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Transgenic Note

Overexpression of a *calcium-dependent protein kinase* gene enhances growth of rice under low-nitrogen conditions

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Abstract The excessive amounts of nitrogen applied in current farming systems can cause environmental problems. There is therefore a need to improve the ability of crop plants to utilize nitrogenous fertilizers. We screened for nitrogen deficiency-tolerant lines among transgenic rice plants that overexpressed full-length cDNAs (FL-cDNAs) corresponding to low-nitrogen response genes, genes related to nitrogen metabolism, and genes related to carbon metabolism. We found that overexpression of *OsCPK12* FL-cDNA, encoding a calcium-dependent protein kinase (CDPK), conferred tolerance to low-nitrogen stress in rice. After two weeks of low-nitrogen treatment, dry weights of shoots from *OsCPK12*-overexpressing plants were greater than those from control plants. Furthermore, total nitrogen contents of *OsCPK12*-overexpressing plants were higher than those of the control plants. Our findings suggest that *OsCPK12* is involved in the signal transduction pathway(s) in the low-nitrogen stress response and may be useful in engineering crop plants with improved tolerance to low nitrogen levels.

Key words: CDPK, low-nitrogen conditions, rice, stress tolerance, transgenic plant.

Nitrogen (N) is an essential macronutrient for plant growth. Plants utilize mainly NH_4^+ and NO_3^- as sources of N. Approximately 85 to 90 million metric tons of nitrogenous fertilizers are currently applied to soils worldwide (Frink et al. 1999). However, crop plants can utilize only 30–50% of the applied N (Good et al. 2004). The remaining N from the fertilizer is lost by denitrification or by leaching into groundwater. There is therefore a need for improved crops that can make more efficient use of N, that are more adaptable to conditions where N is limited, and that show an enhanced uptake and assimilation of N. Several studies aimed at improving the ability of plants to utilize available N by means of molecular engineering have been performed (review by Good et al. 2004). For example, overexpression of a plant-specific transcription factor, *Dof1*, in *Arabidopsis thaliana* confers improved growth under low-N conditions (Yanagisawa et al. 2004). Also, transgenic rice plants overexpressing an early nodulin gene, *OsENOD 93-1*, show increased efficiency in utilization of N (Bi et al. 2009). However, little is known about the molecular mechanisms of tolerance to N-limiting conditions.

Several gain-of-function strategies have been used to investigate gene functions in plants. A gain-of-function approach named the Full-length cDNA Over-eXpresser (FOX) gene hunting system (Ichikawa et al. 2006) has been applied to systematic functional analysis of rice genes by using ~14,000 independent rice full-length cDNAs (FL-cDNAs), and approximately 12,000 FOX-rice lines have been generated (Nakamura et al. 2007). The FOX hunting system permitted the generation of a number of morphological mutations, including changes in plant height and leaf shape. In the present work, we screened the FL-cDNA-overexpressing rice lines under low-N conditions and found that *OsCPK12*-overexpressing plants showed better growth than did control plants under these conditions. We also examined changes in absorption of external NH_4^+ and contents of amino acids in the *OsCPK12*-overexpressing plants.

Rice (*Oryza sativa* L. cv. Nipponbare) plants were grown in a growth chamber under 60% relative humidity with a 12-h light (25°C)/12-h darkness (20°C) cycle. For growth tests under standard and low-N conditions, transgenic seeds from each FL-cDNA overexpressing lines were surface-sterilized and germinated on a plate containing 40 mg L^{-1} of hygromycin at 30°C. The germinated seeds were grown hydroponically with

Abbreviations: C, carbon; FL-cDNA, full-length cDNA; FOX, full-length cDNA overexpressor; N, nitrogen

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deionized water for two weeks. The seedlings were then transferred to eight-times diluted Yoshida nutrient solution (Yoshida et al. 1976) for standard N treatment (177.6 μM NH_4NO_3) or to 1/8 Yoshida nutrient solution in which ammonium nitrate was diluted to 1/32 for low-N treatment (44.4 μM NH_4NO_3). After one week, the seedlings were transferred to fresh nutrient solution; they were then grown for one week more, then the shoots and the roots were harvested separately for measurement of their dry weights.

For the analysis of amino acid contents, frozen shoots and roots were ground with a cell disruptor (Multi-Beads Shocker, Yasui-kikai Co., Osaka, Japan), and the amino acids were extracted as described by Sato et al. (2004). In brief, the ground tissues were resuspended in 5 volumes of 99.7% methanol per fresh weight and then an equal volume of water containing 400 μM L-methionine sulfone [L-2-amino-4-(methylsulfonyl)butanoic acid] was added as an internal standard. The extract was transferred into an Ultrafree-0.5 centrifugal filter device (Millipore, MA, USA) and centrifuged at 12,000 *g* for 40 min at 4°C. The samples were recovered from the device and analyzed by using a Beckman P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, CA, USA) attached to a Finnegan TSQ Quantum Discovery Max quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). For the analysis of the ammonium concentration, 2-mL samples of nutrient solution were taken at the given times and analyzed spectrometrically (Nanocolor Ammonium Kit; Macherey Nagel, Düren, Germany) according to the manufacturer's instructions. For the measurement of the C and N contents, the shoots and roots were dried for three days or more in an oven at 80°C, and then their C and N contents were measured by using a CN analyzer (vario MAX CN; Elementar Analysensysteme GmbH, Hanau, Germany) according to the manufacturer's instructions.

Recent studies on gene expression analysis by using microarray showed that the expression of 471 rice genes changed in response to a low-N stress (Lian et al. 2006). To identify rice genes that are involved in tolerance to low-N conditions, we searched the FL-cDNA overexpressing lines (Nakamura et al. 2007) for low-N response genes, genes related to nitrogen metabolism, genes related to carbon metabolism, and we generated FL-cDNA overexpressing lines for *calcium-dependent protein kinase* (*CDPK*), covering 21 of the 29 known CDPKs. We finally chose 101 lines of FL-cDNA overexpressing line, including 52 lines of rice plants overexpressing *CDPK* (Supplemental Table 1). For screening, two-week-old seedlings of the control and FL-cDNA overexpressing lines were grown hydroponically in a same bottle for two weeks under low-N conditions (44.4 μM NH_4NO_3). Rice plants overexpressing a *CDPK* (*OsCPK12*) showed increased tolerance to low-nitrogen stress. RT-PCR analysis confirmed that *OsCPK12* cDNA was overexpressed in the *OsCPK12*-overexpressing plants (Figure 1A). Three lines of the *OsCPK12*-overexpressing

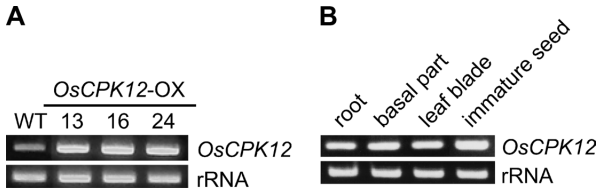

Figure 1. Expression analysis of *OsCPK12*. (A) Expression analysis of *OsCPK12* in wild-type and *OsCPK12*-overexpressing plants grown under normal growth condition. Total RNA was isolated from leaf blades of two-week-old seedlings. RT-PCR analysis (30 cycles for *OsCPK12* or 25 cycles for 18S rRNA) was performed with either *OsCPK12*-specific primers or 18S rRNA specific primers. (B) Expression of *OsCPK12* in four different tissues of wild-type plants. Total RNA was isolated from various tissues [basal parts including meristems, leaf blades, and roots (2 weeks after germination) or immature seeds (5- 10 days after flowering)] of wild-type plants. RT-PCR analysis (35 cycles for *OsCPK12* or 25 cycles for 18S rRNA) was performed with either *OsCPK12*-specific primers or 18S rRNA specific primers.

Table 1. Concentrations of major amino acids (n molg^{-1} fresh weight) in the third leaves of *OsCPK12*-overexpressing plants.

Amino acid	One day after N supply				Two days after N supply			
	VC	#13	#16	#24	VC	#13	#16	#24
Glycine	3329.8 ± 166.4	3452.5 ± 543.8	4580.9 ± 213.5	4453.6 ± 865.8	461.9 ± 31.2	335.1 ± 69.9	389.1 ± 70.0	642.2 ± 87.8
Alanine	3687.0 ± 475.1	2706.3 ± 177.1	3199.0 ± 241.5	3943.3 ± 616.8	1979.5 ± 312.3	1825.6 ± 115.8	2247.1 ± 444.4	2156.4 ± 511.5
Serine	1112.4 ± 99.3	1416.6 ± 49.5	1167.2 ± 87.4	1249.0 ± 62.2	809.1 ± 90.5	1016.9 ± 77.6	727.0 ± 200.8	797.2 ± 43.9
Valine	1116.9 ± 35.0	1079.7 ± 248.3	1065.9 ± 190.3	1505.1 ± 322.6	108.9 ± 19.6	118.4 ± 24.7	84.5 ± 31.0	154.6 ± 40.0
Asparagine	9813.5 ± 1494.6	9085.2 ± 2215.1	10587.5 ± 1420.8	12914.9 ± 2511.2	2147.3 ± 263.0	1262.6 ± 243.2	1495.0 ± 252.0	2444.7 ± 735.6
Threonine	545.3 ± 136.8	461.6 ± 89.9	539.8 ± 33.4	491.9 ± 68.2	492.7 ± 50.3	489.5 ± 36.0	514.7 ± 85.8	633.8 ± 159.4
Glutamine	24890.5 ± 2187.3	24695.3 ± 7192.6	27836.2 ± 5364.3	26434.4 ± 4289.8	5243.9 ± 956.4	2884.3 ± 172.7	4668.8 ± 809.6	6101.5 ± 2172.3
Proline	291.9 ± 33.0	273.0 ± 45.9	267.7 ± 39.0	275.6 ± 31.9	88.8 ± 37.0	100.5 ± 11.8	107.5 ± 22.3	145.4 ± 26.1
Glutamate	3208.1 ± 280.1	4122.2 ± 698.2	3105.7 ± 458.1	3474.3 ± 87.8	2593.0 ± 322.6	2222.4 ± 369.6	2133.6 ± 348.6	2230.7 ± 47.4
Aspartate	1487.9 ± 234.9	1585.7 ± 370.8	1053.9 ± 250.8	1669.2 ± 245.2	890.0 ± 196.7	1068.4 ± 317.0	736.2 ± 272.8	991.5 ± 147.1
Total	49483.3 ± 4772.6	48878.1 ± 10699.7	53404.0 ± 7315.8	56411.3 ± 8123.5	14815.0 ± 1016.3	11323.9 ± 491.6	13103.5 ± 1569.9	16297.9 ± 3603.2

Two-week-old seedlings were treated with 44.4 μM NH_4NO_3.
Concentration of amino acids in the third leaves was analyzed.
Data are means ± SE (n=3).

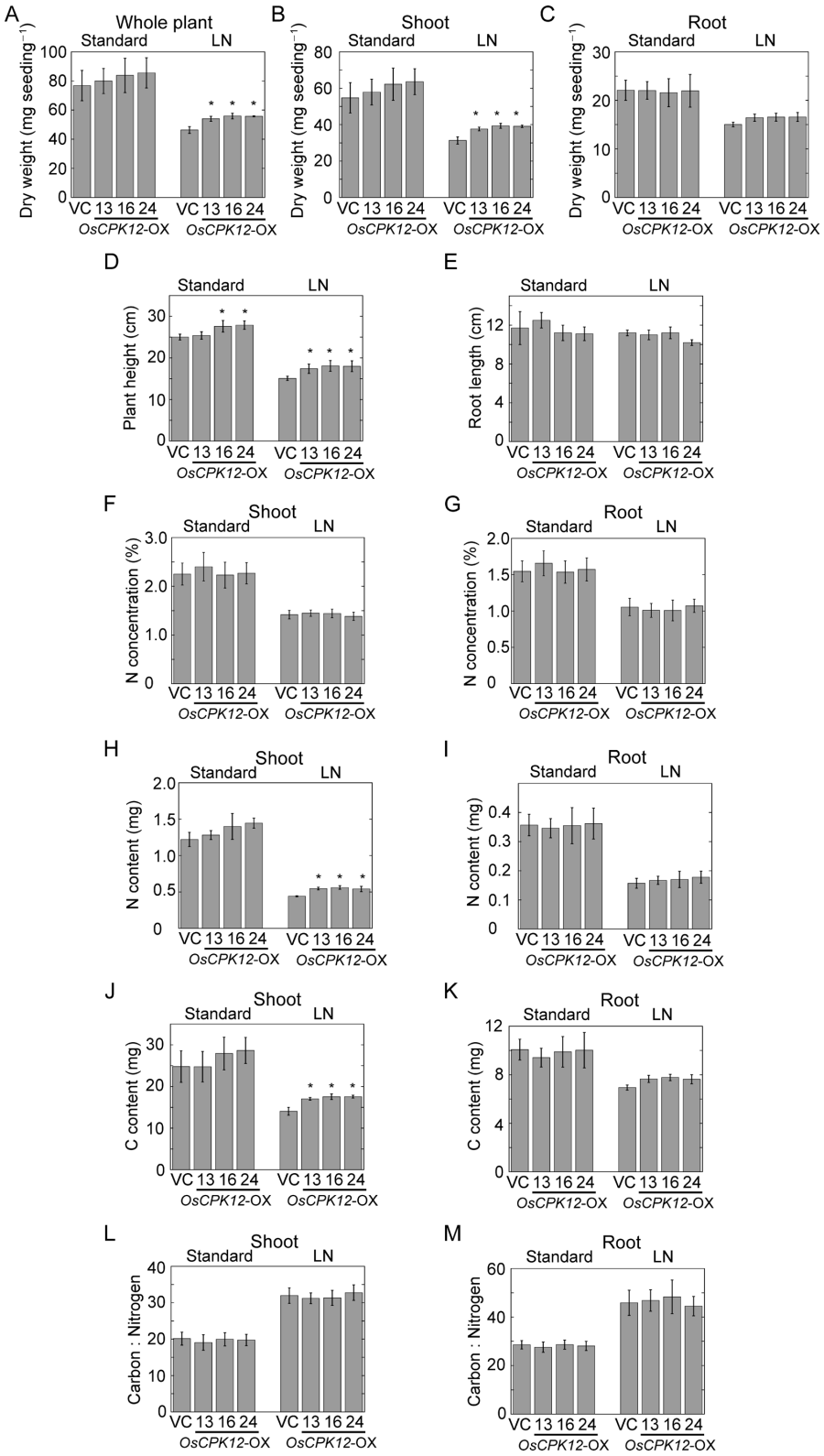

Figure 2. Growth of control and *OsCPK12*-overexpressing plants under low-N conditions. Two-week-old seedlings of control and *OsCPK12*-overexpressing plants were grown hydroponically in the same bottle for two weeks under standard (177.6 μM NH_4NO_3) or low-N conditions (44.4 μM NH_4NO_3). The experiment was performed by using 15–25 plants. Error bars represent SE (n=3). Asterisks indicate a significant difference at $P<0.05$. Whole-plant dry weights (A), shoot dry weights (B), and root dry weights (C) of the control and the *OsCPK12*-overexpressing plants. Plant height (D) and root length (E). N concentrations in shoots (F) and in roots (G). N contents in shoots (H) and in roots (I). C contents in shoots (J) and in roots (K). Carbon–nitrogen ratios in shoots (L) and in roots (M).

plants (#13, #16 and #24) were selected for further analysis. The RT-PCR analysis also indicated that *OsCPK12* was expressed ubiquitously in all tissues analyzed in this study (Figure 1B).

Under the low-N conditions, the average whole-plant dry weight from the *OsCPK12*-overexpressing plants was greater than that from the control plants under low-N conditions (Figure 2A). The dry weight of shoots from the *OsCPK12*-overexpressing plants was greater than that from the control plants (Figure 2B), whereas no obvious difference was observed in the root biomass (Figure 2C). Moreover, no obvious difference in dry weight was observed between the control and the *OsCPK12*-overexpressing plants under standard N conditions (Figure 2A–C). Shoots of the *OsCPK12*-overexpressing plants were longer than those of the control plants under the low-N condition (Figure 2D). In contrast, no significant difference was observed in root lengths between the control and the *OsCPK12*-overexpressing plants (Figure 2E). These results suggest that overexpression of *OsCPK12* can enhance shoot growth under low-N conditions.

Because the *OsCPK12*-overexpressing plants showed better growth than the control plants under the low-N conditions, we suggest that the former group may acquire more nitrogen nutrient than the latter. To test this hypothesis, we measured the total N and C contents of the shoots and roots. The shoots and roots of the control and the *OsCPK12*-overexpressing plants showed similar nitrogen concentrations (nitrogen content/dry weight) under both the standard- and low-N conditions (Figure 2F, G). This suggests that there is no difference in the efficiency of N utilization between the control and the *OsCPK12*-overexpressing plants. In the shoots, however, the *OsCPK12*-overexpressing plants showed higher C and N contents than the control plants under low-N conditions because of their higher biomass (Figure 2H, J). In contrast, no significant difference was observed in the C and N contents of roots between the control and the *OsCPK12*-overexpressing plants under standard and low-N conditions (Figure 2I, K).

Next, we compared NH_4^+ absorption between the control and the *OsCPK12*-overexpressing plants. Two-week-old seedlings of the control and the *OsCPK12*-overexpressing plants were transferred to individual bottles containing 98.8 μM NH_4NO_3. The NH_4^+ concentration in the nutrient solution was measured at each time point after the N treatment. The external NH_4^+ concentration was similar between the control and the *OsCPK12*-overexpressing plants at one day and two days after the N treatment, whereas at three days, it was slightly lower in the *OsCPK12*-overexpressing plants than in the control plants (Figure 3). The *OsCPK12*-overexpressing plants may, therefore, have a higher activity for NH_4^+ absorption than the control plants. This

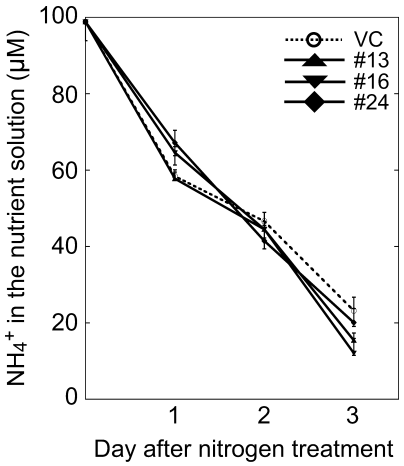

Figure 3. Concentrations of ammonium in the nutrient solution. Two-week-old seedlings of control and *OsCPK12*-overexpressing plants were transferred to individual bottles containing 98.8 μM NH_4NO_3. Ammonium in the solution was analyzed by using a Nanocolor Ammonium kit at the times indicated. Error bars indicate SE (n=3).

suggests that the *OsCPK12*-overexpressing plants could have acquired more nitrogen nutrient than the control plants when the control and the *OsCPK12*-overexpressing plants were grown in the same bottle.

To compare nitrogen assimilation/metabolism between the control plants and the *OsCPK12*-overexpressing plants, two-week-old seedlings were treated with 44.4 μM NH_4NO_3, and the amino acid contents of the third leaves were then determined. At one and two days after the N treatment, the concentrations of amino acids were similar in third leaves of the control and the *OsCPK12*-overepressing plants (Table 1). Furthermore, no significant differences in the time courses of changes in glutamine and asparagine concentrations were found between the control plants and the *OsCPK12*-overexpressing plants. These results suggest that overexpression of *OsCPK12* does not affect the early response in N assimilation. At seven days after the N treatment, however, the total amino acid content of the third leaves was higher in the *OsCPK12*-overexpressing plants than in the control plants (Table 2). In particular, concentrations of glutamine, glutamate, alanine, proline, and threonine in *OsCPK12*-overexpressing plants were higher than those in the control plants; however, no difference was observed in the concentrations of asparagine or aspartate in the control and the *OsCPK12*-overexpressing plants. These results suggest that overexpression of *OsCPK12* may affect the metabolism of N under conditions of N starvation.

CDPKs have been identified throughout the plant kingdom (Harper and Harmon 2005; Ludwig et al. 2004) and in some protozoas (Ward et al. 2004). In rice, *CDPKs* constitute a large multigene family of 29 genes that we identified in a previous study and which we have designated as *OsCPK1* through *OsCPK29* (Asano et al.

Table 2. Concentrations of major amino acids (n mol g^{-1} fresh weight) of *OsCPK12*-overexpressing plants.

Amino acid	VC	*OsCPK12*-OX		
		#13	#16	#24
Alanine	224.6 ± 112.0	584.4 ± 188.6	393.4 ± 64.5	614.0 ± 101.3
Serine	212.0 ± 29.0	334.3 ± 15.0	382.2 ± 17.3	268.2 ± 52.7
Valine	63.6 ± 16.8	78.9 ± 16.7	69.3 ± 2.0	94.3 ± 15.9
Asparagine	79.0 ± 26.3	136.7 ± 52.8	8.5 ± 13.8	76.4 ± 14.0
Threonine	51.2 ± 9.4	101.5 ± 22.1	78.5 ± 9.2	86.0 ± 18.2
Glutamine	27.2 ± 12.6	76.4 ± 14.6	143.6 ± 34.6	117.6 ± 62.8
Proline	33.9 ± 11.0	63.9 ± 22.6	67.2 ± 15.7	54.6 ± 16.8
Glutamate	679.0 ± 131.0	927.2 ± 43.6	891.3 ± 50.5	799.0 ± 130.5
Aspartate	172.7 ± 27.6	248.1 ± 59.9	207.7 ± 41.7	160.6 ± 10.1
Total	1543.3 ± 264.1	2551.5 ± 309.7	2321.9 ± 117.5	2270.8 ± 397.3

Two-week-old seedlings were treated with 44.4 μM NH_4NO_3.

Concentration of amino acids in the third leaves was analyzed at 7 days after N supply.

Data are means ± SE (n=3).

2005). CDPKs are composed of a variable N-terminal domain, a protein kinase domain, an auto-inhibitory region, and a calmodulin-like domain (Cheng et al. 2002; Harper et al. 1991), and they are directly activated by binding of Ca^{2+} to the calmodulin-like domain (Harper and Harmon 2005). CDPKs are thought to be regulators of calcium signaling in various physiological processes in plants, because calcium is a universal second messenger in these signal-transduction pathways. Although the patterns of *CDPK* expression and CDPK enzyme activities in response to various stimuli have been reported in a variety of plant species [for a review, see Ludwig et al. (2004)], little is known about the molecular identity of the CDPKs that regulate N metabolism.

We observed that overexpression of *OsCPK12* conferred improved growth under low-N conditions. After 14 days of N treatment, the total N content of the *OsCPK12*-overexpressing plants was higher than that of the control plants. Seven days after the N treatment, the total amino acid content of the *OsCPK12*-overexpressing plants was higher than that of the control plants, whereas no obvious difference was observed at one day or two days after the N treatment. The overexpression of *OsCPK12* showed little effect on nitrogen absorption. This suggests that overexpression of *OsCPK12* may affect the metabolism under conditions of N starvation, rather than the nitrogen absorption and the early response in N assimilation. Although the precise causal relationship between the overexpression of *OsCPK12* and the increase in dry weight under low-N conditions remains unknown, *OsCPK12*-overexpressing plants might be beneficial in achieving sustainable agriculture with low inputs of N fertilizer.

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