Plant Biotechnology 23, 47–54 (2006)

Original Paper

Improvement of nitrate- and nitrite-dependent growth of rice by the introduction of a constitutively expressing chloroplastic nitrite transporter

Sustiprijatno[1], Miwa Sugiura[1], Ken'ichi Ogawa[2], Masaaki Takahashi[1]*

[1] Department of Applied Biological Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1, Gakuen-cho, Sakai, Osaka 599-8531, Japan; [2] RIBS (Research Institute for Biological Sciences, Okayama), 7549-1 Yoshikawa, Kibichuo-cho, Koga-gun, Okayama 716-1241, Japan
* E-mail: mtakaha@biochem.osakafu-u.ac.jp Tel & Fax: +81-72-254-9451

Received November 14, 2005; accepted January 30, 2006 (Edited by T. Kohchi)

Abstract Loading of cytosolic nitrite into the chloroplast in nitrate assimilation of rice was modified by introducing a gene for chloroplastic nitrite transport from cucumber. Rice (*Oryza sativa* L. japonica cv. Nipponbare) was transformed with a cDNA (*CsNitr1-L*) that encodes a nitrite transporter of cucumber under the control of the *CaMV35S* promoter. *CsNitr1-L* transgenic rice grown by hydroponics with nitrate supplied as the sole nitrogen source grew as well as plants grown with ammonia. In contrast the growth of the untransformed or mock-transformed control rice was retarded on a nitrate-containing medium by about 60% compared to plants grown with ammonia. Nitrite was not beneficial to the growth of the untransformed or mock-transformed control rice but sustained that of *CsNitr1-L* transgenic rice. Low steady-state concentration of nitrite in leaves as well as delayed non-photochemical quenching of chlorophyll fluorescence in *CsNitr1-L* transgenic rice seems indicative of higher nitrite use in chloroplasts.

Key words: Metabolic engineering, nitrate assimilation, nitrite transporter, *Oryza sativa*, transgenic rice.

Nitrate is the most oxidized nitrogenous compound. In the global nitrogen cycle many kinds of unicellular organism utilize nitrate in their respiration as a terminal oxidant (Stewart 1988) and plants use it as a starting substrate for the synthesis of biological nitrogenous compounds such as amino acids (and amino acid-derived biological molecules) (Marschner 1995). During the conversion of nitrate to amino acids by plants nitrate is reduced to nitrite by cytosolic assimilatory nitrate reductase (NR). Resulting nitrite is reduced further to ammonia in chloroplast stroma by nitrite reductase (NiR) with the help of reduced ferredoxins generated by photosynthetic electron transfer.

In nitrate metabolism two transport processes are involved in loadings of nitrate from soil solution to cytoplasm of nitrate-assimilating cells and of nitrite from cytoplasm to chloroplast (plastid) stroma. Flow of nitrite into the chloroplast should relate to the amount of nitrate utilization. We first identified a nitrite transporter from higher plants that functions at the chloroplast envelopes possibly in loading cytosolic nitrite into chloroplast stroma (Sugiura et al. unpublished). Although nitrite-metabolizing unicellular organisms have been reported to contain a nitrite specific transporter (Rexach et al. 2000; Machín 2004), the nitrite transporter of higher plants happened to diverge from a member of proton-dependent oligopeptide transporter (POT) family (Sugiura et al. unpublished). Among POT families of plant, CHL1 functions at root possibly to absorb nitrate from soil solution (Tsay et al. 1993) and NPT1 transfers histidine and oligopeptides into sink organs (Rentsch et al. 1995). In cucumber there are two isoforms of nitrite transporter. One of the nitrite transporter isoform has a chloroplast-targeting signal at its N-terminal and is localized in chloroplast envelopes (Sugiura et al. unpublished). In the nitrate assimilation pathway, the function of the chloroplast nitrite transporter might not be rate-limiting in dicotyledonous plants as deduced from the low level of accumulation of cellular nitrite (Kawamura et al. 1996). When nitrite is supplied to a culture medium of cucumber as a source of nitrogen, the plant takes it up at an acidic pH and utilizes it as a nutrient for its growth (Oji and Okamoto 1981). Accumulation of nitrite, however, is a two-edged sword: cells would become damaged with nitrite, a strong oxidant in neutral and acidic solutions (Hinze and Holzer 1985). Rice could not grow well utilizing nitrite and died with roots damaged possibly due to the toxicity of accumulated nitrite (Oji

Abbreviations: CaMV35S, cauliflower mosaic virus 35S RNA; Chl, chlorophyll; GUS, β-glucuronidase; NR, nitrate reductase; NiR, nitrite reductase; POT, proton-dependent oligopeptide transporter; WT, wild type.
This article can be found at http://www.jspcmb.jp/

and Okamoto 1981). Rice cultivars were bred in wet and slightly acidic grasslands and have been cultivated in paddy field where ammonium may be the major nitrogen species. Although rice could not metabolize nitrate well, the activities of NR and NiR are comparable to those of the plants that prefer nitrate as a source of nitrogen (Oji and Okamoto 1981). The accumulation of nitrite in rice might arise from inefficiency in the nitrite incorporation into chloroplasts and/or plastids.

Rice is a major source of dietary food which sustains the life of about half of the world's population. To increase crop yield, approximately 85 million tons of nitrogen fertilizers are applied annually over the world (Crawford and Glass 1998). Recently-issued contamination of water from unabsorbed nitrogen fertilizer increases nitrate concentration in soil solution to several millimolars l^{-1} (Andrews et al. 2004). The nitrate flowing into paddy fields is a source of nitrogen for rice cultivation and more than 10% of total nitrogen in rice comes from the nitrate in irrigation water. If nitrate is effectively utilized by rice as a nutrient, ground water nitrate will be taken up reducing both the need for nitrogen fertilizer, and the detrimental effects of nitrate on our health.

Cellular nitrate concentration can be increased with the increase of nitrate concentration in soil solution, however, with no marked growth stimulation (Marschner 1986). In this study, we focused on the transport process of nitrite from cytosol to chloroplast stroma in the nitrate assimilation pathway and tried a metabolic engineering of a japonica rice to change its preference to the nutrient by improving nitrite transport into chloroplasts and plastids. Genetic engineering of plants successfully improved production of secondary metabolites (for review, see Della Penna 2001). The acceleration of the primary nitrogen metabolism by genetic modification of participating enzymes, however, turned out to be less than one would expect (Andrews et al. 2004). Enhanced function of a transcription factor is more effective on the activation of a polygenic system like nitrate assimilation (Yanagisawa et al. 2005) than the overexpression of an individual enzyme. In this study, transformation with cucumber nitrite transporter cDNA results in making rice growing normally with nitrate and nitrite as the sole source of nitrogen.

Materials and methods

Cultivation of rice

Rice was cultivated on rockwool (Airrich; Taiyo Kogyo, Tokyo) or vermiculite (GS; Nittai, Aichi) wetted with Yoshida medium (Yoshida et al. 1971) that contained 1.4 mM NH_4NO_3, 0.32 mM NaH_2PO_4, 0.51 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.7 mM $MgSO_4$, 12 μM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 19 μM H_3BO_3, 0.15 μM $ZnSO_4$, 0.15 μM $CuSO_4$, 36 μM $FeCl_3$, and 78 μM citric acid throughout this work in a growth chamber at 28°C unless otherwise specified. The medium was made by mixing 800-times concentrated stock solutions and adjusted to pH 5.0 just before the renewal of the medium every 2 days. Light was given at an intensity of 250 μmol photons $m^{-2}s^{-1}$ by mercury lamps (NHR360L-N; Toshiba, Tokyo) under a 16-h photoperiod.

Transformation of rice

A japonica rice (*Oryza sativa* L. cv. Nipponbare) was transformed with a cucumber nitrite transporter cDNA (*CsNitr1-L*; accession Z69370; Sugiura et al. unpublished) by the use of a method of *Agrobacterium tumefaciens* infection (Hiei et al. 1994). To construct a binary vector for plant transformation full length of *CsNitr1-L* cDNA that had been inserted at the *Not*I site of *pBluescript* KS (Stratagene, La Jolla, CA, U.S.A.) was digested by *Xba*I and *Sac*I and ligated to the same site of pIG121Hm in place of intron β-glucuronidase (GUS) gene (Hiei et al. 1994). The resulting vector, *pBI121Hm::CsNitr1-L*, was maintained in *Eschelichia coli* DH5α and used to transform *A. tumefaciens* strain EHA101 by an electro-poration as described by Walkerpeach and Velten (1994).

Scutellum-derived calli of the rice were infected with the *A. tumefaciens* harboring *pBI121Hm::CsNitr1-L* or orginal *pIG121Hm* vector. Transformed rice plants were regenerated from 50 mg l^{-1} hygromycin-resistant calli according to the method of Hiei et al. (1994). GUS-transformed rice was made as above, in which incorporation of GUS gene was certified by histochemical staining of GUS activity (Herrera-Estrella et al. 1994).

Southern blotting

Genomic DNA was isolated from the leaves of rice with Isoplant (Takara) according to the manufacture's instruction. Southern blot analysis was performed as described by Sambrook and Russel (2001). Ten micrograms of DNA was digested with 10 units of *Pst*I (Takara) and subjected to a 0.8% agarose gel electrophoresis. The bands were transferred onto a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech, Buckinghamshire, England) under alkaline condition for 12 h, prehybridized in a phosphate-SDS buffer (0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, 1% bovine serum albumin) at 65°C for 2 h, and hybridized with ^{32}P-labeled probes in the same solution for 12 h at 65°C. Probes were synthesized in a Ready-to-Go labeling reaction mixture (Pharmacia Biotech, Uppsala, Sweden) containing [α-^{32}P]-dCTP (Amersham Pharmacia Biotech) and a 1-kb *Hind*III-*Sac*I fragment of *CsNitr1-L* from the derivative of *Bluescript* KS described above. After being washed with 0.1×SSC (3.0 M NaCl

and 0.3 M sodium citrate) with 0.1% SDS for 4 h at 65°C the membrane was exposed to an Imaging Plate (Fuji film, Tokyo) for 2 days and image of radioactivity was captured by LAS3000 (Fuji film).

Assay of NR and NiR activities

The second leaf of rice grown for a month with Yoshida medium was used for the source of enzyme preparation. The leaves were homogenized by a mortar and pestle with 4 volumes of the extraction buffer that contained 50 mM Tris-Cl^-, pH 8.5, 1 mM dithiothreitol, 10 mM leupeptin at 4°C. After being centrifuged at 30,000×*g* for 10 min at 4°C, the supernatants were used as crude enzyme preparations for assays of NR and NiR.

NR reaction was conducted in a reaction (2 ml) that contained 50 μmol of potassium phosphate, pH 7.5, 20 μmol of KNO_3, 0.8 μmol of NADH, and 0.1 ml of the crude enzyme preparation at 28°C for 15 min. The reaction was terminated by the addition of 0.1 ml of 1 M zinc acetate and remaining NADH was oxidized by phenazine methosulfate. Nitrite formation was determined colorimetrically (Hageman and Hucklesby 1971).

NiR activity was assayed according to Losada and Paneque (1971). Nitrite reduction was initiated by the addition of 0.2 ml of 0.1 M sodium bicarbonate solution containing 1.6 mg of sodium dithionite to a reaction mixture (1.1 ml) that contained 40 μmol of KNO_2, 0.04 mg of methylviologen, and 0.1 ml of the crude enzyme preparation and terminated by the removal of electron donors by aeration after 15-min incubation at 28°C. Remaining nitrite in the reaction mixture was determined as above.

Analysis of Chlorophyll (Chl) fluorescence kinetics

WT, *GUS* transgenic control, and *CsNitr1-L* transgenic rice were cultivated with NH_4NO_3 as the nitrogen source for 1 month from the end of August in a green house at room temperature around 35°C under natural light. Prior to the fluorescence analysis the plants were transferred into dark room at 10:00 am and dark-adapted for longer than 30 min. Chl fluorescence kinetics was determined at the middle of the second leaf by using a MINI PAM photosynthetic yield analyzer (Waltz; Effeltrich, Germany).

Determination of nitrate and nitrite contents

Fresh leaves (100 mg) were sampled at 10:00 am, ground with 50 μl of 0.1% (w/v) SDS, and extracted with 200 μl of chloroform. After centrifugation at 18,000×*g* for 10 min, 50 μl of the aqueous layer was subjected for nitrate and nitrite determination by a capillary electrophoresis as described by Kawamura et al. (1996).

Results and discussion

Putative nitrite transporters of rice

Database searches revealed that *CsNitr1-L* sequence is homologous to POT families showing highest homology to four members. Three are found in the genome of rice (The rice full-length cDNA consortium 2002) and one in the genome of an *Arabidopsis* (Arabidopsis genome initiative 2000). The genes are actually expressed since corresponding mRNAs were captured as cDNA clones: AK070558, AK120596, and AK110441 of rice and an EST from At1g68570 of *Arabidopsis*. AK070558, AK120596, and At1g68570 are homologous in both length and sequence to the chloroplastic nitrite transporter of cucumber (*CsNitr1-L*): sequence identity to *CsNitr1-L* is 53.9% both for AK070558 and AK120596 (Figure 1) whereas 30–40% for other POT family members such as nitrate transporter and peptide transporters (data not shown). The widespread presence of putative nitrite transporter gene over the taxa of monocotyledonous and dicotyledonous plants suggests a universal importance of nitrite transport in plant metabolisms although their expression levels and the activity of nitrite transport have not been determined yet.

Transformation of rice with CsNitr1-L

Rice (*Oryza sativa* L. japonica cv. Nipponbare) was transformed with a cDNA of cucumber nitrite transporter (*CsNitr1-L*) to promote the incorporation of cytosolic nitrite into chloroplast. By the *A. tumefaciens* infection method we obtained 20 lines of hygromycin-resistant plantlet (T_0) from regenerated scutellum-derived calli. The plants were allowed to flower and the incorporation of transgene was evaluated from the hygromycin resistance of the self-pollinated progenies (T_1) by dipping their leaf discs in 50 mg l^{-1} hygromycin-containing medium. Chl bleaching became visible at the edge of leaf discs of hygromycin sensitive plants including untransformed plant (hereafter referred to as WT) after 5 days of treatment and spread over the leaf discs after 8 days whereas leaf discs from *CsNitr1-L* transgenic lines were kept green and not severely damaged (data not shown). We analyzed incorporation of *CsNitr1-L* cDNA in the genome of 6 lines among those hygromycin-resistant lines by Southern blotting to *Pst*I-digested genomic DNAs of WT and the hygromycin-resistant lines. The vector, *pBI121Hm::CsNitr1-L*, has four *Pst*I sites in the T-DNA region, one of which locates inside the *CsNitr1-L* at position 954/955. *Pst*I digestion gives two *CsNitr1-L* sequence-containing fragments with franking sequence of T-DNA. A ~4.8-kb fragment containing 3′ half of *CsNitr1-L* sequence hybridizes to the probes. Figure 2 shows the presence of a 4.8-kb positive band in the genome of all hygromycin-resistant lines except line 9, indicating the presence of the

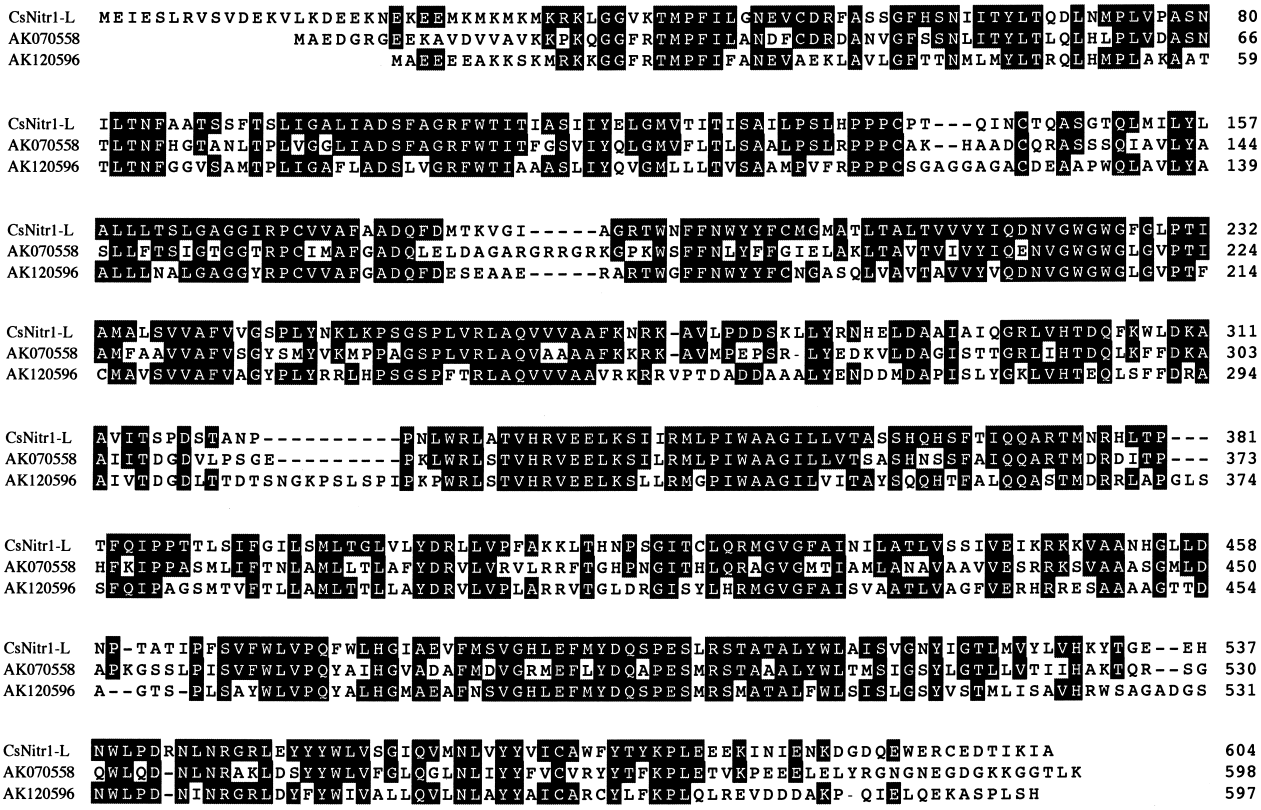

Figure 1. Alignment of amino acid sequence of *CsNitr1-L* with AK070558 and AK120596. Amino acid residues conserved between *CsNitr1-L* and rice homologs are shown in reverse.

CsNitr1-L transgene in the genome of T_1-1, T_1-2, T_1-5, T_1-6, and T_1-8. Since line 9 showed a phenotype of hygromycin resistance, the sequence including *Pst*I site near the left border might be partially defected in the line 9 with *hpt2* gene left functional in the T-DNA. Line 8 gave a more intense signal than other transgenic lines suggesting multiple insertion of T-DNA in the genome (Figure 2 lane 5).

Growth of CsNitr1-L transgenic rice with nitrate

Seeds of WT and seven T_1 lines including *GUS* transgenic control were germinated on blocks of rockwool wetted with water. Seedlings that had been grown for 2 weeks only with water under 16-h photoperiod became 12–15 cm in height showing no marked difference in their growth between *CsNitr1-L* transgenic plants and WT or GUS transformed control. Then, the seedlings were supplied with modified Yoshida medium that contained either nitrate or ammonia as the sole source of nitrogen.

Using 3 mM NH_4Cl instead of 1.4 mM NH_4NO_3 in the original Yoshida medium rice seedlings, all lines including WT and *GUS*-transformed control rice grew at nearly the same rate (Figure 3(a)). However, the growth rate varied from one line to another when 3 mM KNO_3 was used as the nitrogen source (Figure 3(b)). Comparing the nitrate-dependent growth of WT and

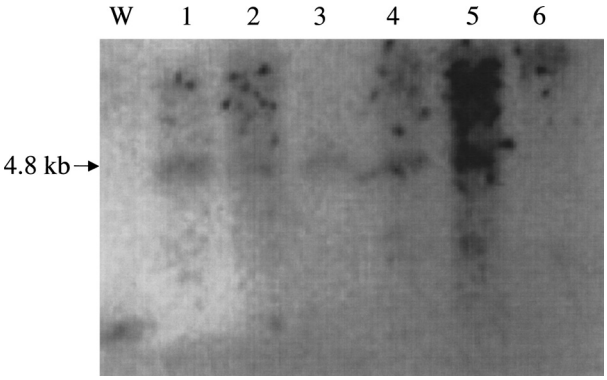

Figure 2. Southern blotting of DNA from rice transformed by *A. tumefaciens* infection. Separation of *Pst*I-digested genomic DNA (10 μg) and southern blotting was carried out as described in text. Lane W, WT; lanes 1–5, *CsNitr1-L* transgenic lines T_1-1, T_1-2, T_1-5, T_1-6, T_1-8, and T_1-9.

GUS transformed control rice with ammonia-dependent ones nitrate is shown to be less effective than ammonia as a nutrient for rice: the growth with nitrate was delayed by about 2 weeks from ammonia-fed rice during the cultivation of 5 weeks (Compare the solid lines in (a) with those in (b) of Figure 3). With ammonia all lines of rice including two control plants grew at almost the same rate. In contrast, the nitrate-dependent growth curves of *CsNitr1-L* transgenic rice were somewhat scattered but faster than those of the control plants. The growth of T_1-

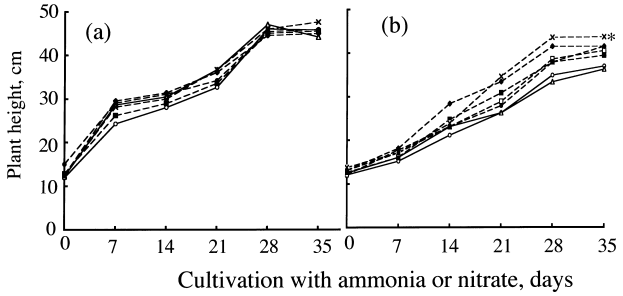

Figure 3. Effects of nitrogen sources on the growth of *CsNitr1-L* transgenic rice. Plant heights were measured every 7 days after 14-day old rice seedlings were fed with 3 mM NH_4^+ (a) or 3 mM NO_3^- (b). Nutrients were renewed every two days. Growth of *CsNitr1-L* transgenic lines are shown by broken lines and those of WT and *GUS*-transformed control rice by solid lines with following symbols: WT (○), *GUS*-transformed control (△), T_1-1 (□), T_1-2 (■), T_1-6 (X), T_1-8 (◆), and T_1-9 (●). Difference in height indicated by asterisk was statistically significant to WT by Student's *t*-test ($P<0.05$, $n=3$).

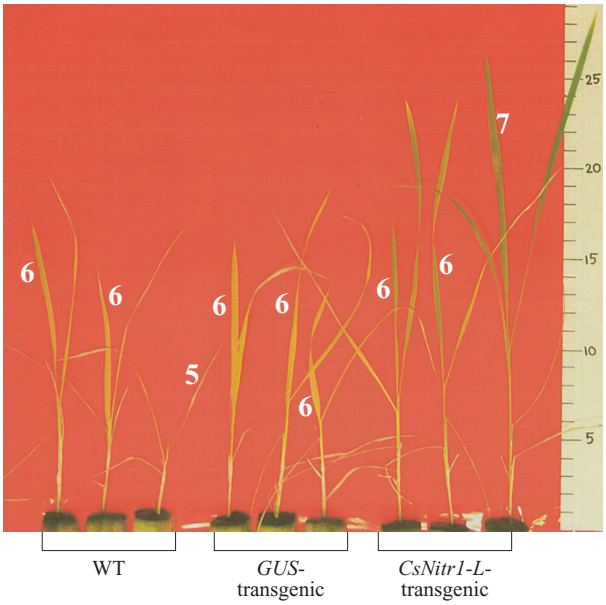

Figure 4. Nitrate-dependent growth of *CsNitr1-L* transgenic rice. Picture was taken when 2-week old rice grown without any nutrients were cultivated for a further week with Yoshida medium that contained 30 mM KNO_3 as the sole source of nitrogen. Scale stands for height in cm. Numbers in reverse indicate the stage of leaf development. (Left to right) WT, *GUS* transgenic control, and *CsNitr1-L* transgenic rice (T_1-6).

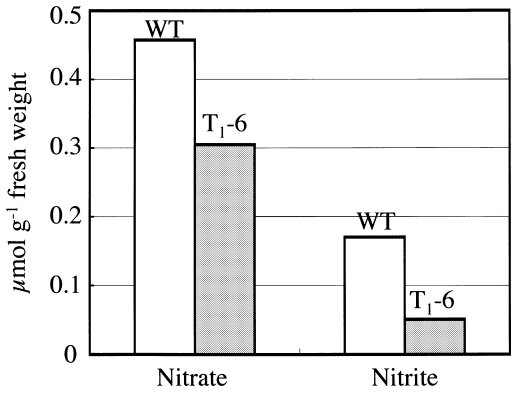

Figure 5. Contents of nitrite and nitrate in *CsNitr1-L* transgenic rice. Nitrate and nitrite contents of WT and *CsNitr1-L* transgenic rice (T_1-6) are shown as open and closed boxes, respectively.

6 line shown by a symbol × in (b) was obviously the fastest among those of *CsNitr1-L* transgenic lines and comparable to the ammonia-dependent growth.

Plants assimilate nitrate both in leaves and roots. In a plant grown with a low external concentration of nitrate most of the nitrate assimilation occurs at the roots. With external nitrate of high concentration the uptake of nitrate overcomes the reduction of nitrate in root and the resulting surplus of nitrate is metabolized in leaves (Marschner 1995). We examined the effect of high concentration of external nitrate on the growth of *CsNitr1-L* transgenic rice to see if enhanced transfer of nitrite could improve the nitrate use efficiency in the whole body of rice plants with constitutively expressing *CsNitr1-L*. Until the 3 to 4-leaf age, rice grows heterotrophically using endosperm as a nitrogen source. WT, *GUS* transgenic control, and *CsNitr1-L* transgenic rice grew without any difference in appearance before the rice were fed with 30 mM KNO_3 at 2 weeks after germination. A week after the addition of NO_3^- all lines of rice including WT and *GUS* transgenic control emerged 6th leaf although the growth and leaf color were different between *CsNitr1-L* transgenic rice and others. *CsNitr1-L* transgenic rice grew as usual to 6th leaf stage and were higher making differences in height of about 10 cm taller than that of WT and *GUS* transgenic control (Figure 4, compare right to left and middle). Leaf blades were dark green, normal in width, and longer than 10 cm indicating a little nitrogen excess for their growth (Hoshikawa 1989). WT (left three in Figure 4) and *GUS* transgenic rice (middle three in Figure 4) had grown to 6th leaf stage with leaf blades of normal length of about 6 cm or less, but colored yellowish green. Shortage of nutrient gives similar chlorosis to rice seedlings (Hoshikawa 1989). Delayed growth of rice with 30 mM nitrate (Figure 3) can be due to nitrate toxicity rather than to nutrient deficiency. These clear differences in appearance suggest that rice could avoid such impairment caused by nitrate by the expression of heterologous chloroplastic nitrite transporter.

Since accumulation of nitrite might give rise to chlorosis on plants (Vaucheret et al. 1992), chlorosis on the leaves of WT and *GUS* transgenic control may be a sign indicating that the processing of nitrite could be limiting the overall rate of nitrate assimilation in WT and *GUS* transgenic rice fed with high concentration of nitrate. The amount of nitrite in leaves of rice was indeed lower in a *CsNitr1-L* transgenic line than in WT and *GUS* transgenic control (Figure 5). The leaves of a month-old *CsNitr1-L* transgenic line, T_1-6, that had been

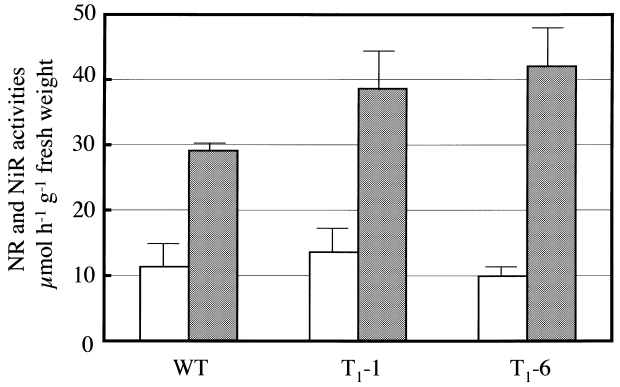

Figure 6. NR and NiR activity of *CsNitr1-L* transgenic rice. NR and NiR activities are shown as open and closed boxes, respectively.

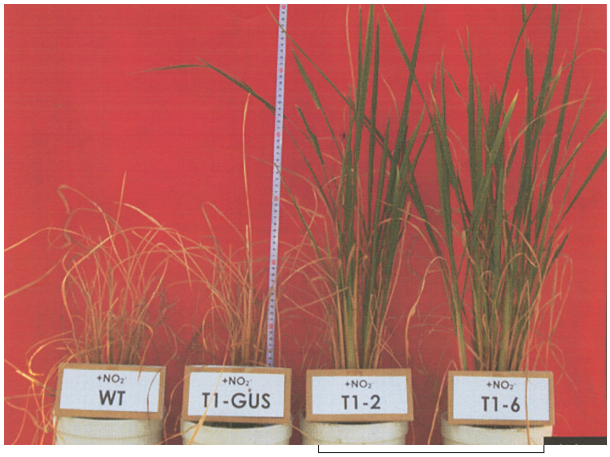

Figure 7. Utilization of nitrite by *CsNitr1-L* transgenic rice. After *CsNitr1-L* transgenic rice, WT, and *GUS* transgenic control had been cultivated for 2 months with complete Yoshida medium, plants were rinsed with water, cultivated for a week without nutrient and then with Yoshida medium containing 20 mM KNO_2 as sole source of nitrogen for 2 weeks. (Left to right) WT, *GUS* transgenic control, and two *CsNitr1-L* transgenic T_1-2 and T_1-6 rice.

cultivated with 3 mM nitrate, contained nitrite at an amount of ca. 50 nmol g^{-1} fresh weight, which was 30% of the nitrite content of WT (ca. 170 nmol g^{-1} fresh weight). Nitrite level is lower than 10 nmol g^{-1} fresh weight in dicotyledonous plants (Kawamura et al. 1996) where intermediary nitrite rarely accumulates due to faster consumption by NiR than the production by NR. The nitrite content of WT of rice, however, is at a critical level to allow the growth of rice callus (Ogawa et al. 1999). Accumulation of nitrite, in spite of the similar balance between NR and NiR in rice (Figure 6) to that in dicotyledonous plants, suggests that the transfer of nitrite into chloroplast is rate-limiting in rice. Nitrite accumulation in *CsNitr1-L* transgenic rice dropped to the safety level for the growth of nitrate-grown plants. Nitrite transporter of chloroplast envelopes is a low affinity type of POT having K_m of 100–200 μM (Sugiura et al. unpublished). In WT cells nitrite concentration is half-saturated, therefore, the increase of nitrite transporter molecules would improve the rate of nitrite uptake by chloroplasts.

Figure 5 also shows the nitrate level was lowered in the transgenic rice. Estimating roughly the average concentration of nitrate in the cytosol to be sub mM from its amount, 457 nmol g^{-1} fresh weight, the concentration is as low as the K_m of rice NR (0.7 mM; Sharma and Dubey 2005). In the concentration range of nitrate around K_m the rate of nitrate reduction, i.e., the production of nitrite should linearly correlate to the concentration of nitrate. Low accumulation of nitrite in the *CsNitr1-L* transgenic rice arose partly from lowered nitrate concentration. Since steady state concentration of nitrate is also determined by the balance between the supply of nitrate and the reduction by NR, nitrate transfer to the leaf tissues might be lowered in *CsNitr1-L* transgenic rice as long as the NR activity is constant (Figure 6). Multiple forms of nitrate transporter drive the transfer of nitrate from soil solution to the site of assimilation (Crawford and Glass 1998). Expression of a high affinity nitrate transporter is down-regulated by the accumulation of glutamine or ammonia even in the presence of nitrate (Crawford and Glass 1998). Although the content of such reduced nitrogen species was not determined in this experiment, higher production might be possible in *CsNitr1-L* transgenic lines. Nitrate-induced expression of NR and NiR has been reported not to be suppressed by either ammonia or glutamine (Crawford and Arst 1993), which is consistent with our finding that NiR activity was enhanced a little with NR activity being remained constant in *CsNitr1-L* transgenic rice (Figure 6).

Nitrite-dependent growth of CsNitr1-L transgenic rice

From the above findings, we speculated that nitrite is metabolized much faster in the *CsNitr1-L* transgenic rice than in WT. We tested if nitrite can be utilized as a source of nitrogen in the *CsNitr1-L* transgenic rice. It has been considered that plants have no uptake-type nitrite transporter at plasma membranes, however, nitrite in an acidic culture medium is able to permeate to plasma membranes as a form of nitrous acid (Vaucheret et al. 1992). KNO_2 (20 mM) was added to the hydroponic culture medium of WT, GUS transgenic control, and 2 lines of *CsNitr1-L* transgenic rice after they had been grown for 2 months with NH_4NO_3. Growth stage of all lines of rice were almost the same at the time of nitrogen source replacement, however, drastically changed during the cultivation with nitrite for following one week (Figure 7). *CsNitr1-L* transgenic rice continued to grow showing normal and healthy appearance whereas WT and *GUS* transgenic control died with chlorosis affecting

the whole area of leaves. In the *CsNitr1-L* transgenic rice the heterologous nitrite transporter could be constitutively expressed in root tissues as well as in leaves because of *CaMV35S* promoter-controlled expression. The root of *CsNitr1-L* transgenic rice looked normal without any possible nitrite-borne damages such as reddish-brown necrosis (data not shown) as described for WT rice by Oji and Okamoto (1981). Nitrite accumulation in leaves and/or roots may be the cause of chlorosis on the leaves. Nitrite-dependent growth of *CsNitr1-L* transgenic rice suggested that nitrite could be utilized by *CsNitr1-L* transgenic rice as a source of nitrogen and furthermore nitrite was not accumulated to a level that causes photooxidative damage to leaves.

Enhanced photosynthetic productivity of CsNitr1-L *transgenic rice*

Assimilation of nitrate to amino acids is an endothermic reaction requiring potent reductants for the conversions of nitrate to ammonia and of glutamine to glutamate and ATP for the glutamine synthesis. Nitrite transfer through the chloroplast envelopes may be driven by a pH gradient across the membrane, which could be also an ATP requiring process. Photosynthetic electron transfer supplies reductants indirectly to NR and directly to NiR and glutamate synthase and also generates ATP through photophosphorylation. If a plant utilizes more photosynthetically generated energies for processes other than CO_2 fixation in chloroplast, photon costs for the production of pH gradient across thylakoid membranes would become smaller as shown by delayed or low non-photochemical quenching of Chl fluorescence emission (Schreiber et al. 1989). We analyzed fluorescence emission kinetics to evaluate photosynthetic productivity for an individual line of *CsNitr1-L* transgenic rice. Figure 8 shows Chl fluorescence kinetics of two types of rice. Photosystem II activity judged from F_m/F_0 was equal for both types of rice: 5.61 for *GUS* transgenic rice (blue) and 5.63 for *CsNitr1-L* transgenic T_1-1 line (red). Non-photochemical quenching of *CsNitr1-L* transgenic rice occurred much slower than that of *GUS* transgenic rice indicating that reaction(s) having a higher demand of photosynthetic electron transfer might occur in *CsNitr1-L* transgenic rice. It is thus suggested that nitrite reduction could be enhanced by the increased supply of nitrite in the rice engineered with *CsNitr1-L* gene.

On the vegetative growth of rice the overexpression of chloroplastic nitrite transporter is shown to be effective. Low nitrate concentration in leaves is another favorable property for food materials. New rice varieties have been bred to have good taste and consequently lost active nitrite metabolism (Ogawa et al. 1999; Nishimura et al. 2005). Contents of storage proteins and taste of the seed of *CsNitr1-L* transgenic rice is now being tested.

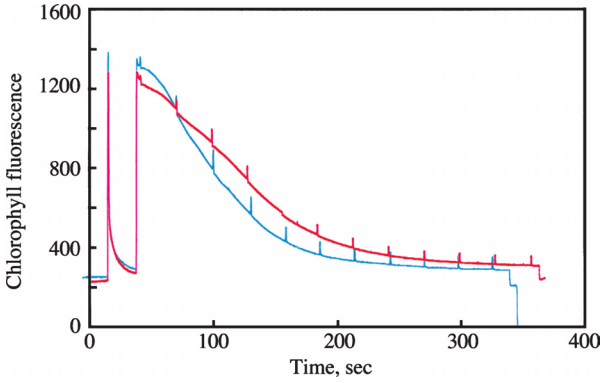

Figure 8. Chl fluorescence kinetics from *CsNitr1-L* transgenic T_1-1 rice (red) and *GUS* transgenic rice (blue) showing delayed non-photochemical quenching of *CsNitr1-L* transgenic rice.

Acknowledgements

We thank Yoshihumi Kawamura for capillary electrophoresis. We also thank Grant Griffith for his assistance in preparing this manuscript.

References

Andrews M, Lea PJ, Raven JA, Lindsey K (2004) Can genetic manipulation of plant nitrogen assimilation enzymes result in increased crop yield and greater N-use efficiency? An assessment. *Ann Appl Biol* 145: 25–40

Arabidopsis genome initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796–815

Crawford NM, Arst Jr HN (1993) The molecular genetics of nitrate assimilation in fungi and plants. *Annu Rev Genet* 27: 115–146

Crawford NM, Glass ADM (1998) Molecular and physiological aspects of nitrate uptake in plants. *Trends Plant Sci* 3: 389–395

Della Penna D (2001) Plant metabolic engineering. *Plant Physiol* 125: 160–163

Hageman RH, Hucklesby DP (1971) Nitrate reductase from higher plants. *Methods Enzymol* 23: 491–503

Herrera-Estrella L, León P, Olsson O, Teeri TH (1994) Reporter genes for plants. In: Gelvin SB, Schilperoort RA (eds) *Plant Molecular Biology Manual*. Kluwer Academic Publishers, Dordorecht, pp C2: 1–32

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J* 6: 271–282

Hinze H, Holzer H (1985) Accumulation of nitrite and sulfite in yeast cells and synergistic depletion of the intracellular ATP content. *Z Lebensm Unters Forsch* 180: 117–120

Hoshikawa K (1989) *The Growing Rice Plant.* Nobunkyo, Tokyo

Kawamura Y, Takahashi M, Arimura G, Isayama T, Irifune K, Goshima N, Morikawa H (1996) Determination of levels of NO_3^-, NO_2^- ad NH_4^+ ions in leaves of various plants by capillary electrophoresis. *Plant Cell Physiol* 37: 878–880

Losada M, Paneque A (1971) Nitrite reductase. *Methods Enzymol* 23: 487–491

Machín F, Medina B, Navarro FJ, Pérez MD, Veenhuis M, Tejera P, Lorenzo H, Lancha A, Siverio JM (2004) The role of Ynt1 in

nitrate and nitrite transport in the yeast *Hansenula polymorpha*. *Yeast* 21: 265–276

Marschner H (1995) *Mineral Nutrition of Higher Plants*, Second Edition. Academic Press, London

Nishimura A, Ashikari M, Lin S, Takashi T, Angeles ER, Yamamoto T, Matsuoka M (2005) Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. *Proc Natl Acad Sci USA* 102: 11940–11944

Ogawa T, Fukuoka H, Yano H, Ohkawa Y (1999) Relationships between nitrite reductase activity and genotype-dependent callus growth in rice cell cultures. *Plant Cell Rep* 18: 576–581

Oji Y, Okamoto S (1981) Nitrite utilization in barley plants as compared with nitrate and ammonium utilization. *Sci Rep Fac Agr Kobe Univ* 14: 349–353

Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S, Frommer WB (1995) NTR1 encodes a high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett* 370: 264–268

Rexach J, Fernadez E, Galván A (2000) The *Chlamydomonas reinhardtii Nar*1 gene encodes a chloroplast membrane protein involved in nitrite transport. *Plant Cell* 12: 1441–1453

Sambrook J, Russel DW (2001) *Molecular Cloning*. Cold Spring Harbor Laboratory Press, New York

Schreiber U, Neubauer C, Klughammer G (1989) Devices and methods for room-temperature fluorescence analysis. *Phil Trans R Sci Lond B* 323: 241–251

Sharma P, Dubey RS (2005) Modulation of nitrate reductase activity in rice seedlings under aluminum toxicity and water stress: role of osmolytes as enzyme protectant. *J Plant Physiol* 162: 854–864

Stewart V (1988) Nitrate respiration in relation to facultative metablism in eubacteria. *Microbiol Rev* 52: 190–232

The rice full-length cDNA consortium (2002) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice clones. *Science* 301: 376–379

Tsay Y-F, Schroeder JI, Feldmann KA, Crawford NM (1993) The herbicide sensitivity gene CHL1 of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. *Cell* 72: 705–713

Vaucheret H, Kronenberger J, Lepngle A, Vilaine F, Boutin J-P, Caboche M (1992) Inhibition of tobacco nitrite reductase activity by expression of antisense RNA. *Plant J* 2: 449–569

Walkerpeach CR, Velten J (1994) Agrobacterium-mediated gene transfer to plant cells: cointegrate and binary vector systems. In: Gelvin SB, Schilperoort RA (eds) *Plant Molecular Biology Manual*. Kluwar Academic Publishers, Dordrecht, pp B1: 1–19

Yanagisawa S, Akiyama A, Kisaka H, Uchimiya H, Miwa T (2004) Metabolic engineering with Dof1 transcription factor in plants: Improved nitrogen assimilation and growth under low-nitrogen conditions. *Proc Natl Acad Sci USA* 101: 7833–7838

Yoshida S, Forno DD, Cock JH (1971) Routine procedure for growing rice plants in culture solution. In: *Laboratory Manual for Physiological Studies of Rice*. The International Rice Research Institute, Philippines, p 53