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A Fungal Cytochrome P-450nor Confers Denitrifying Ability to Tobacco BY-2 Cells

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Abstract: Reactive nitrogen gases progressively contribute to the global warming. Development of gas-gas denitrifying plants that can efficiently reduce reactive nitrogen gases to dinitrogen (N_2) could help to mitigate the effect of these gases. Taking the advances in gene manipulation technology, tobacco BY-2 cells were transformed with the fungus *Cylindrocarpon tonkinense* cytochrome P-450nor2 (Cnor2) gene. The product of this gene acts as nitric oxide reductase (nor). Transgenic BY-2 cell clones cultured in ^{15}N-labelled nitrate ($^{15}NO_3^-$) actively evolved $^{15}N_2O$ gas up to 35-folds compared to the wild-type cells. In ^{15}N-labelled ammonium ($^{15}NH_4^+$), the transgenic and wild-type cells produced comparable amounts of $^{15}N_2O$. This indicates that ammonium is not a direct substrate for nor and the small amount of N_2O observed may be due to the nitrification of ammonium to nitrite. Addition of tungstate (a nitrate reductase inhibitor) and cyanide to the transgenic cell cultures strongly inhibited $^{15}N_2O$ production. Activity of nor enzyme was also confirmed by *in vitro* activity assay. These observations together suggest that Cnor2 is actively expressed and enhanced the reduction of nitrate to N_2O in plant cells. This finding indicates that plant cells are capable to tackle the denitrification pathway.

Key words: Denitrification, nitrous oxide, nitric oxide reductase (P-450nor), tobacco BY-2 cells

INTRODUCTION

Plants play a critical role in regulating the chemical and physical state of the atmosphere. Most notable are plant-atmosphere exchanges of CO_2, O_2 and H_2O; however; leaves also emit a variety of carbon- and nitrogen-based trace gases involved in climate alteration processes (Smart and Bloom, 2001). Plants have the ability to sense internal and external nitrogen status and to adapt to changing nitrogen condition by modifying gene expression, enzymes activities and metabolite contents, thus providing an efficient and integrated sensory transduction and regulatory network (Sakakibara *et al*., 2006). Recent studies on nitrogen metabolism by plants revealed a novel finding that exogenously supplied NO_2 functions as an airborne signal in plants (Morikawa *et al*., 2004, 2005; Takahashi *et al*., 2005, 2006). Furthermore, it has been reported that endogenously produced nitrogen oxides (NO_x) such as nitric oxide (NO) function as a vital plant signal (Delledonne *et al*., 1998; Wendehenne *et al*., 2001; Neill *et al*., 2003; Sakamoto *et al*., 2004). On the other hand, plant-mediated mitigation of atmospheric NO_2 pollution and the use of NO_2 as an alternative nitrogen fertilizer were also investigated (Morikawa *et al*., 1998; Goshima *et al*., 1999; Takahashi *et al*., 2001; Morikawa *et al*., 2004). Hence, there are several indications that NO_x involved in regulation of plant signaling and physiological processes. However, the anthropogenic reactive nitrogen species (RNS) are increasing dramatically worldwide and mostly accumulating in the environment. An increasing demand for food and energy supplies and the lack of effective measures to improve the efficiency of fertilizer nitrogen use, as well as effective measures for the prevention of NO_x emissions from fossil-fuel combustion, are the principal drivers behind the environmental nitrogen-enrichment problem (Zheng *et al*., 2002). In this regard, it is urgent to find or develop highly-efficient RNS metabolizing plants. Such a strategy depends largely upon advances in molecular biology and biological technology.

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One of the management strategies to mitigate RNS or reactive nitrogen intermediates in the environment is to develop plants that have high capacity to serve as sinks for nitrate and NO_2 (Morikawa *et al*., 1998; Takahashi *et al*., 2001) or to reduce RNS to an inert state. This approach can be achieved by genetically engineer plants with the denitrifying genes to enhance conversion of nitrate, RNS and NO_2 ultimately to dinitrogen gas.

To our knowledge, no denitrification gene of plant origin has been characterized and also there is no evidence for the involvement of any of the bacterial or fungal denitrification enzymes in nitrous oxide (N_2O) emission from plants (Goshima *et al*., 1999; Smart and Bloom, 2001; Hakata *et al*., 2003). Moreover, the *Arabidopsis* genome database contains no single homologous gene to the denitrification genes in bacteria or fungi. Therefore, it is necessary to engineer the denitrification pathway in plant cells. For this reason we manipulated in tobacco cells a gene encoding a fungal denitrifying enzyme, a cytochrome P-450 nitric oxide reductase (P-450nor) from *Cylindrocarpon tonkinense* (Usuda *et al*., 1995; Kudo *et al*., 1996). The enzyme belongs to the cytochrome P-450 superfamily on the basis of primary and tertiary structures (Kizawa *et al*., 1991; Park *et al*., 1997). P-450nor is unique among P-450 proteins in the enzymatic properties. It accepts two electrons directly from NADH or NADPH [(NAD(P)H] and catalyzes the following reaction without the aid of other protein components such as a flavorprotein reductase (Kaya *et al*., 2004):

$$2NO + NAD(P)H + H^+ \rightarrow N_2O + H_2O + NAD(P)^+$$

Two isoforms of P-450nor genes from *C. tonkinense*, namely P-450nor1 and P-450nor2 (Cnor1 and Cnor2), were isolated and cloned (Usuda *et al*., 1995; Kudo *et al*., 1996). Products of the two genes differ in their intracellular localization and specificity to the electron donors. A putative signal sequence is present only in Cnor1 suggest that it is located in mitochondria, whereas Cnor2 is located in cytosol. Cnor1 is specific to the electron donor NADH, while Cnor2 can utilize both electron donors, NADH and NADPH (Nakahara *et al*., 1993; Usuda *et al*., 1995; Kudo *et al*., 1996). Here we show that *Nicotiana tabacum* L. cv. Bright Yellow No. 2 (BY-2) cells (Kato *et al*., 1972) harboring Cnor2 gene emit a large quantity of N_2O, which demonstrate that genetic engineering with the denitrifying genes confers the denitrifying ability to plant cells. This approach helps to understand the mechanism of N_2O production by plants, a phenomenon observed years ago but enzymatically not characterized yet.

MATERIALS AND METHODS

This study was done at the Department of Mathematical and Life Science, Graduate School of Science, Hiroshima University, Japan in the period from April 2004 to March 2007.

Cloning of the cytochrome P-450nor gene into the plant transformation vector: *C. tonkinense* cytochrome P-450nor2 (Cnor2) cDNA has an open reading frame coding for 408 amino acid residues and lacks the putative signal peptide (Kudo *et al*., 1996). The gene was originally cloned in pET17b vector. The cDNA was amplified by polymerase chain reaction (PCR) to generate *Bam*HI and *Sna*BI sites at the 5′ and 3′ ends, respectively, by using the following primers: *Cnor2FBam*HI, 5′-aggatccccgggtggtcagtcc-cttatgcacgctaccgaagac-3′ and Cnor2R*Sna*BI, 5′-ctacgtatcagtcagcatctaccaccagcttgctcagc-3′. The PCR products were cloned into the *Bam*HI and *Sna*BI sites of the vector pB1221 (Jefferson *et al*., 1987) to generate pBCnor2 and transformed into *Escherichia coli* strain DH5α. Recombinant pBCnor2 plasmids were purified from the *E. coli* and digested with *Xba*I and *San*BI restriction enzymes to excise the Cnor2 gene. The gene then inserted into the sites of the same restriction enzymes of the binary vector, pIG121-Hm, under the CaMV 35S promoter to generate pIGCnor2 plasmid. The binary vector also contains *npt* II and *hpt* genes for kanamycin and hygromycin selection of transgenic plant cells. The plasmid pIGCnor2 was transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw transformation method (Chen *et al*., 1994).

Generation of transgenic tobacco BY-2 cells and molecular analysis of the transgenic cells: BY-2 cells were maintained in LS medium (pH 5.6) (Linsmaier and Skoog, 1965). The cells were transformed by the *Agrobacterium*-mediated method (Yoshioka *et al*., 1996). Fresh culture of BY-2 cells (4 mL) in Petri dishes was co-cultured with 100 μL of the recombinant agrobacteria ($OD_{660} \approx 1\ mL^{-1}$) in presence of 100 μM acetosyringone (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and mixed. The dishes were sealed with parafilm and incubated vertically for 24 h at 25°C. Then the co-culture was dispensed by pipette into 50 mL sterile tubes and washed 4 times with LS medium. The tubes were centrifuged at 1000 rpm for 1 min and the supernatant was decanted after each washing step. Cells were suspended to about 0.1 mL packed cell volume/mL culture. One mL of the suspension was transferred on LS medium solidified with 0.8% agarose L03 (TaKaRa Bio Inc., Japan) and 0.1%

$MgSO_4 \cdot 7H_2O$. The solid LS medium contains 200 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin for selection. Transformation and selection processes were done regularly in three-week intervals. Independent kanamycin-resistant cell colonies were used for molecular analyses.

The presence of the Cnor2 gene in transgenic cells was confirmed by PCR using genomic DNA isolated with the DNeasy Plant Mini Kit (QIAGEN) and the gene-specific primers (Cnor286.FOR, 5′-gatgctcccgcacacatg-3′ and Cnor1020.REV, 5′-acggtgcatgtcgaacac-3′) yielding the target product of 734 bp. Furthermore, the Cnor2 gene sequence integrity in the transformed cells was confirmed by sequencing.

Suspension culture conditions: Transgenic BY-2 cell colonies were individually picked from the selection dishes and cultured in 300 mL conical flasks containing 100 mL LS medium (pH 5.6) at a constant temperature of 25°C on a rotary shaker (130 rpm). The medium for transgenic cells was supplemented with the selection antibiotics as mentioned above and the cell suspension cultures were sub-cultured every two weeks in newly prepared LS medium.

Cell suspension cultures for gas phase N_2O measurements: Two-weeks and one-week old, respectively, transgenic and wild-type BY-2 cell suspension cultures were harvested by brief centrifugation (1,000 rpm for 2 min) to remove the excess medium and resuspended in 50 mL LS medium containing either 20 mM $^{15}NO_3^-$ (51.1 atom % ^{15}N) or 40 mM $^{15}NH_4^+$ (99.4 atom % ^{15}N) in 300 mL conical flasks and tightly covered with rubber stoppers. The internal gas was purged with argon and the cell cultures were maintained as mentioned above for 5 days after which the evolved gas was analyzed by gas chromatography-mass spectrometry (GC-MS) equipped with a preparation and concentration (PreCon) apparatus as described previously (Goshima *et al*., 1999) with some modifications. Gas sample (50 mL) was taken with a gas-tight syringe connected to a plastic hose and a needle and placed into the PreCon gas sampling bottle (120 mL volume). The remaining volume of the sampling bottle was filled up with helium and subjected to analysis by the GC-MS machine. Estimates of the quantity of ^{15}N-labelled N_2O emitted were calculated from the results of GC-MS. To assess the effect of tungstate and cyanide on N_2O production, wild-type and Cnor2 transformed BY-2 cells were maintained in LS medium containing 20 mM $K^{15}NO_3$ (51.1 atom % ^{15}N) supplemented with either 0.5 mM sodium tungstate or 2 mM KCN. For ammonium effect evaluation, cells were cultured in a medium containing 40 mM $^{15}NH_4Cl$ (99.4 atom% ^{15}N) as a sole nitrogen source. The internal gas was purged with argon and the cell suspensions were cultured for 3 days (+KCN) and 5 days for the other treatments and the gas phase was examined as detailed above.

***In vitro* nitric oxide reductase assay:** Electron donor consumption method detailed in refs (Shiro *et al*., 1995; Kaya *et al*., 2004) was applied to measure NO reductase activity. Cells were frozen in liquid nitrogen, ground into fine powder and then suspended in 50 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol and 0.3 mM phenylmethylsulfonyl fluoride. The suspension was centrifuged at 14,000 rpm for 25 min to obtain the cell-free extract. The concentration of proteins in the extract was determined using the dye-binding assay (Bio-Rad Protein Assay; Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin (BSA; Sigma Chemicals, St. Louis, MO, USA) as standard.

NO reductase specific activity was measured by monitoring the absorption decrease at 340 nm derived from NAD(P)H (Shiro *et al*., 1995; Kaya *et al*., 2004). The molar absorptivity of NAD(P)H is 6.22 mM^{-1} cm^{-1} at 340 nm. N_2/NO gas and the NO donor, 3-(2-Hydroxy-1- (1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC 5) (Dojindo, Kumamoto, Japan), were used as substrates for the assay. NOC 5 was dissolved in 0.1 M NaOH solution, because it is relatively stable in alkaline solution. An NO release can begin from the point of addition of the stock solution to the sample solution. A mixture of enzyme solution, 50 mM sodium phosphate buffer (pH 7.2) and 0.16 mM NAD(P)H in 1 mL cuvette, fitted with rubber stopper designed for serum bottles, was anaerobically incubated at 25°C. After injection of the NO donor solution (0.3 mM), the absorbance decrease at 340 nm was recorded at 25°C using a UV/Vis light spectrophotometer (BioSpec-1600; Shimidzu, Kyoto, Japan). Alternatively, a suitable volume of the assay buffer and 0.16 mM NAD(P)H was placed in the cuvette and purged with N_2/NO gas for saturation with NO. The reaction was initiated by injecting the enzyme to a final volume of 1 mL and the absorbance decrease was recorded as above.

RESULTS AND DISCUSSION

Cnor2 is involved in N_2O production by tobacco BY-2 cell suspension: Denitrification activity, the process of anaerobic respiration, was considered to be characteristic of prokaryotes until Shoun and Tanimoto (1991) and Shoun *et al*. (1992) found that the fungi *Fusarium oxysporum* and *C. tonkinense* could undertake denitrification to form nitrous oxide (N_2O) from nitrate or nitrite. The most distinctive characteristic of the fungal

denitrification pathway is the involvement of cytochrome P-450 (P-450nor), which acts as nitric oxide reductase (nor). Both denitrifying systems of *F. oxysporum* and *C. tonkinense* contain two isoforms of P-450nor. At least one isoform of the two systems is localized in the mitochondria and is coupled with the synthesis of ATP (Kobayashi *et al*., 1996).

We have been investigating the potential denitrification pathway in plants for several years (Goshima *et al*., 1999; Hakata *et al*., 2003) to understand the plants' phenomenon of emitting some nitrogen oxides that are mainly the intermediate products of the denitrification pathways of prokaryotes and fungi. In the same context, we have discovered that 17 wild-type plant species cultured aseptically and fed with ^{15}N-labelled NO_3^- emitted various amounts of $^{15}N_2O$. The result indicates that plants are potentially capable to convert NO_3^- to N_2O with huge variation in potentiality among plant species (Hakata *et al*., 2003). Moreover, we found that transgenic tobacco plant expressing antisense nitrite reductase (NiR) mRNA (clone 271), in which the constitutive nitrite reductase was extremely suppressed, emits a considerable amount of N_2O. Yet, it is not clear whether this N_2O production is catalyzed enzymatically (Goshima *et al*., 1999). Thus, we used a eukaryotic gene to study the denitrification pathway in plant cells. Notably, *C. tonkinense* P-450nor2 (Cnor2) gene, which catalyzes the reduction of NO to N_2O in the fungus, was overexpressed in tobacco BY-2 cells under the control of the CaMV 35S promoter (Fig. 1a). Colonies of

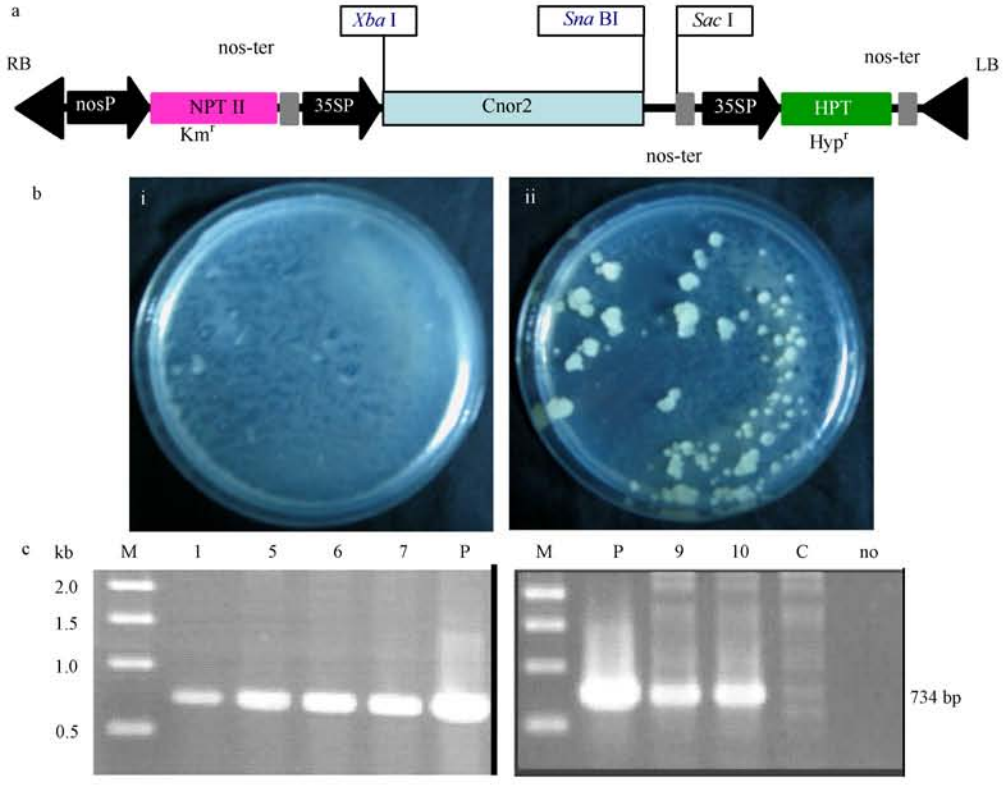

Fig. 1: Transformation of BY-2 cells by Cnor2 gene. (a) T-DNA region of the construct used for transformation. nosP, nopaline synthase promoter; NPT II, neomycin phosphotransferase type II gene; Km^r, kanamycin resistance; 35SP, CaMV 35S promoter; HPT, hygromycin phosphotransferase gene; Hyg^r, hygromycin resistance; nos-ter, nopaline synthase terminator. (b) A typical photograph of two-weeks old (i) wild-type and (ii) Cnor2 transgenic BY-2 cells plated on LS medium containing 200 mg L^{-1} kanamycin and maintained at 25°C. Kanamycin resistant cell colonies grows well on Cnor2 transgenic plate, whereas in the control plate cells did not survive. (c) Integration of Cnor2 gene was confirmed by PCR. Amplification was carried out by gene specific primers for Cnor2 and the genomic DNA extracted from the respective BY-2 cell clones to produce a 734-bp fragment. M, marker; lanes 1, 5, 6, 7, 9 and 10, the template is the genetic DNA from transgenic BY-2:Cnor2 clones, respectively, (1), (5), (6), (7), (9) and (10); P, binary plasmid pIGCnor2; C, the template is the genomic DNA from wild-type BY-2 control cells; no, no template

Table 1: Amount of $^{15}N_2O$ and atom percentage of ^{15}N of N_2O in the gas phase of BY-2 cell suspension cultures

Genotype	Amount of $^{15}N_2O^a$ (ng g^{-1} FW $week^{-1}$)	Atom%^{15}N of N_2O^a
BY-2 WT	2.24±0.71	0.39±0.02
BY-2:Cnor2 (1)	11.14±3.59**	2.54±1.38*
BY-2:Cnor2 (5)	67.56±3.74***	3.90±1.44**
BY-2:Cnor2 (6)	79.26±4.95***	4.99±2.06**

The differences between the wild-type and transgenic cell clones in production of $^{15}N_2O$ gas were statistically significant (t-test, *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$). [a]: Values are means±SD of a minimum of three assays

transgenic cells were recovered using selection regimes of 200 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin (Fig. 1b) and the stable integration of the gene into the cells was confirmed by sequencing and also by PCR with the transgenic cells genomic DNA as template (Fig. 1c).

To examine *in vivo* activity of Cnor2 in tobacco cells, we cultured both transgenic and wild-type control cells in a medium containing ^{15}N-labelled KNO_3 and the gas phase was cryogenically trapped and analyzed by GC-MS. Transgenic cell clones produced $^{15}N_2O$ up to 35-folds more than their wild-type counterpart (Table 1). It is noteworthy that the N_2O values in Table 1 reflect only the ^{15}N-labelled $^{45}N_2O$ (isoforms of $^{15}N^{14}N^{16}O$ and $^{14}N^{15}N^{16}O$) fraction, but not the total N_2O that evolved by the cells. Thirty-five folds increase in the isotopic signature of some transgenic cell clones, compared to the wild-type cells, clearly indicate that the $^{15}N_2O$ is produced during the reduction of $^{15}NO_3^-$ that was taken up from the medium. Each transgenic cell clone is statistically reproducible in generating $^{15}N_2O$ compared to wild type; though there are variations among transgenic cell clones in emitting $^{15}N_2O$. These variations may be due to the co-suppression of the Cnor2 gene expression and/or due to allele silencing in the low $^{15}N_2O$ producing transgenic clones. Comparing this result with a previous report on $^{15}N_2O$ emissions from wild-type plant species (Hakata *et al*., 2003) indicates that Cnor2 transgenic BY-2 cell clones acquired a much higher capacity in N_2O production. *Hibiscus cannabinus*, the highest N_2O producer among the 17 wild-type plant species investigated, evolved 0.45±0.20 ng $^{15}N_2O$ g^{-1} fresh weight in a week (Hakata *et al*., 2003). This value is, respectively, 25 - and 176-folds less than that from the lowest and highest $^{15}N_2O$ producing transgenic tobacco cell clones investigated here (Table 1). It is likely that Cnor2 catalyzed the reduction of the intermediates of nitrate assimilation to generate N_2O in BY-2 cells in a similar manner to the fungal denitrification pathway (Kizawa *et al*., 1991; Usuda *et al*., 1995; Kudo *et al*., 1996; Kaya *et al*., 2004). Wheat leaves (Smart and Bloom, 2001), soybean and winged bean (Dean and Harper, 1986) were also reported to produce N_2O. Intact chloroplasts and NiR extracted from wheat produced N_2O, but nitrate reductase (NR) did not. This indicates that N_2O produced by the leaves occurred during photoassimilation of NO_2^- in the chloroplasts and NR is not directly catalyzing the enzymatic reduction to N_2O (Smart and Bloom, 2001). In soybean, N_2O production from NO_2^- was catalyzed by NAD(P)H-NR (a side reaction of NR) (Dean and Harper, 1986). Klepper (1987) concluded that NO_2^- is the predominant product which accumulates during the *in vivo* NR assays of soybean leaves and that further conversion of NO_2^-, whether by chemical or enzymatic reaction, produces NO. However, Dean and Harper (1986) showed that a soybean nr_1 mutant that lacks the constitutive NR enzyme did not produce N_2O. All these reports provided no evidence for direct involvement of NR in production of N_2O from NO and no evidence for the existence of a specific enzyme that reduces NO to N_2O in plant cells.

In this study, we used a non-photosynthetic tobacco cells and, therefore, N_2O production by the transgenic cells is not enhanced by photoassimilation. Recently, it was reported that tobacco cell suspensions (wild-type and NR-deficient) generate considerable quantities of NO with possible involvement of the mitochondrial electron transport system in the reduction of nitrite to NO (Planchet *et al*., 2005). Most probably the NO generated during this reaction acts as a substrate for nor. Since plants are sessile they have to cope with their surrounding environment by an efficient use of their cellular machinery. Therefore, they undergo diverse biological activities to detoxify xenobiotics and the secondary metabolites that are generated within the cells. NO is an important plant signaling molecule and its production must be tightly controlled. Excessive NO production within plant cells may require efficient systems to reduce it into some other products, namely N_2O and N_2. Possible candidates that enhance nitrate reduction steps ultimately to these gaseous forms are the denitrification enzymes from either bacteria or fungi (Zumft, 1997; Shoun *et al*., 1992).

***In vitro* activity of recombinant Cnor2 protein from BY-2 cells:** The activity of Cnor2 protein from transgenic BY-2 cells was further confirmed by *in vitro* enzymatic assay. Figure 2a and b show the result of enzymatic activity assayed with N_2/NO gas purging and the electron donors NADH (Fig. 2a) and NADPH (Fig. 2b). For further confirmation, the assay was done using NOC 5, an NO donor, as a substrate and NADH (Fig. 2c) and NADPH (Fig. 2d) as electron donors. The specific activities of proteins from representative transgenic clones were higher than those obtained from wild-type cells under the same experimental conditions. These results indicate that

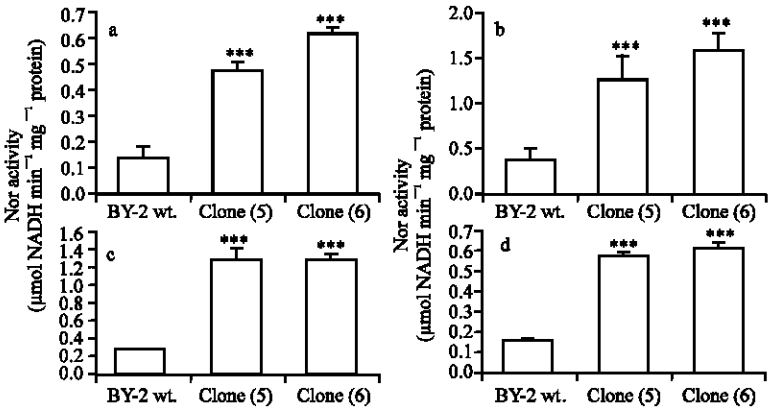

Fig. 2: Functional analysis of Cnor2 from BY-2 cells. Nitric oxide reductase (nor) activity was assayed anaerobically in 50 mM potassium phosphate buffer (pH 7.2) with N_2/NO gas purged into a 1 mL cuvette, fitted with rubber stopper and added (a) 0.16 mM NADH or (b) 0.16 mM NADPH as electron donors. Alternatively, NOC 5, an NO donor, was used as substrate for assays with either (c) 0.16 mM NADH or (d) 0.16 mM NADPH as electron donors. The reaction was started after the addition of soluble fractions (7-23 µg protein in 1 mL assay) from wild-type or transgenic BY-2 cells and incubated at 25°C and the decrease in the absorbance at 340 nm was recorded. Asterisks indicate very highly significant differences from the wild-type cells in nitric oxide reductase activity (t-test, ***$p < 0.001$)

Table 2: Effect of tungstate, cyanide and ammonium on $^{15}N_2O$ production

	Amount of $^{15}N_2O$ (ng g^{-1} FW $week^{-1}$)		
Genotype	$^{15}NO_3^- + [WO_4]^{2-a}$	$^{15}NO_3^- + KCN^a$	$^{15}NH_4^{+a}$
BY-2 WT	1.20±0.56	0.76±0.56	1.35±0.55
BY-2:Cnor2 (1)	1.54±0.94	1.49±0.55	1.54±0.36
BY-2:Cnor2 (5)	2.04±1.54	1.34±0.91	0.99±0.38
BY-2:Cnor2 (6)	3.07±1.25	1.62±0.27	1.27±0.22

[a]: Values are means±SD of a minimum of three independent assays

the recombinant enzyme from the transgenic cells is a functional nitric oxide reductase and support the observation of high N_2O production by the intact transgenic cells.

Effect of tungstate, cyanide and ammonium on N_2O production: Tungstate, the NR inhibitor, exerts a strong inhibition to N_2O production. In the presence of tungstate, $^{15}N_2O$ production from BY-2:Cnor2 clones (6), (5) and (1) was declined to 3.9, 3 and 13.8%, respectively, (Table 1, 2). This may support the notion that P-450Cnor2 is not directly involved in the respiratory electron transfer system (Usuda *et al*., 1995; Watsuji *et al*., 2003), but it requires NR and mostly NiR enzymes (Planchet *et al*., 2005) to generate NO, the substrate for nor.

Production of $^{15}N_2O$ from transgenic BY-2 cells cultured in a medium containing cyanide was declined dramatically (Table 2). Amounts of $^{15}N_2O$ from BY-2:Cnor2 clones (6), (5) and (1) were, respectively, 2, 2 and 13% of the values estimated when cells were cultured without a cyanide supplement (Table 1). Cell-free extracts assayed

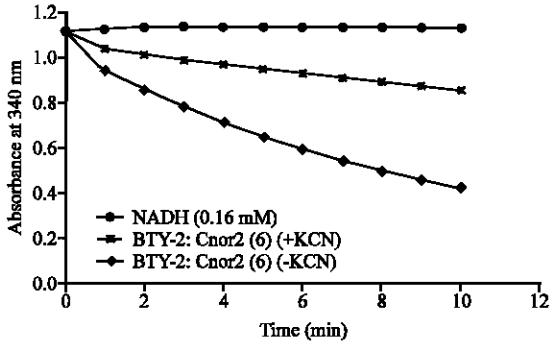

Fig. 3: Effect of cyanide on the activity of Cnor2. The assay was essentially done as described in Fig. (2) with 0.16 mM NADH as electron donor and NOC 5 as substrate in presence or absence of 5 mM KCN. A typical result from three independent experiments is shown

with NADH in the presence of cyanide (Fig. 3) showed a major decline in Cnor2 activity (retained activity about 68% in 5 min and 49% in 10 min). Cell-free extract assay results are slightly different from those reported from the assay of the authentic Cnor2 isolated from *C. tonkinense*, in which the respiratory inhibitors such as CO, cyanide, azide and antimycin A showed a negligible effect on the nitric oxide reductase activity (Usuda *et al*., 1995). While antimycin A inhibited the total denitrifying activity of intact cells but it did not inhibit the cell-free NiR and isolated Cnor2 activities (Usuda *et al*., 1995).

The presence of ammonium in addition to nitrate and the use of a fermentable sugar as an electron donor were key condition for inducing the denitrifying activity in *C. tonkinense* (Watsuji *et al*., 2003). When ammonium ($^{15}NH_4^+$) was used as a sole nitrogen source, the amount of $^{15}N_2O$ obtained from transgenic clones (6), (5) and (1) was estimated to be 1.6, 1.5 and 13.8% of the values from the same clones cultured in the presence of $^{15}NO_3^-$ containing medium (Table 1, 2). Ammonium as a sole nitrogen source for cell growth does not favor the denitrification pathway in plant cells (Table 2), instead, it may be involved in the nitrification process to generate nitrate (Hipkin *et al*., 2004), which in turn could be reduced by the authentic NR and NiR to generate NO, the substrate for Cnor2. To our knowledge, this is the first enzymatic evidence of functional analysis of a denitrification gene in plant cells. It is now clear that NR is a major player in the plant NO production game in addition to several other enzymes. NR-catalyzed reduction of nitrate or nitrite can produce large amounts of NO, or at least more than the amount needed for signaling (Meyer *et al*., 2005). Furthermore, NO production in plants can occur non-enzymatically (Bethke *et al*., 2004). But the process of N_2O production in plants is a matter of broad speculations. This study provides evidence that plant cells expressing fungal cytochrome P-450nor2 generate N_2O gas during the reduction of NO_3^- in a similar manner to the denitrification pathway in fungal systems. Further studies are necessary to address the process of N_2O reduction step to dinitrogen (N_2) by plant cells.

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