Physiological and Molecular Responses of Rice to N, P, K and Mg Deficiencies

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Abstract

Nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) constitute the most important nutrient elements of plant cells. Their physiological functions have been intensively investigated in the past. However, interactions between these nutrients are not fully understood and the underlying processes are largely unclear at the molecular level. In this study, we detected both physiological and molecular responses of rice (*Oryza sativa* L. ssp. *Japonica* cv. *Nipponbare*) to N, P, K and Mg starvation. Deficiencies of these nutrients, particularly N and P, resulted in the accumulation of soluble sugar and starch in the leaves. The root to shoot biomass ratio was increased by N and P deficiencies, but decreased by K and Mg deficiencies. In addition, our data showed that deficiency of either K or Mg induced the accumulation of the other. Moreover, K starvation markedly decreased both K and soluble sugar concentrations in the roots. Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that expression of two sugar transporter (*SUT)* genes in the leaves was orchestrated with the sugar accumulation induced by the nutrient shortages. Expression of a putative high affinity K transporter gene (*OsHAK1*) and a putative Mg transporter gene (*OsMGT*) showed opposite up- or down-regulation by K and Mg supply status. These findings suggest that deficiencies of the major nutrients suppressed the export of carbohydrates from source leaves, and that the regulated sugar and nutrient transporter genes detected in this study could be used to elucidate the molecular mechanism of plants in their adaption to varied nutrient supply.

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Introduction

Nitrogen (N), phosphorus (P), potassium (K) and magnesium (Mg) constitute the most important nutrient elements in plant cells. Their physiological functions have been intensively investigated in the past (Hermans *et al.*, 2006; Maathuis, 2009). At the cellular level, N is the main nutrient constituent of protein which makes up a high percentage of the plant organic material. Compared with N, a much lower percentage of P is present in the cellular composition. However, P plays a predominant role in genetic materials such as DNA and RNA. Moreover, it is also essential in energy metabolism and in regulating cellular activity (Maathuis, 2009).

In contrast to N and P, K is not an integral constituent of any plant metabolite and is present as a cation, making up the highest cation concentration in the plant cell (Karley and White, 2009; 2010). Besides its action as an osmoticum, K functions in phloem loading of sucrose, facilitates photosynthesis and sustains water content (Karley and White, 2010). Plants suffering from deficiency of K are vulnerable to biotic stress such as disease infection or abiotic stress including mechanical pressure, and fruit quality is reduced (Tuncay *et al.*, 1999). Mg is another macro element which is essential for photosynthesis in the chloroplast. In addition, it functions as an activator of many enzymes during chemical reactions in the cell (Berkowitz and Wu, 1993).

Previous research has demonstrated that plants utilize different strategies in response to deficiencies of the various nutrients. Lee (1982; 1993) showed that uptake of N, P or S in barley is dependant on plant nutrient status, with efflux playing only a minor part in the regulation of nutrient uptake. In 1994, Cakmak *et al.* reported differences in partitioning of dry mass and carbohydrate between shoot and root in beans suffering from P, K, Mg deficiencies. In the model plant *Arabidopsis*, translocation of carbohydrate and sugar differed between plants deprived of N, P, K and Mg (Hermans *et al.*, 2006). Under N deficiency, carbohydrates were accumulated in shoots, whereas root systems expanded to increase their potential for nutrient acquisition. Starvation of P resulted in similar changes in *Arabidopsis*. By contrast, deficiency of K did not increase plant root systems, and carbohydrate was accumulated in the shoot to a much lesser degree than that under N and P deficiencies (Hermans *et al.*, 2006).

In addition to these physiological adaptations, a micro-array analysis by Hermans *et al.* showed that related categories of gene are over-represented or down-regulated in the processes. For example, photosynthesis and sucrose synthesis related genes change their expression when plants become P-deficient (Hermans *et al.*, 2006). Sucrose is usually the main form in which carbohydrate is moved in long distance transport in plants (Hayashi and Chino, 1990). It also acts as a signal to control gene expression and plant development (Li *et al.*, 2003; Gibson, 2005). Increasing data from tomato, *Arabidopsis* and *Plantago major* demonstrate that SUT/C genes play pivotal roles in sucrose transport (Stadler *et al.*, 1995; Barker *et al.*, 2000; Matsukura *et al.*, 2000; Barth *et al.*, 2003; Carpaneto *et al.*, 2010). In rice, five SUC transporter genes have been cloned, and these genes show different expression profiles in embryo, pollen, and tonoplast (Matsukura *et al.*, 2000; Hirose *et al.*, 2010; Sun *et al.*, 2010; Eom *et al.*, 2011). Whether these genes are involved in translocation of sugar in

Research Findings

plants under nutrient starvation is an intriguing question. There is some support for the idea in that several lines of evidence have shown that expressions of high-affinity K transporters can be enhanced under K deficiency (Okada *et al.*, 2008; Qi *et al.*, 2008; Horie *et al.*, 2010). It is not clear, however, whether monocot plants, e.g. rice, respond to the starvation of different nutrients in a similar way to *Arabidopsis* and which changes in gene expression accompany the physiological changes taking place in the plants.

In *Arabidopsis*, two gene families might relate to Mg uptake, one is *AtMGT*s, a group of 10 family members that complement the mutant of the Mg transporter in yeast or bacterium (Li *et al.*, 2001). The other is *AtMHX*, which encodes a protein located on the tonoplast and acts as an antiporter of H^+ and Mg^{2+} (Galili, 1999; Shaul *et al.*,1999). By a similarity search in the rice database with amino acids of AtMHX and AtMGT as the queries, we retrieved their homologs, OsMGT and OsMHX, respectively. However, little is known about the functions of OsMGT and OsMHX.

In this study, we investigated physiological changes of rice grown hydroponically, deprived of different nutrients with the aim of identifying the interaction between nutritional status and related gene expression, to increase our understanding of plant mineral nutrition.

Materials and methods

Growth conditions

Rice (*Oryza sativa* L. *ssp japonica*) seeds were sterilized in 10% H_2O_2 for 30 mins, then subsequently washed five times before being immersed in deionized water in the dark for 24 hours. The seeds were transferred into plastic trays to germinate in a growth cabinet, with a light/dark cycle of 14 h/10 h and a day/night temperature regime of 32°C/22°C. After 15 days the seedlings were transplanted into 3-L plastic boxes and cultivated gradually from 1/4, 1/2, to full strength nutrient solutions (modified based on the protocol of International Rice Research Institute) (Yoshida *et al.*, 1976). The seedlings were cultivated for one month before the P, K and Mg starvation treatments were introduced, while the seedlings for the N deficiency were grown in the full strength nutrient solution for one more week before beginning the treatment. Under the different nutrient starvation treatments, $NaNO_3$, $KHPO_4$, KCl and $MgSO_4$ were replaced by equivalent molar concentrations of NaCl, KCl, and $CaSO_4$ for four weeks (three weeks for the N treatment). Plant samples for RNA extraction were stored at -70°C immediately after harvest.

Measurements of plant samples

Plant samples were harvested after four weeks (three weeks for the N treatment) of the nutrient starvation treatment. Roots were washed with de-ionized water three times to remove adhering nutrients, followed by desiccation in a forced-air oven at 70°C for about 48 h to a constant weight. The dried samples were ground into powder. About 0.05 g of dried powder was digested by 5 ml of 98% H_2SO_4 and 1 ml of 30% H_2O_2 at 270°C. After cooling, the digested sample was diluted to 100 ml with distilled water. The P, K, Mg concentrations were determined by an ICP-emission spectrometer (Perkin Elmer Optima 2100DV), N concentration was determined by colorimetric continuous flow analysis (AutoAnalyser 3, Bran+Luebbe, Germany) as described by Ding *et al.* (2006).

Soluble sugar and starch concentration

The measurement of starch and soluble sugar concentration of shoot and root was based on the method of Hansen and Møller (1975), as described in detail by Ding *et al.* (2006).

To visualize the distribution of starch in rice plants, iodine staining of whole plants was performed. After nutrient starvation treatments, the rice samples were immersed in 70% ethanol and incubated overnight at 70°C to remove chlorophyll. Afterwards, they were stained in iodine solution (0.5% KI+0.1% I_2) for five hours before observation.

Determination of active root surface area

Root activity can be evaluated by determination of active root surface area and percentage of the active root surface area of the total root surface area. Active root surface is the root surface capable of assimilating the ions from the outside of the plasma membrane into the cytosol. In this study, active root surface area was determined based on Zhang's methods (1994) by use of the dye Methylene Blue ($C_{16}H_{18}N_3SCl•3H_2O$) which is adsorbed to the surface of roots. The principle of the method is that by treating the roots with a given amount of methylene blue then determining how much of it remains in solution after the treatment, the dye adsorbed by the roots was worked out and the active root surface area was calculated according to the adsorbed dye mass. The rice roots were immersed in three beakers in sequence, each beaker containing 10 ml 2 mM Methylene Blue solution. The time in each beaker was 1.5 min. Afterwards, 1 ml solution from each beaker was extracted and diluted to 10 ml. Absorbance at 660 nm (A_{660}) of the diluted solutions were determined by a universal Microplate spectrometer (SpectraMax M5). The mass of Methylene Blue adsorbed by the roots was calculated according to a standard curve on the base of the A_{600} of a series of Methylene Blue solutions with different concentrations. Three replicates were conducted for each treatment of the nutrient starved plants.

Research Findings

Expression of sugar, Mg and K transporter genes

Total RNAs of roots and leaves were extracted from a mixture of three separate groups of plants in each treatment by use of the Trizol reagent (Invitrogen). DNAse I (TaKaRa) was applied to remove residue genomic DNA in the total RNA. First-strand cDNA was synthesized with 5 µg total RNA as templates. To detect sugar transporter gene expression under the different nutrient starvation regimes, forward and reverse gene specific primers of the rice SUT family members (listed in Table 1) were synthesized according to their sequence on genebank (Os03g07480; Os12g44380; Os10g26470; Os02g 58080; Os02g36700, equal to SUT1-5, respectively). RT-PCR was performed in parallel with the samples under normal nutrient conditions as a control in triplicate. A housekeeping gene, Actin (*OsRac1*, accession number AB047313) was amplified in parallel as their internal control. The PCR primers were used to amplify putative Mg transporter genes *OsMHX* (Os11G43860), *OsMGT* (Os06g44150) and a high affinity K transporter gene *OsHAK1* (Os04G32920) as listed in Table 1.

Table 1. Primer pairs for amplification of sucrose transporters, Mg transporters *OsMGT, OsMHX* and K transporter *OsHAK1*, respectively.

Gene	Sequence
	Sucroze transporters
OsSUT1	F: CAGCCCTCCCAACAAAATCAA
	R: CGAGAACTACAAAGCTCACCA
OsSUT2	F: TTCCTCGCCGACCTCACCGAG
	R: CACCAGCCCACCGATAAAAGA
OsSUT3	F: TCTGTTCTTGGATGGCATTAGG
	R: GCACGACGATGGAGATGTTGA
OsSUT4	F: TTGGCTTTGTGGACCTATT
	R: GTCCCATCCAGTCAGTATCAA
	Mg transporters
OsMGT	F: TCACCCACAGAATCACGG
	R: TCAACAGCGTAGACGACAAT
OsMHX	F: TGGCAGATACTGTTCCTT
	R: CCTCCCATTTGTTCTTTA
	K transporters
OsHAK1	F: TCTACACCCTCATCATCATCCC
	R: TACACCTGCCCCTCGTACTTCT

increased by N and P starvation, but decreased by K and Mg starvation (Fig. 2).

Deficiencies of N, P, and K resulted in a decrease of the percentage (%) of active root surface (Fig. 3), representing an impairment of root activity. However, active root surface of the plants was larger under N and Mg deficiencies but smaller under P and K deficiencies in comparison with plants receiving adequate nutrient supply (Fig. 3). It is interesting to note that deficiency of Mg did not change the percentage of active root surface, although it increased the total active root surface of rice (Fig. 3).

Accumulation of carbohydrates in the nutrient deficit shoot and shortage of soluble sugar in the K starved roots

Deficiency of the four major nutrients resulted in a noticeable increase of soluble sugar in the shoot, particularly under N and P starvation conditions (Fig. 4). The soluble sugar in rice roots was increased slightly by N deficiency, but seems not to be affected by P and Mg deficiency (Fig. 4). In contrast, there was only very low soluble sugar in the K starved roots, representing the distinct impairment of sugar translocation from shoot source to the root sink (Fig. 4).

A similar trend of increases of starch concentration as that for soluble sugar in the shoot of rice was observed for the four nutrient deficiency treatments (Fig. 1; Fig. 4). The starch accumulation in the P deficient shoot was the largest among the four treatments. The difference of starch concentration in the roots between the four treatments was much less significant than that in the shoot (Fig. 4).

Results

Different effects of N, P and K, Mg starvation on root to shoot biomass ratio and active root surface

Removal of N for three weeks and P, K and Mg for four weeks from the culture solution decreased biomass production and changed root morphology in comparison to adequate nutrient supply (Fig. 1). Shoot growth was decreased much more significantly by deficiency of N and P than by deficiency of K and Mg (Fig. 1; Fig. 2). However, there was only a small difference of total root biomass among the four treatments (Fig. 2). The root to shoot biomass ratio expressed on a fresh weight basis was

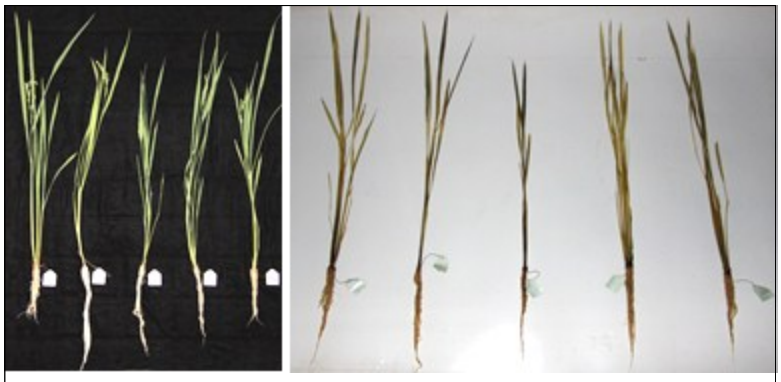

Fig. 1. Morphological comparison of rice plants grown in a complete nutrient solution or different nutrient-deficient solutions for three or four weeks. Period of N-deficiency treatment was three weeks; for P-, K- and Mg- deficiency four weeks. In the right panel, plants were stained with iodine solution (0.5% KI+0.1% I_2) for five hours to visualize starch accumulation. Before iodine solution staining, plants were incubated in 70% ethanol overnight to remove chlorophyll. From left to right: Control; -N; -P; -K; -Mg, respectively.

Research Findings

Interactive effects of N, P, K and Mg deficiency on their concentrations in rice

As expected, temporary removal of N, P, K and Mg supply in the culture solution decreased their concentrations respectively (expressed on a dry weight basis) both in the roots and shoots (Fig. 5). Total P concentration in the shoot was also decreased by lack of N, K and Mg supply (Fig. 5). The largest variation of K concentration occurred in the roots. Interestingly, under K starvation conditions, the K concentration in the shoot was much higher than in the roots, which showed very low concentrations of K implying rapid transfer of any K acquired from the outer medium from root to shoot (Fig. 5).

In comparison to Mg sufficient plants, removal of Mg supply resulted in lower K in the roots and higher K in the shoots, representing enhanced K transport from the roots to shoots (Fig. 5), similar to the effects of K starvation.

Notably, when rice plants grew under K starvation for four weeks, their Mg concentration increased markedly in the shoots but not significantly in the roots, compared with that of the control and N and P starvation treatments (Fig. 5). Likewise, plants under Mg starvation had a much higher K concentration than the plants deprived of N and P. The same was also true in comparison to the control. The data demonstrated a strong complementary relationship between K and Mg in plants. That is to say, deficiency of K enhanced uptake of Mg, and deficiency of Mg enhanced the uptake of K. In addition, K significantly inhibited Mg uptake in rice.

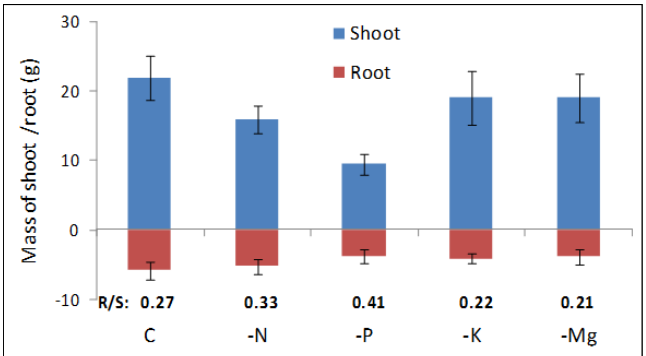

Fig. 2. Root and shoot biomass (fresh weight) and root to shoot biomass ratios of rice plants grown in a complete nutrient solution or in nutrient solutions deficient in N for three weeks or deficient in P, K and Mg for four weeks, respectively. Columns are means (±SD) of mass determined from four replicates.

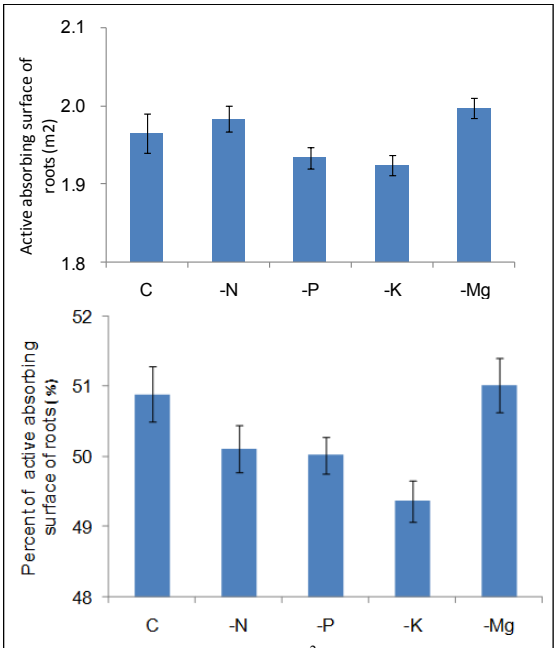

Fig. 3. Active root surface area (m^2) and percentage of active root surface area of rice plants grown in a complete nutrient solution or nutrient-deficient solutions for three weeks (N-deficiency) or four weeks (P-, K-, Mg-deficiencies), respectively. Columns are means (±SD) of active root surface or percentage of active root surface determined from four replicates.

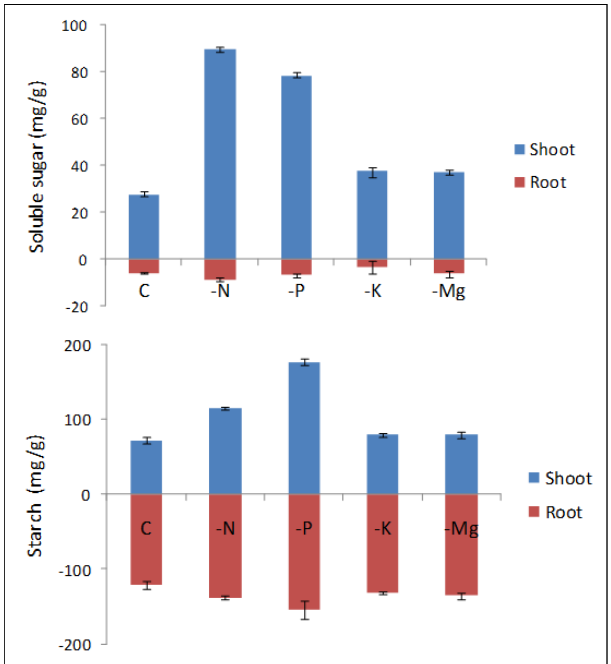

Fig. 4. Soluble sugar and starch concentrations in shoots and roots of rice plants (expressed on a dry weight basis) after growth in a complete nutrient solution or a nutrient solution deficient in N for three weeks, or deficient in P, K or Mg for four weeks, respectively. A: Soluble sugar; B: Starch. Columns are means (±SD) of soluble sugar or starch concentrations determined from four replicates.

Research Findings

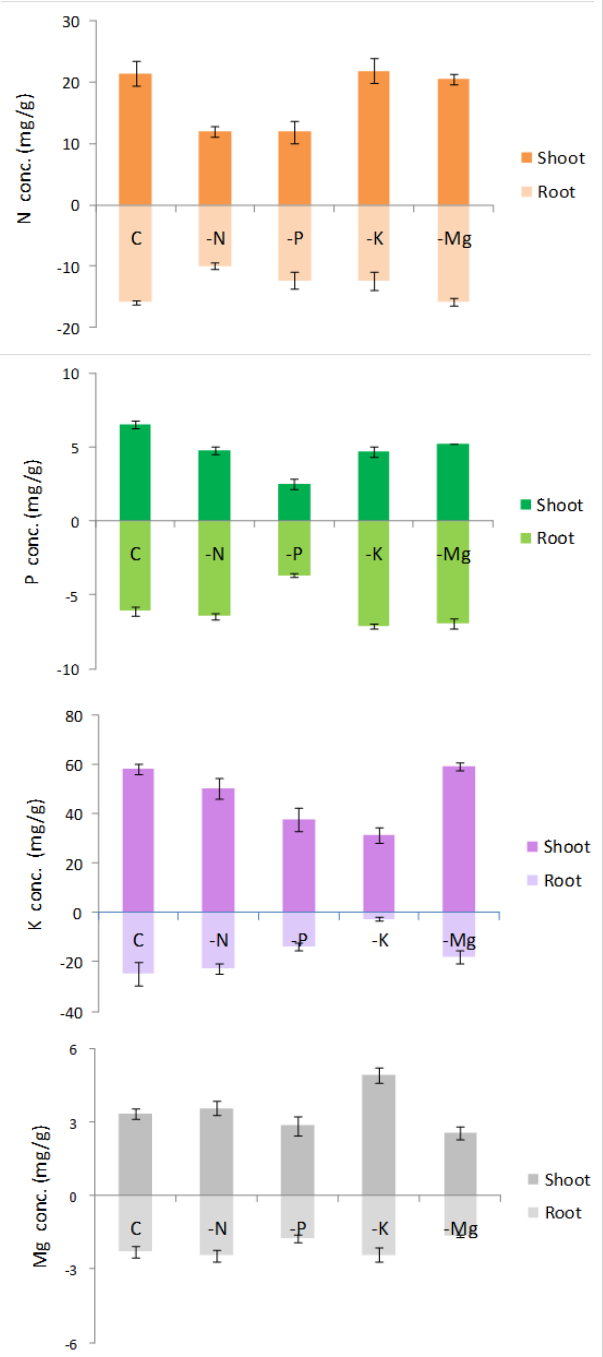

Fig. 5. N, P, K, Mg concentrations of rice plants (expressed on a dry weight basis) after growth in a complete nutrient solution or nutrient-deficient solutions for three weeks (N deficiency) or four weeks (for P-, K-, Mg- deficiencies). Columns are means (±SD) of ion concentrations determined by ICP-emission spectrometer (Perkin Elmer Optima 2100DV) from four replicates. N concentration was determined by colorimetric continuous flow analysis (AutoAnalyser 3, Brank Luebbe, Germany).

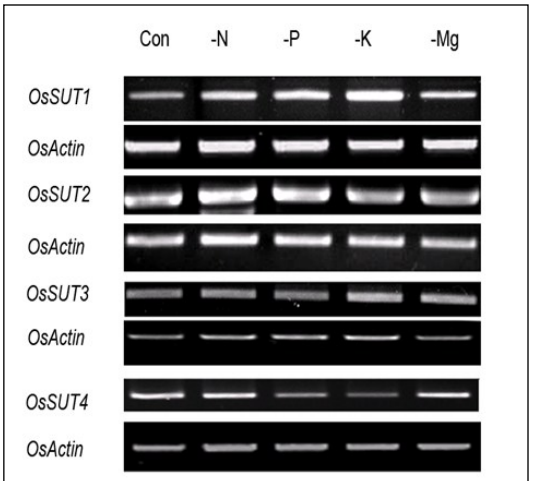

Fig. 6. Rice SUT genes expression detected by RT-PCR for a complete nutrient solution or deprived of different nutrients for three days. RT-PCR was performed in triplicate with a housekeeping gene, Actin (*OsRac1*, accession number AB047313) in parallel as their internal control.

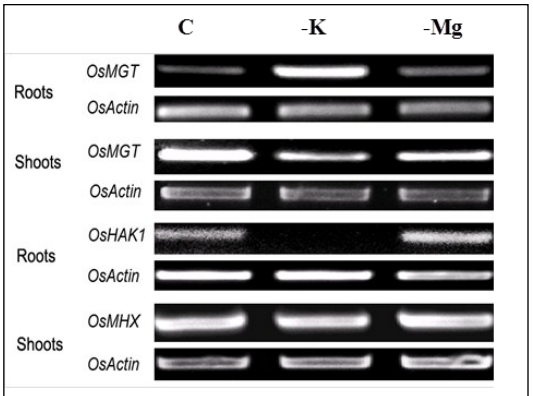

Fig. 7. Several rice K^+ and Mg^{2+} transporter genes expression detected by RT-PCR under complete nutrient solution or deprived of K^+ or Mg^{2+} for three days. RT-PCR was performed in triplicate with a housekeeping gene, *Actin* (*OsRac1*, accession number AB047313) in parallel as their internal control.

Research Findings

Effects of N, P, K and Mg starvation on expression of sugar transporter (SUT) genes in shoots

In order to test if the sugar accumulation in the nutrient starved leaves was related to inhibition of sugar export to the root sinks, five sugar transporter genes were examined for their expression in rice leaves by RT-PCR. Interestingly, the expression of *OsSUT1* was up-regulated in the nutrient deficient leaves, particularly under K starvation (Fig. 6). In contrast, the transcripts of *OsSUT4* were decreased under deficiencies of P and K (Fig. 6). *OsSUT2* and *OsSUT3* were not apparently regulated by nutrient supply status. Expression of *OsSUT5* was not detected in our experimental conditions.

K and Mg starvation regulated putative K and Mg transporter genes expression

Since a strong interaction of K and Mg was shown by physiological data, we analyzed transcriptional expression of a putative high affinity K transporter gene (*OsHAK1*) and two putative Mg transporter genes (*OsMGT* and *OsMHX*). Very noticeably, *OsHAK1* expression was suppressed by K deficiency and up-regulated by Mg deficiency in the roots (Fig. 7). In contrast, *OsMGT* expression was up-regulated in the roots and down-regulated in the shoots by shortage of K supply (Fig. 7). Its expression was not apparently regulated by Mg supply status. No difference of *OsMHX* expression was observed under different K and Mg treatments (Fig. 7).

Discussion

Plants have developed various strategies to deal with different nutrient deficiencies, which are frequently encountered as environmental stresses. Using hydroponic cultivation, we investigated the response of rice to starvation of different nutrients. The consistency in carbohydrate partitioning and root to shoot ratio between rice and *Arabidopsis* suggests that dicots and monocots are comparable in their responses to different nutrient deficiencies. In addition, interaction revealed by physiological data between these nutrients and underlying genes expression provides new information to understand activities *in planta* during nutrient deficiencies.

In our experiment, soluble sugar and starch accumulated markedly in rice shoots under P and N deficiency (Fig. 4). By contrast, much lower amounts of these compounds were accumulated under K^+ and Mg^{2+} deficiency (Fig. 4), and their root to shoot biomass ratios decreased only slightly compared with the control (Fig. 2). These findings are consistent with the conclusions drawn by Hermans *et al.* (2006) from experiments with *Arabidopsis*. The photosynthetic rate of rice decreased notably under N and P deficiencies while it was affected only slightly under K^+ and Mg^{2+} deficiencies (Fig 8). According to Hermans *et al.* (2006), the arrest of photosynthesis under N and P deficiencies is due to accumulation of carbohydrates in the shoot.

In *Arabidopsis*, a Ca^{2+} signaling pathway has been shown to regulate a K^+ channel for low-K response (Li *et al.*, 2006), implying an interaction between different cations in plants. Interestingly, Mg^{2+} concentration increased under K^+ starvation and *vice versa* (Fig. 5). This shows a complementary relationship between K^+ and Mg^{2+} concentrations in plants which confirmed our previous research (Ding *et al.*, 2006). A plausible explanation is that K^+ and Mg^{2+} are the most abundant uni-/divalent cations that have a

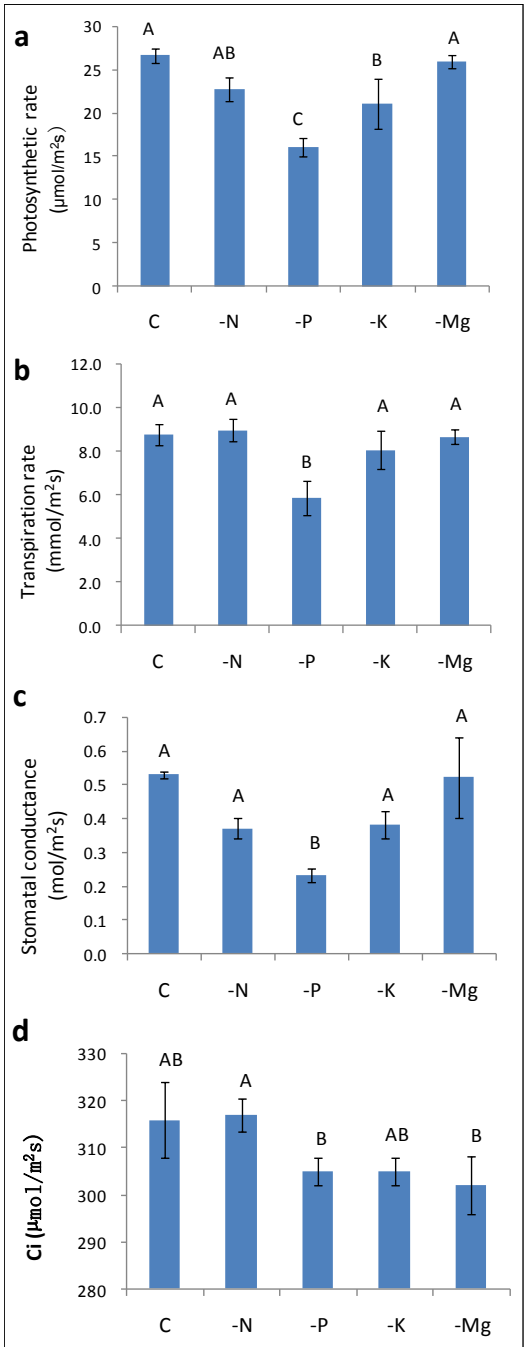

Fig. 8. Photosynthetic parameters including photosynthetic rate (a), transpiration rate (b), stomatal conductance (c), and intercellular CO_2 concentration (Ci) of rice plants (d). Rice plants were measured with a portable infrared gas exchange system (Li-6400) according to the manufacturer's instruction. The measurements were performed between 10 and 11 am in rice plants grown in nutrient deficient solutions for three weeks (for -N treatment) or four weeks (for control and -P, -K, -Mg treatments). The measurements were made in three repeated experiments. Different letters in large case in the figure indicated statistical differences among means by Duncan's multiple range test ($p \leq 0.01$).

Research Findings

pivotal role in sustaining ion homeostasis of the cell (Li *et al.*, 2001). Therefore, deficiency of one ion will induce accumulation of the other. Consistent with this assumption, our RT-PCR results showed that under Mg starvation, *OsHAK1* expression in roots was up-regulated notably. Likewise, under K starvation, expression of *OsMGT* in rice roots was up-regulated slightly (Fig. 7). These facts suggest that *OsHAK1* and *OsMGT* may be important for the interaction of Mg and K *in planta*.

Notably, under K^+ starvation of rice, K^+ concentration in the root was extremely low compared with the concentration of K^+ in the shoot (Fig. 5). This difference implies that the translocation efficiency from root to shoot is very high during K^+ starvation. Since *OsHAK1* decreased its expression in roots under K^+ starvation as compared with the control growing in a complete nutrient condition, it means that other transporter(s) may be involved in this process. Moreover, *OsHAK1* has previously been identified to increase its expression markedly in the roots of rice seedlings under K^+ starvation or Na^+ stress (Okada *et al.*, 2008; Horie *et al.*, 2010), whereas in our experiment, its expression decreased in six-week rice plants under K^+ starvation. This finding implies that the expression of this gene is differently regulated during the plant developmental stages.

It has long been established that loading of sucrose into the phloem of plants needs K^+ (Doman and Geiger, 1979). Increasing data show that SUT family members have an important function in long distance transport of sucrose (Stadler *et al.*, 1995; Matsukura *et al.*, 2000; Scofield *et al.*, 2007; Sun *et al.*, 2010). It has been suggested that OsSUT1 is a potential sucrose transporter in phloem loading (Hirose *et al.*, 1997; Matsukura *et al.*, 2000; Scofield *et al.*, 2007). Our data showed that expression of *OsSUT1* in shoot was up-regulated significantly under K^+ starvation. In accordance with the increase, soluble sugar concentration in the K^+-deficient rice was lower compared with that of the N and P-deficient plants. It should be noted that *OsSUT1* also increased its expression level slightly under N and P deficiencies. However, soluble sugar and starch concentration remained very high in the shoot of these plants which suggests that other regulators may participate in the process.

OsSUT4 is identified to be preferentially expressed in sink leaves (Aoki *et al.*, 2003). Decrease of *OsSUT4* expression by P and K starvation (Fig. 7) suggests that its down-regulation might be tuned to adapt to the situation since its substrate, sugar, was accumulated in source leaves.

In conclusion, we showed that rice, a monocot, gave a similar response to nutrient deficiencies as that of the dicot *Arabidopsis*, which suggests that higher plants adopt analogous means of dealing with nutrient deficiencies. Moreover, our physiological data revealed clearly that K^+ and Mg^{2+} interacted with each other during deficiency, and that K^+ was unique in partitioning between root and shoot during K^+ starvation. Detected expression regulation of both sugar transporter and K and Mg transporter genes under these nutrient deficiencies further support these physiological findings and facilitate understanding of activity of plants at the molecular level.

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