The Effects of Fluctuations in the Nutrient Supply on the Expression of Five Members of the AGL17 Clade of MADS-Box Genes in Rice

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Abstract

The ANR1 MADS-box gene in Arabidopsis is a key gene involved in regulating lateral root development in response to the external nitrate supply. There are five ANR1-like genes in Oryza sativa, OsMADS23, OsMADS25, OsMADS27, OsMADS57 and OsMADS61, all of which belong to the AGL17 clade. Here we have investigated the responsiveness of these genes to fluctuations in nitrogen (N), phosphorus (P) and sulfur (S) mineral nutrient supply. The MADS-box genes have been shown to have a range of responses to the nutrient supply. The expression of OsMADS61 was transiently induced by N deprivation but was not affected by re-supply with various N sources. The expression of OsMADS25 and OsMADS27 was induced by re-supplying with NO₃⁻ and NH₄NO₃, but downregulated by NH₄⁺. The expression of OsMADS57 was significantly downregulated by N starvation and upregulated by 3 h NO₃⁻ re-supply. OsMADS23 was the only gene that showed no response to either N starvation nor NO₃⁻ re-supply. OsMADS23 and OsMADS57 was the only gene not regulated by P fluctuation whereas the expression of OsMADS23, OsMADS25 and OsMADS27 were downregulated by P starvation and P re-supply. In contrast, all five ANR1-related genes were significantly upregulated by S starvation. Our results also indicated that there were interactions among nitrate, sulphate and phosphate transporters in rice.

Introduction

Nitrogen, phosphorus and sulfur are three major macronutrients essential for plant growth and development [1]. Nitrogen (N) deficiency is a vital factor limiting agricultural quality and productivity. In aerobic soil conditions, nitrate is the major source of nitrogen for many higher plant species [2–4]. Nitrate acts not only as a nutrient but also as a signal to regulate gene expression, energy transfer, protein activation, metabolic and physiological activities, and plant growth and development [5–8]. Phosphorus (P) is a component of many key biomolecules and participates in various enzymatic reactions and metabolic pathways [9]. Due to low availability of phosphate (P) in soil, it acts as the second most important limiting macronutrients for plant growth and development [10–12]. Plants can modify their root architecture to improve phosphate acquisition in phosphate deficient soil. Many studies have demonstrated that P deficiency affects root development, including root hair elongation, reduced primary root length and increased number and length of lateral roots in Arabidopsis thaliana [13–19]. Sulfur (S) is also an essential macronutrient, and its deficiency adversely affects plant growth and development as well as the quality of crops [20,21].

Plants can modify their root architecture to forage for sources of N that are distributed unevenly in soil [6]. One important kind of foraging response involves increased proliferation of lateral roots within soil patches enriched in certain nutrients, such as NH₄⁺ and NO₃⁻ [22,23]. There are signaling mechanisms in roots to measure the levels of intrinsic and extrinsic nutritional factors so that they can modify their growth and development [24,25]. In Arabidopsis, localized nitrate treatment stimulated a localized increase in lateral root (LR) numbers and elongation. The stimulation of LR elongation was found to be the result of a signaling effect of external NO₃⁻ ion itself rather than downstream metabolites [26,27]. An important breakthrough in understanding NO₃⁻ in stimulating LR growth was the identification of the ANR1 gene, which is a vital component in regulatory signaling pathway [26]. ANR1 is a member of the plant MADS-box family of transcription factors, which covers more than 100 members in Arabidopsis [28–30]. In addition to their roles in regulation of reproductive development, the MADS-box transcription factors are also widely expressed in vegetative tissues [28,31]. Recent research has demonstrated that at least 50 MADS-box genes are expressed in roots of Arabidopsis, with ANR1 as the only member so far to have a known function in lateral root development in Arabidopsis [32,33]. Previous reports

Citation: Yu C, Su S, Xu Y, Zhao Y, Yan A, et al. (2014) The Effects of Fluctuations in the Nutrient Supply on the Expression of Five Members of the AGL17 Clade of MADS-Box Genes in Rice. PLoS ONE 9(8): e105597. doi:10.1371/journal.pone.0105597

Editor: John Schiefelbein, University of Michigan, United States of America

Received May 28, 2014; Accepted July 23, 2014; Published August 20, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: The research was supported by the International Scientific and Technological Cooperation Project of the Ministry of Science and Technology of China (grant number 2010DFA34430), International Scientific and Technological Cooperation Project of Science and Technology Department of Zhejiang Province (grant number 2013C3402010017), National Natural Science Foundation of China (Grant No. 31370215; 31228002), and Zhejiang Provincial Natural Science Foundation of China (Grant No. Z3110004). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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revealed that ANR1 is a positive regulator of lateral root growth and is not present in the primary root tip [34]. Initial studies using Arabidopsis root cultures had demonstrated that ANR1 gene was NO₃⁻ inducible [26]. Subsequent results obtained from hydroponically culture experiments illustrated that the expression of ANR1 was induced by N deprivation and rapidly downregulated when NO₃⁻ or other N source was re-supplied [29]. OsMADS23, OsMADS25, OsMADS27, OsMADS37 and OsMADS61 are five ANR1-like homologs in rice and their functions have not been well understood [35,36]. Recent work from Meng et al. (2013) has suggested that OsMADS27 could play a key role in the response to cold and salt stress [37]. To gain further insight into the possible regulatory functions of ANR1-like genes in roots, we have investigated their root expression patterns in response to N, P, and S fluctuation using quantitative real-time PCR (qPCR).

### Materials and Methods

#### Plant Materials

Rice seeds (Oryza sativa L. cv. Nipponbare) were used for all experiments.

#### Hydroponic culture

Rice seeds were surface-sterilized by treatment with 70% ethanol for 1 min and 10% sodium hypochlorite for 20 min, followed by five rinses with sterile distilled water. Seeds were germinated in the dark by placing into the incubator at 28°C for 2 days (d). Uniform seedlings were selected and transferred to black plastic buckets containing 4 L nutrient solution, where the growth conditions were 30/28°C day/night temperature with a 14/10 h light/dark at a relative humidity of 65–70%. The complete

### Table 1. List of primers used for Real-time PCR.

| Gene     | Forward primer (5'–3')       | Reverse primer (5'–3')       |
|----------|------------------------------|------------------------------|
| OsMADS23 | TCTTCTCCAGCACCAGCGGCTCT     | TGCTGCCCTCGTGTCAGAGGC        |
| OsMADS25 | CCAGTCTGCAAGCTATAGAATCAA     | AAGTTCGCCTGTGGTGCTGGT        |
| OsMADS27 | GAAGGGGAGGACGGGATCTTCAA      | TGCCATACGGATCTAATCGACT       |
| OsMADS57 | ACGGAACGGGACAGTTG             | ACTCATAGGGCGGCTGGTGCT        |
| OsMADS61 | GGGAGGGCGAAGATAGATGAT         | TGGTGCAGGACTGCTGGTAG         |
| OsActin  | CTTCATAGAAATGGAAGCTGCCGGT    | CGACCACCTTGATCTATGCTGCT      |

doi:10.1371/journal.pone.0105597.t001

Figure 1. Effect of N deprivation and nitrate resupply on the expression of five ANR1 related genes in rice roots. Ten-day old rice seedlings grown in complete nutrient solutions were transferred to modified nutrient solutions during which 2.88 mM KNO₃ was the sole nitrogen source for 4 days. Transcript abundance was assayed by qPCR and was expressed relative to the abundance in roots of plants of the same age grown under continuous N (CK). The OsNAR2.1 gene, a known nitrate-regulated gene, was included for comparison. Treatments: CK: continuous KNO₃; –N4h: starved of N for 3 d and resupplied with KCl for 4 h; +N4h: resupplied with KNO₃ for 4 h; –N6h: starved of N for 3 d and resupplied with KCl for 6 h; +N6h: resupplied with KNO₃ for 6 h. The mRNA of OsActin was used as the reference. A Student’s t-test was calculated at the probability of either 5% (*, p<0.05) or (**, P<0.01). doi:10.1371/journal.pone.0105597.g001
nutrient solution contained: 1.44 mM \( \text{NH}_4\text{NO}_3 \), 0.32 mM \( \text{NaH}_2\text{PO}_4 \), 0.5 mM \( \text{K}_2\text{SO}_4 \), 1 mM \( \text{CaCl}_2\cdot2\text{H}_2\text{O} \), 1.6 mM \( \text{MgSO}_4\cdot7\text{H}_2\text{O} \), 50 \( \mu \)M Fe-EDTA, 15 \( \mu \)M \( \text{H}_3\text{BO}_3 \), 0.12 \( \mu \)M \( \text{CuSO}_4\cdot5\text{H}_2\text{O} \), 0.12 \( \mu \)M \( \text{ZnSO}_4\cdot7\text{H}_2\text{O} \), 40.5 \( \mu \)M citric acid and, 0.39 \( \mu \)M \( \text{Na}_2\text{MoO}_4\cdot2\text{H}_2\text{O} \) [38]. 1 M HCl or NaOH was added to adjust pH to 5.5 and the nutrient solution was replaced every 2 d.

Nitrogen treatments
For the analysis of gene expression in response to nitrate, rice seedlings were grown in liquid culture for 14 d with 1.44 mM \( \text{NH}_4\text{NO}_3 \) as the N source. The solution was changed every 2 d. After 10 d, the medium was changed to 2.88 mM \( \text{KNO}_3 \) as the sole N source for 4 d. Then, the seedlings were deprived of N for 3 d before being re-supplied with \( \text{KNO}_3 \) or \( \text{KCl} \) to a final concentration of 2.88 mM. The control plants were grown in continuous 2.88 mM \( \text{KNO}_3 \) as the only N source. The plants from different treatments were harvested at 4 h, 6 h and 8 h after re-supply separately. The roots were frozen in liquid \( \text{N}_2 \) and stored at \(-80^\circ\text{C}\) for later analysis.

For analyses of gene expression in response to different N sources, the plants were grown in complete nutrient solution with 2.88 mM \( \text{KNO}_3 \) as the sole nitrogen source for 14 d [39]. Then, the plants were starved for N for 3 d before being transferred to fresh complete nutrient solution at the same time. For re-supply, the appropriate nutrient, 0.32 mM \( \text{PO}_4^{2-} \) or 2.1 mM \( \text{SO}_4^{2-} \) was added in the light period for 3 h before the roots were harvested and frozen in liquid \( \text{N}_2 \) and stored at \(-80^\circ\text{C}\) for gene expression analyses [38].

RNA extraction and qPCR
Primers for \( \text{OsMADS23} \), \( \text{OsMADS25} \), \( \text{OsMADS27} \), \( \text{OsMADS57} \) and \( \text{OsMADS61} \) for qPCR (see Table 1) were designed using Primer Premier 5. Total RNA was extracted using the RNAiso Plus reagent (Takara) according to the manufacturer’s instructions. The first-strand cDNA was synthesized using PrimeScript RT reagent Kit with gDNA Eraser (Takara). The qPCR was performed in 96-well plates using SYBR Premix Ex Taq II (Takara) according to the manufacturer’s instructions. The qPCR was conducted in the following cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Melt curve analysis was used to confirm the absence of non-specific amplification products. Relative expression levels were calculated by subtracting the threshold cycle (Ct) values for \( \text{OsActin} \) (Os03g0718100) from those of the target gene (to give \( g\text{Ct} \)) and then calculating \( 2^{-g\text{Ct}} \) as we described before [40–42]. RT-PCR experiments were performed with three biological replicates with the representative being shown.

Statistics
The results were analyzed for variance by IBM SPSS Statistics 20. Student’s t-test was calculated at the probability at either 1% (P<0.01 with significant level ***) or 5% (P<0.05 with significant level **) as described before [43,44].
Results

Five ANR1-like genes have different expression patterns in response to nitrate

The Arabidopsis ANR1 gene was identified as a key gene controlling lateral root growth through NO$_3^-$ signaling [26]. To understand whether these five ANR1 homologous genes in rice could play similar roles to ANR1 in Arabidopsis, we begin by investigating their expression patterns and levels in response to nitrate. Rice seedlings were grown in complete nutrient solution for 14 d with 2.88 mM KNO$_3$ as the sole N source. They were then deprived of N for 3 d before being re-supplied with 2.88 mM KNO$_3$ (or 2.88 mM KCl as control). The rice nitrate transporter gene OsNAR2.1, which is known to be nitrate-inducible [45], was used as a positive control. As shown in Figure 1, the expression of OsNAR2.1 was very strongly upregulated by nitrate re-supply as expected. Each of the five ANR1-related genes was found to have a different expression pattern in response to nitrate starvation and re-supply (Figure 1). Similar to ANR1 gene in Arabidopsis, the expression of OsMADS61 was significantly induced by nitrate starvation at 4 h, 6 h and 8 h and significantly suppressed by nitrate re-supply at 6 h in comparison to the seedlings continuously supplied with nitrate. In contrast, the expression of OsMADS25 was not significantly affected by nitrate starvation but was induced by nitrate re-supply at 4 h, 6 h and 8 h in comparison to the continuous nitrate treatment. Similarly, the expression of OsMADS27 was not significantly affected by nitrate starvation but was induced by nitrate re-supply at 4 h and 8 h in comparison to the continuous nitrate treatment. The expression of OsMADS57 was downregulated by nitrate starvation at 4 h, 6 h and 8 h and was upregulated by nitrate re-supply at 4 h in comparison to the continuous nitrate treatment (Figure 1). OsMADS23 was the only gene to show no significant response to either nitrate starvation or nitrate re-supply (Figure 1).

Five ANR1-like genes respond differently to different N sources

To investigate whether these five ANR1 homologous genes in rice were also regulated by the other N sources, we further investigated their expression patterns in response to different N sources. The plants were grown in the complete nutrient solution for 10 d before changing media with 2.88 mM KNO$_3$ as the sole nitrogen source for 4 d. Then, the plants were starved for N nutrient for 3 d followed by re-supply with the same concentrations of different N sources. As shown in Figure 2, the expression of rice transporter OsNAR2.1 was down-regulated in the roots of N-starved rice plants and then rapidly upregulated when supplied with 2.88 mM KNO$_3$ for 3 h which was consistent with a previous report [39]. OsMADS61 and OsMADS57 were the only genes that were affected by nitrate not by any other N sources, whereas OsMADS23 was the only gene regulated by various different N sources but not by nitrate. In contrast, OsMADS25 and OsMADS27 were upregulated by both nitrate and ammonium nitrate but downregulated by ammonium chloride.

Effect of P and S deprivation and re-supply on the expressions of five ANR1-like genes in rice roots

To analyze whether ANR1-like homologous genes are involved in the regulation of gene expression in response to other nutritional stresses, we investigated the effect of P and S deprivation and re-supplementation. As shown in Figure 3, in contrast to the phosphate transporter OsIPS1, OsMADS23, OsMADS25 and OsMADS27 were all downregulated by P starvation and P re-supply whereas OsMADS57 and OsMADS61 were not significantly affected. For the S treatment, OsMADS23, OsMADS25, OsMADS27 and OsMADS57 were all upregulated by S starvation but not by S re-supply whereas OsMADS61 was upregulated by both S starvation and re-supply in comparison to the continuous S nutrient treatment.

Effect of N-deprivation and re-supply on the expressions of OsNRT2.1, OsNAR2.1, OsIPS1 and OsSULTR1;1 in rice roots

We have investigated whether there is crosstalk between the N, P and S regulatory pathways in the regulation of expression of the
the OsNRT2.1, OsNAR2.1, OsIPS1 and OsSULTR1;1 genes. We first investigated whether the expression of phosphate transporter OsIPS1 and sulphate transporter OsSULTR1;1 was regulated in response in nitrate starvation and nitrate re-supply. The OsNRT2.1 and OsNAR2.1 genes, which encode components of the high affinity nitrate transport system (HATS) in rice, were used as the positive controls OsNRT2.1 and OsNAR2.1, were previously shown to be upregulated by nitrate and suppressed by NH$_4^+$. As expected, OsNRT2.1 and OsNAR2.1 were downregulated by nitrate starvation and rapidly upregulated by nitrate re-supply (Figure 4), confirming the results of previous studies [45,46]. However, the expression of phosphate transporter OsIPS1 was significantly downregulated by both nitrate starvation and nitrate re-supply at both 4 h and 6 h time points, whereas OsSULTR1;1 was only significantly downregulated by both nitrate starvation and re-supply at 4 h and not at 6 h.

Effect of P, S deprivation and re-supply on the expression of OsNRT2.1, OsNAR2.1, OsIPS1 and OsSULTR1;1 in rice roots

In this experiment two-week old rice seedlings grown in complete nutrient solution were deprived of phosphate or sulfate for 3 d and re-supplied with phosphate or sulfate for 3 h. OsIPS1 was used as P control gene and OsSULTR1;1 was used as S control gene. As shown in Figure 5, the expression of OsIPS1 was notably upregulated by P deprivation and downregulated by P re-supply, which was consistent with previous study [14]. Surprisingly, the gene expression patterns of OsNRT2.1 and OsNAR2.1 is very similar to the expression pattern of OsIPS1, which were upregulated by both starvation and P re-supply in comparison to the continuous P treatment (Figure 5). However, the expression of OsSULTR1;1 was significantly downregulated by P starvation and P re-supply in comparison to the continuously P supply treatment. For the S fluctuation treatment, as shown in Figure 6, the mRNA level of OsSULTR1;1 was significantly increased by sulfate starvation, which was consistent with previous study [47]. The expression patterns of OsNRT2.1, OsNAR2.1 and OsIPS1 were the same as OsSULTR1;1, being upregulated by both S starvation and S re-supply in comparison to the continuous S treatment (Figure 6).

Discussion

Five ANR1-like genes have different expression patterns in response to nitrogen

It was previously demonstrated that expression of ANR1 in roots of hydroponically grown Arabidopsis plants was induced by N deprivation and rapidly downregulated by N re-supply [29]. This pattern of N responsiveness differs from the NO$_3^-$ inducibility of ANR1 as previously obtained in Arabidopsis root cultures [26]. In the present study we have shown that the five ANR1-related genes in rice have diverse expression patterns in response to N starvation and N re-supply. The expression of
OsMADS61 was only significantly induced by N starvation and significantly down regulated by N re-supply, which is very similar to the expression pattern of ANR1 in Arabidopsis. In contrast, the expression of OsMADS57 was significantly downregulated by N starvation and significantly upregulated by nitrate re-supply. Furthermore, OsMADS25 and OsMADS27 were only significantly upregulated by nitrate re-supply not by N starvation. These results were partly consistent with the results from [48], in which the rice seedlings were grown in half-strength liquid Murashige and Skoog medium without N and re-supplied with 3 mM KNO3. They found that OsMADS23 and OsMADS61 were not significantly affected by N fluctuation and that OsMADS57, OsMADS25, OsMADS27 were all significantly upregulated by nitrate re-supply [48]. They therefore suggested that the AGL17-like related genes in rice had specific functions differing from those of their *A. thaliana* homologs. However, our finding found that the expression pattern of OsMADS61 is very similar to that of ANR1 pattern in Arabidopsis.

It has recently been shown that microRNA444 (miR444), which targets three OsMADS-box genes (OsMADS23, OsMADS27, OsMADS57) in rice, has multiple roles in the NO3– signaling pathway [49]. In this study, OsMADS23, OsMADS27 and OsMADS57 were downregulated under conditions of N-deprivation and were unaffected following 0.5, 1, or 2 h of 5 mM KNO3 supplementation [49]. The different sampling times may account for the partial differences between their results and ours.

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The phenotype of transgenic lines overexpressing miR444 provided evidence that OsMADS23, OsMADS27 and OsMADS57 could have a role in regulating the lateral response to localised nitrate in rice [49], as ANR1 does in Arabidopsis [26]. In addition, there was evidence that this group of genes is involved in controlling the root architecture in response to P starvation [49]. However, it has been noted that miR444 has additional target genes (non-MADS box) in rice and there could be other unknown genes involved in the root developmental responses to the nutrient supply [50].

The five ANR1-like homologous gene in rice are regulated by P and S fluctuations

Previous results from Arabidopsis had demonstrated that expression of ANR1 was not regulated by fluctuations in P and S supplies and that and SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) was the only type-II MADS-box gene that responded to phosphate and sulfate deprivation and re-supplementation [29,41]. A later study found that AGL12, AGL18 and AGL19 were downregulated by P and S re-supply [41]. In this study, the OsMADS23, OsMADS25 and OsMADS27 genes were downregulated by both P starvation and P re-supply whereas expression of OsMADS57 and OsMADS61 was not significantly regulated by P fluctuations, which was consistent with what was observed for ANR1 in Arabidopsis [29]. These results were partly
consistent with the results from [49], in which phosphate starvation reduced the mRNA levels of two miR444 targets (OsMADS27 and OsMADS57). In our result, the expression level of OsMADS23 and OsMADS27 were downregulated by P starvation, however in [49], the mRNA abundance of OsMADS27 and OsMADS57 were downregulated by phosphate starvation. We used Yoshida [38] rice nutrient solution and Yan chose 1/2 MS culture solution [49] and the time course of phosphate-deprivation was different, which may account for the different gene expression patterns. However, for the S treatment, the expression of all five ANR1-related genes in rice was modulated by S starvation, in contrast to what was seen with ANR1 in Arabidopsis [29], suggesting that these rice genes have specific functions differing from their Arabidopsis homologs. Furthermore, the result of the expression of OsMADS25 regulated by phosphate deprivation was consistent with previous report by [51]. Like SOC1 in Arabidopsis, rice OsMADS25, OsMADS27 and OsMADS57 in roots were responsive to nitrate, phosphate and sulfate fluctuations, which suggest that these three genes may be involved in a general stress response pathway to these three macronutrients.

Crosstalk among OsNRT2.1, OsNAR2.1, OsIPS1 and OsSULTR1;1

As already discussed, depriving seedlings of one mineral nutrient may lead to the disruption of the metabolism of other nutrients [29,52,53]. For example, molybdenum deficiency had positive impacts on genes involved in nitrate and sulfate assimilation and phosphate transport [54]. Our results show that OsNRT2.1 and OsNAR2.1 were regulated by both P and S starvation and re-supply, which are partly consistent with previous research in which the gene expression level of AtNRT2.1 in Arabidopsis was found to be upregulated by P and S re-supply [44]. Our results also indicated that OsIPS1 was sensitive to N and S fluctuation and that the expression of OsSULTR1;1 was downregulated by phosphate re-supply, indicating that there is crosstalk between the signaling pathways regulating expression of nitrate, phosphate and sulfate transporters. This is similar to the finding that some nitrate-inducible genes are involved in sulfate metabolism [51,55]. These results suggest that there is a complex regulatory network among these three macronutrients and their transporters. Further work using mutants or other genomic approach needs to be done to identify the mechanisms of the crosstalk among nitrate, phosphate and sulfate transporters.

Acknowledgments

We thank Prof. Hao Yu from the National University of Singapore for critical reading of this manuscript.

Author Contributions

Conceived and designed the experiments: CY SS YG. Performed the experiments: CY SS YX. Analyzed the data: CY SS YZ. Contributed reagents/materials/analysis tools: CY SS LH. Contributed to the writing of the manuscript: CY AY IA YG.
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