddy field conditions with different N applications, and the nitrogen-deficiency tolerance (NDT) traits were evaluated. Dimorphic root system varieties that have both shallow and deep root system showed less yield reduction when the fertilizer application was reduced, compared to both monomorphic deep and shallow varieties (Fig. 4.1). We concluded that dimorphic rooting system would be helpful to enhance NDT trait in yield under paddy filed conditions.

To gain a better understanding about the genetic basis of relationships between RSA and agronomic performance, we evaluated a set of CSSLs derived from crosses between two genotypes of contrasting root plasticity, Curinga and *Oryza rufipogon* (accession IRGC105491) under three experimental settings similar to the above mentioned experiments.

QTL analysis was conducted with average data of RSA traits, agronomic traits and NDT traits using CSSL finder v. 0.84 computer program (Lorieux 2005). Following QTLs analysis of each experiment, we identified a total of 18 QTLs; including five QTLs for RSA traits on chromosomes 1 and 12 (Fig. 3.5),

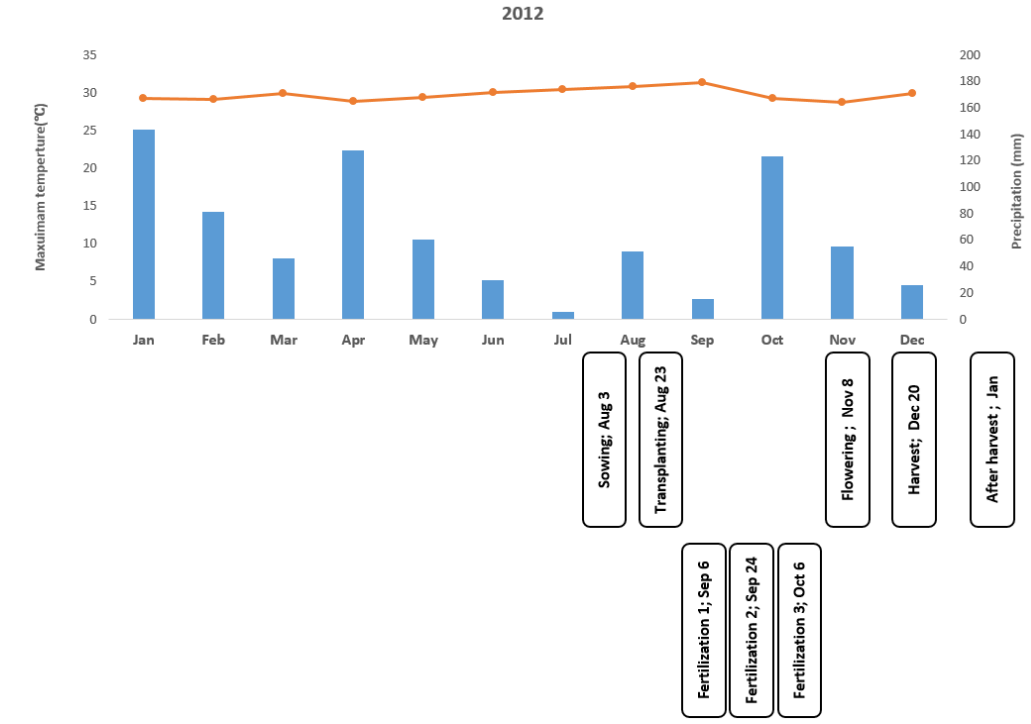
three QTLs for NDT on chromosomes 1, 7, 8, and 10 QTLs for agronomic traits on chromosomes 3, 4, 5, 7, 9, 10 and 12 (Table 5.3). Even if we should take the undesirable genetic linkage and pleiotropy into account, the identified QTLs could be used as target region for future breeding because of the possibility of simultaneous improvement in NDT traits.

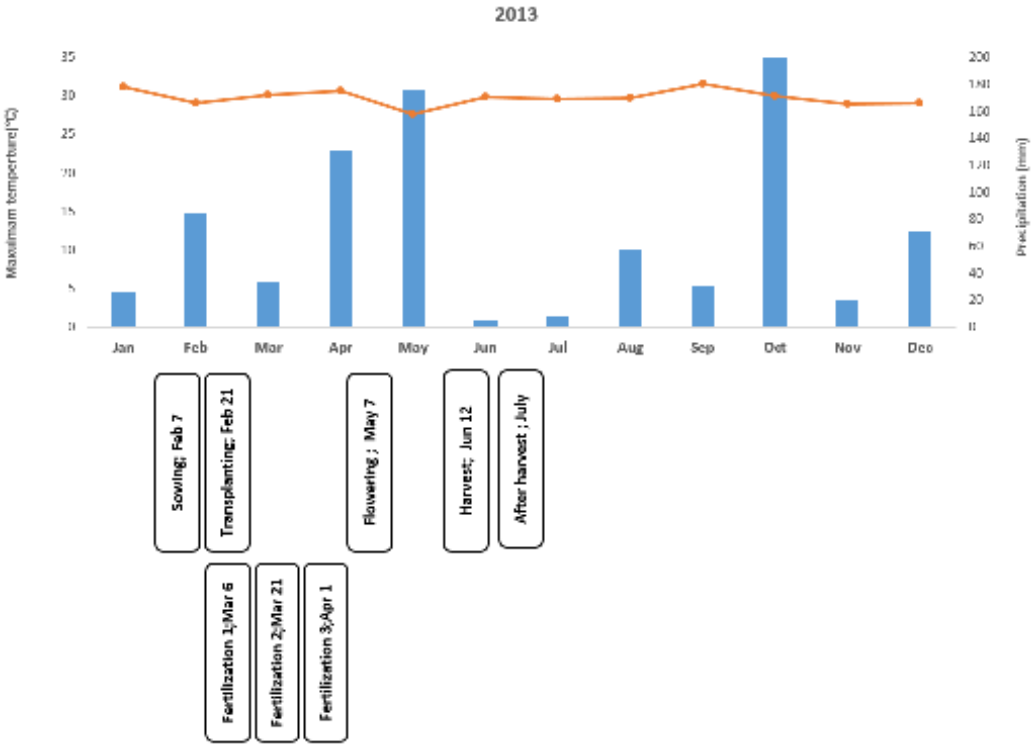
Interestingly, we found that a QTL for deeper root number identified in the region of SNP marker between id1012330 and id1021697 on chromosome 1 under hydroponic conditions overlapped with a QTL for NDT trait of relative grain yield (RGY) (Table 3.6; Table 5.3). These results suggest that there are some relationship and/or recombinant effect between deeper rooting trait and grain yield, although we cannot yet say that these QTLs are controlling those two traits. The QTL associated root system architecture could potentially be used in future breeding efforts to increase agronomic performance and to maintain grain yield under nitrogen-deficient conditions.

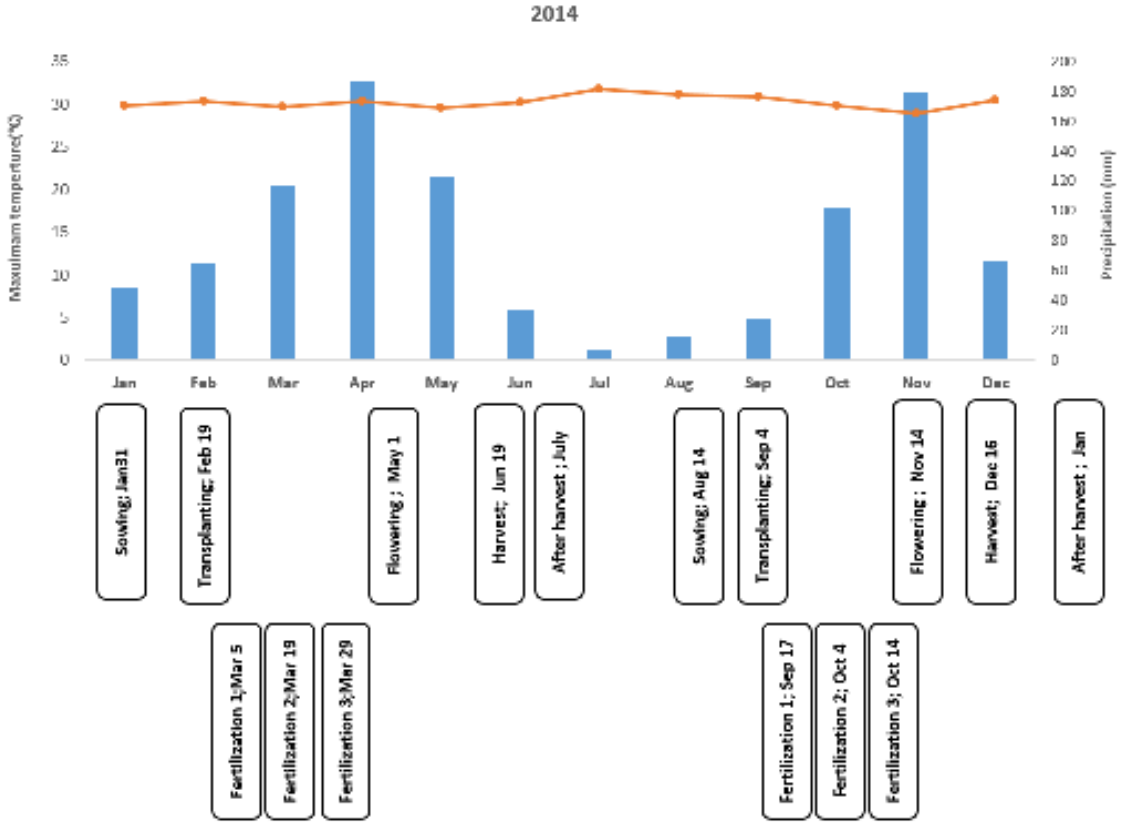
Genetic variation in RSA and its plasticity to nutrient conditions may be an appropriate targets for marker-assisted selection to improve rice nutrient acquisition efficiency. However, RSA is a complex trait that combines root length and root growth angle (Abe and Morita 1994). Our challenge is to discover useful RSA that improve NAE and to identify relevant gene that control interesting RSA traits for future rice breeding. Future studies would be to pyramid useful RSA QTLs effectively in single genetic background using advanced molecular tools and understanding interactions of Genotype x Genotype and Genotype x Environment for the development of rice varieties suitable for N deficit conditions.

ANEXO 1

Climate data and agronomical schedule






2014
Maxuimam temperture(°C)
Precipitation (mm)
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Sowing; Jan31
Transplanting; Feb 19
Fertilization 1;Mar 5
Fertilization 2;Mar 19
Fertilization 3;Mar 29
Flowering ; May 1
Harvest; Jun 19
After harvest ; July
Sowing; Aug 14
Transplanting; Sep 4
Fertilization 1; Sep 17
Fertilization 2; Oct 4
Fertilization 3; Oct 14
Flowering ; Nov 14
Harvest; Dec 16
After harvest ; Jan

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