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Functional Analysis of *Fusarium oxysporum* Nitric Oxide Reductase Expressed in Plant Suspension-Cultured Cells

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Abstract: The biological function of Reactive Nitrogen Species (RNS) is not well understood, however, they actively contribute to the effect of green house gases. Development of plants that could efficiently denitrify intermediates of the RNS to the dinitrogen (N_2) is a rationale that could help amelioration the effect of these gases. *Fusarium oxysporum* cytochrome P-450 nor gene (Fnor) was constitutively expressed in tobacco BY-2 cells. The gene product functions as nitric oxide reductase (nor), which catalyzes the reduction of nitric oxide (NO) to nitrous oxide (N_2O) in the fungal denitrification pathway. Intact transgenic BY-2 cells cultured in ^{15}N-labeled nitrate ($^{15}NO_3^-$) actively produced $^{15}N_2O$ gas up to 59 folds higher than the wild-type cells. Activity of the enzyme was also confirmed by an *in vitro* nor activity assay. Tungstate (a nitrate reductase inhibitor) and cyanide (an inhibitor of the last protein complex of electron transport chain) strongly inhibited $^{15}N_2O$ production. These observations together suggest that Fnor 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, nitric oxide reductase, Fnor, tobacco BY-2 cells

INTRODUCTION

The chemical and physical state of the atmosphere is regulated by plants mostly through exchange of water, carbon dioxide and oxygen. However, plant leaves also emit a variety of nitrogen-based trace gases such as nitrogen dioxide (NO_2), nitric oxide (NO) and nitrous oxide (N_2O) (Dean and Harper, 1986; Smart and Bloom, 2001). Due to their reactive nature, nitrogenous gases are harmful to living organisms that are exposed to these free radicals (Wellburn, 1990) as well as being involved in climate change. Plants are able to sense the internal and external nitrogen status and adapt to the 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). Therefore, it is plausible to consider plants as sinks for atmospheric NO_2 through foliar uptake and subsequent assimilation into amino acids after spontaneous break down of NO_2 into inert nitrate and nitrite (Lerdau *et al*., 2000). Although, the above notion is still controversial, nonetheless, evidence is now accumulating that exogenously supplied NO_2 as well as the endogenously produced nitrogen oxides function as vital plant signals (Delledonne *et al*., 1998; Wendehenne *et al*., 2001; Neill *et al*., 2003; Morikawa *et al*., 2004, 2005; Sakamoto *et al*., 2004; Takahashi *et al*., 2005, 2006; Adam *et al*., 2008a, b).

Anthropogenic Reactive Nitrogen Species (RNS) are increasing dramatically worldwide and mostly accumulating in the atmosphere. Plants were also reported to emit considerable amount of N_2O with no evidence for the involvement of any bacterial or fungal denitrification enzyme in N_2O emission (Goshima *et al*., 1999; Smart and Bloom, 2001; Hakata *et al*., 2003). An increasing demand for food and energy supplies and the lack of effective measures for prevention of RNS emission from fossil-fuel combustion are the principal drivers behind the atmospheric nitrogen-enrichment (Zheng *et al*., 2002). One possible way to mitigate atmospheric RNS is through developing transgenic plants that have high capacity to

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serve as sinks for nitrate and RNS (Morikawa *et al*., 1998; Takahashi *et al*., 2001) or to reduce nitrate and the metabolic intermediates of RNS to the inert state. This approach could be achieved by manipulating plants with denitrifying genes that could enhance conversion of nitrate to N_2O or finally to dinitrogen (N_2). In an attempt to achieve this goal we transformed a cytochrome P-450 nitric oxide reductase (P-450 nor) from *Cylindrocarpon tonkinense* (Cnor2) into tobacco BY-2 cells (Abdel-Banat *et al*., 2008) since there was no denitrification gene of plant origin has been identified. The Cnor2 enzyme accepts two electrons directly from either NADH or NADPH during reduction of NO to N_2O.

Another, fungal P-450 nor that was involved in the reduction of NO to N_2O was characterized from the fungus *Fusarium oxysporum* (Shoun and Tanimoto, 1991; Nakahara *et al*., 1993). The *F. oxysporum* P-450 nor enzyme (Fnor) did not require a flavoprotein for electron transfer to the heme, but it used NADH directly for the reduction reaction according to the sum equation (Averill, 1996; Daiber *et al*., 2005; Zhang and Shoun, 2008):

$$2NO + NADH + H^+ \rightarrow N_2O + NAD^+ + H_2O$$

There was no little doubt that this NO reductase (Kizawa *et al*., 1991), participated in the reduction of nitrate/nitrite leading to N_2O, since P450nor could be induced by nitrate and nitrite (Shoun *et al*., 1989), thus completing the nitrate reduction pathway where nar, nir and nor stand for nitrate, nitrite and nitric oxide reductase, respectively (Daiber *et al*., 2005):

$$NO_3^- \xrightarrow{nar} NO_2^- \xrightarrow{nir} {}^{\bullet}NO \xrightarrow{nor} N_2O$$

Here, we report the expression and functional analysis of Fnor in plant suspension culture. Unlike Cnor2, Fnor is absolutely specific for NADH, thus we were interested to examine its function in plant cells and to know how it differ from Cnor2. The expression of Fnor in *Nicotiana tabacum* BY-2 cells significantly enhanced the conversion of the ^{15}N-labeled ^{15}NO, which was the intermediate of assimilation of the ^{15}N-labeled nitrate ($^{15}NO_3^-$), to N_2O as evidenced by the detection of a large quantity of ^{15}N-labeled N_2O. The result further confirms the success of engineering fungal denitrifying pathway in plant cells.

MATERIALS AND METHODS

Cloning of P-450 nor into plant transformation vectors: *Fusarium oxysporum* P-450 nor (Fnor) cDNA has an open reading frame coding for 403 amino acid residues and lacks the putative signal peptide (Kizawa *et al*., 1991). The gene was originally cloned in pET17b vector and received as a generous gift from Professor Hirofumi Shoun at the Department of Biotechnology, University of Tokyo. The cDNA was amplified by Polymerase Chain Reaction (PCR) to generate *Xba*I and *Sac*I sites at the 5' and 3' ends, respectively, by using the following primers: FnorXba, 5'-ctctagaggatccccgggtggtcagtcccttatggcttctggtgctc-3' and FnorRSacI, 5'-cgagctcttaaaaaatgacaggaagatccacgatt ccgacatctcg-3'. The PCR products were cloned into the *Xba*I and *Sac*I sites of the binary vectors pIG121-Hm (Ohta *et al*., 1990) and pE7113-GUS (Mitsuhara *et al*., 1996) to generate pIG-Fnor2 and pE7113-Fnor, respectively. Fnor expression in the binary vector pIG-Fnor, was under the control of CaMV 35S promoter, while the pE7113-Fnor contains in addition to the CaMV 35S promoter enhancer sequences for the promoter. The binary vector pIG-Fnor also contains *npt* II and *hpt* genes for kanamycin and hygromycin selection of transgenic plant cells. However, the pE7113-Fnor contains only *npt* II gene for kanamycin resistance selection. The plasmids pIG-Fnor and pE7113-Fnor were transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw transformation method (Chen *et al*., 1994).

Molecular analysis of the transgenic BY-2 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 milliliter of the suspension was transferred on LS medium solidified with 0.8% agarose L03 (TaKaRa Bio Inc., Japan) and 0.1% $MgSO_4.7H_2O$. 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 3 week intervals. Independent kanamycin-resistant cell colonies were used for molecular analysis.

The presence of the Fnor gene in transgenic cells was confirmed by PCR using genomic DNA isolated with the DNeasy Plant Mini Kit (QIAGEN) and the gene-specific primers (Fnor286-FOR, 5'-catcagaggagcatggtg-3' and Fnor914-REV, 5'-accagcttgtcaccgatc-3') yielding the target

product of 629 bp. Furthermore, the Fnor2 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 controlled 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 2 weeks in newly prepared LS medium.

Cell suspension cultures for gas phase N_2O measurements: Two weeks and 1 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 20 mM $^{15}NO_3^-$ (51.1 atom % ^{15}N) in 300 mL conical flasks and the flasks were tightly covered with rubber stoppers. The internal gas was purged with argon and the cell cultures were maintained as mentioned above for 5 days. Then the evolved gas was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS) equipped with a preparation and concentration (PreCon) apparatus as described earlier (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-labeled N_2O emitted were calculated from the results of GC-MS.

***In vitro* nitric oxide reductase assay:** An electron donor consumption method, detailed elsewhere (Shiro *et al*., 1995; Kaya *et al*., 2004) was used to measure the Fnor 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 NADH (Shiro *et al*., 1995; Kaya *et al*., 2004). The molar absorptivity of NADH is 6.22 mM^{-1} cm^{-1} at 340 nm. An NO donor, 3-(2-Hydroxy-1-(1-methylethyl)-2-nitrosohydrazino)-1-propanamine (NOC 5) (Dojindo, Kumamoto, Japan), was used as substrate 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 NADH was prepared in a 1 mL cuvette fitted with rubber stopper designed for serum bottles and the mixture was anaerobically incubated at 25°C. After injection of the NO donor solution (0.3 mM), the absorbance decrease at 340 nm was recorded using a UV/Vis light spectrophotometer (BioSpec-1600; Shimidzu, Kyoto, Japan).

RESULTS AND DISCUSSION

Fnor is involved in N_2O production by tobacco BY-2 cell suspension: Potential denitrification pathway in plants has been addressed to understand the phenomenon of nitrogen oxides emission by plants (Goshima *et al*., 1999; Hakata *et al*., 2003). The emitted nitrogen oxides are mainly the intermediate products of the denitrification pathways of prokaryotes and fungi. Plants are potentially capable to convert NO_3^- to N_2O (Hakata *et al*., 2003), though they do not inherently encode functional nitric oxide reductase (nor). Thus, we used eukaryotic genes encoding nitric oxide reductase to study the denitrification pathway in plant cells.

Earlier, we have demonstrated that *C. tonkinense* P-450nor2 (Cnor2) gene product, which accepts two electrons directly from either NADH or NADPH, could catalyze the reduction of NO to N_2O in tobacco BY-2 cells (Abdel-Banat *et al*., 2008). Here, we show the expression of *F. oxysporum* Fnor gene in tobacco BY-2 cells. Fnor is absolutely specific for NADH showing little or no activity with NADPH (Averill, 1996). The gene was cloned in two expression vectors under the control of CaMV promoter (Fig. 1a). Transgenic cell colonies were recovered using either selection regime of 200 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin for the integrant plasmid pIG-Fnor or 200 mg L^{-1} kanamycin for the integrant plasmid pE7113-Fnor. Representative transgenic colonies together with a control plate were shown in Fig. 1b. 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 Fnor in tobacco cells, we cultured both transgenic and wild-type control cells in

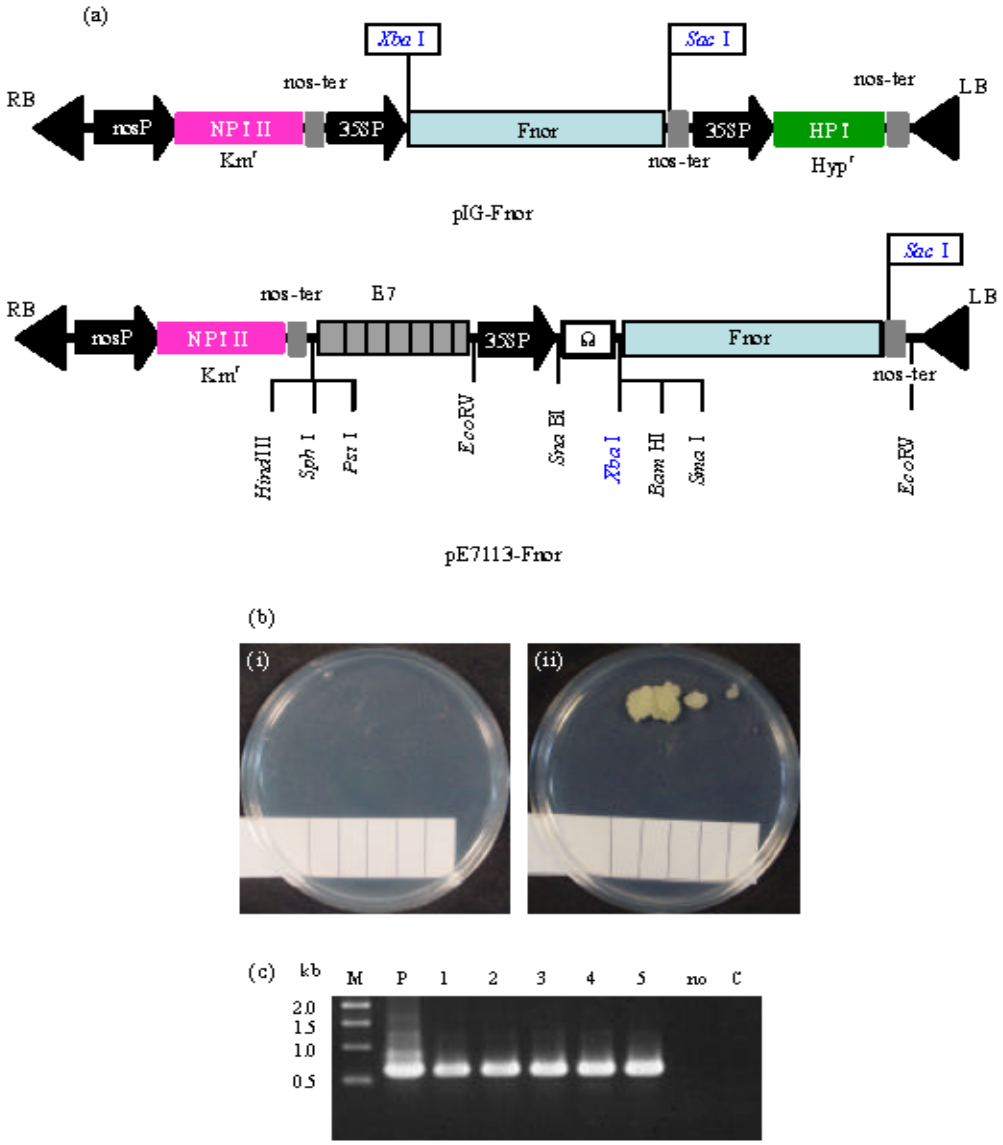

Fig. 1: Transformation of BY-2 cells by Fnor gene (a) T-DNA region of the pIG-Fnor and pE7113-Fnor constructs used for transformation. nosP, nopaline synthase promoter; NPT II: Neomycin phosphotransferase type II gene; Km^R: Kanamycin resistance ; HPT: Hygromycin phosphotransferase gene; Hyg^R: Hygromycin resistance; nos-ter: nopaline synthase terminator; 35SP: 5-upstream sequence of CaMV 35S promoter (-90 to -1); E7, enhancer sequence from the 5-upstream sequence of CaMV 35S promoter (-940 to -290) and (-290 to -90) × 7; Ω: 5'-utranslated sequence of Tomato Mosaic Virus (TMV), (b) A typical photograph of two-weeks old (i) wild-type and (ii) Fnor transgenic BY-2 cells plated on LS medium containing 200 mg L^{-1} kanamycin and incubated at 25°C. Kanamycin resistant cell colonies grows well on the plate transformd with an Fnor harbouring plasmid, whereas in the control plate cells do not survive and (c) Integration of Fnor gene was confirmed by PCR. Amplification was done by using Fnor gene specific primers and the genomic DNA extracted from the respective BY-2 cell clones to produce a 629 bp fragment. M: Marker; Lanes 1~5: Templates are the genomic DNA from the transgenic clones, respectively, BY-2:pE7113.Fnor (1), BY-2: pE7113.Fnor (3), BY-2:pE7113.Fnor (4), BY-2:pIG.Fnor (1) and BY-2:pIG.Fnor (2); P, binary plasmid pIGCnor2; C: The template is the genomic DNA from the wild-type BY-2 cells; no: No template

Table 1: Amount of $^{15}N_2O$ and atom percentage of ^{15}N of N Ω in the gas phase of BY-2 cell suspension cultures fed with ^{15}N-labeled KNO_3

Genotypes	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	0.385±0.022
BY-2:pE7113.Fnor (1)	51.39±22.93*	10.074±3.036**
BY-2:pE7113.Fnor (3)	110.27±67.50*	7.469±4.254*
BY-2:pE7113.Fnor (4)	131.00±20.83***	8.354±1.795***
BY-2:pIG.Fnor (1)	91.70±7.07***	6.378±2.774*
BY-2:pIG.Fnor (2)	76.18±27.78*	6.803±3.053*

Abdel-Banat *et al.* (2008). Production of $^{15}N_2O$ gas by the transgenic BY-2 cell clones was statistically significant compared to the Wild-Type (WT) (t-test, *p<0.05; **p<0.01; ***p<0.001). [a]Values are expressed as Mean±SD of a minimum of three independent assays

Table 2: Influence of tungstate and cyanide on $^{15}N_2O$ production by transgenic BY-2 cells expressing the Fnor gene

Genotypes	Atom % ^{15}N of N_2O (ng g^{-1} FW $week^{-1}$)	
	$^{15}NO_3^- + [WO_4]^{2-a}$	$^{15}NO_3^- + KCN^a$
BY-2 WT	0.369±0.006	0.367±0.006
BY-2:pE7113.Fnor (1)	1.366±0.175	0.612±0.273
BY-2:pE7113.Fnor (3)	0.576±0.111	0.694±0.311
BY-2:pE7113.Fnor (4)	1.914±0.355	0.746±0.319
BY-2:pIG.Fnor (1)	0.802±0.263	0.645±0.308
BY-2:pIG.Fnor (2)	0.637±0.251	0.627±0.242

Abdel-Banat *et al.* (2008). [a]Values are expressed as Mean±SD of a minimum of three independent assays

a medium containing ^{15}N-labeled KNO_3 and the gas phase was cryogenically trapped and analyzed by GC-MS. Depending on the transgenic cell clones being investigated, $^{15}N_2O$ production in transgenic cells was 23 to 59 folds higher than in wild-type counterpart (Table 1). Apparently the enhancer sequences in the plasmid pE7113-Fnor (the 5'-upstream sequences of the CaMV promoter and the 5'-untranslated sequence also called Ω sequence of tobacco mosaic virus) (Mitsuhara *et al*., 1996) made the Fnor gene product more active. It is noteworthy that the N_2O values in Table 1 reflect only the ^{15}N-labeled $^{45}[N_2O]$ (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. Fifty nine folds increase in the isotopic signature of some transgenic cell clones compared to the wild-type cells clarified that the ^{15}N-labeled N_2O was produced during the reduction of $^{15}NO_3^-$ that was taken up from the medium. There was no other source for the ^{15}N-labeled nitrogen. Each transgenic cell clone generates a statistically reproducible $^{15}N_2O$ compared to the wild-type. Fnor 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; 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 nitrite reductase (nir) extracted from wheat produced N_2O, but nitrate reductase (nar) did not. This indicates that N_2O produced by the leaves occurred during photo assimilation of NO_2^- in the chloroplasts, but nar does not directly catalyze the enzymatic reduction to N_2O (Smart and Bloom, 2001). In soybean, N_2O production from NO_2^- was catalyzed by NAD(P)H-nar (a side reaction of nar) (Dean and Harper, 1986). Klepper (1987) concluded that NO_2^- is the predominant product, which accumulates during the *in vivo* nar 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 nar_1 mutant that lacks the constitutive nar enzyme did not produce N_2O. All these reports provide no evidence for direct involvement of nar in production of N_2O from NO.

Here, we used a non-photosynthetic tobacco cells and, therefore N_2O production by the transgenic cells is not enhanced by photo assimilation. Recently, it was reported that tobacco cell suspensions (wild-type and nar-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. NO is an essential plant signaling molecule for disease resistance (Delledonne *et al*., 1998) but its production must be tightly controlled. Excessive NO production within plant cells require efficient systems to reduce NO into less harmful products through biological activities of plants xenobiotics and secondary metabolites detoxification machineries. Possible candidates that enhance nitrate reduction steps ultimately to the inert gaseous forms are the denitrification enzymes from either bacteria or fungi (Zumft, 1997; Shoun *et al*., 1992).

***In vitro* activity of recombinant Fnor protein from BY-2 cells:** The activity of Fnor protein from transgenic BY-2 cells was further confirmed by *in vitro* enzymatic assay. Enzymatic activity was assayed with an NO donor, NOC 5 and NADH as electron donor (Fig. 2a, b). The specific activities of proteins from representative transgenic clones were higher than those obtained from wild-type cells under the same experimental conditions. This result indicates that the recombinant enzyme from the transgenic cells is functional nitric oxide reductase and supports the observation of high $^{15}N_2O$ production by the intact transgenic cells (Table 1). This is in a general agreement with the findings reported on the assay of Cnor2 expression in BY-2 cells (Abdel-Banat *et al*., 2008).

Effect of tungstate, cyanide and ammonium on N_2O production: Tungstate is a known nitrate reductase (nar) inhibitor. It exerts a strong inhibition of N_2O production. In the presence of tungstate, $^{15}N_2O$ production from the transgenic clones BY-2:pE7113-Fnor (1), BY-2:pE7113-Fnor (3), BY-2:pE7113-Fnor (4), BY-2:pIG-Fnor (1) and BY-2:pIG-Fnor (2) was declined to about 2.7, 0.5, 1.5, 0.9 and 0.8%, respectively, (Table 1, 2). This may support the

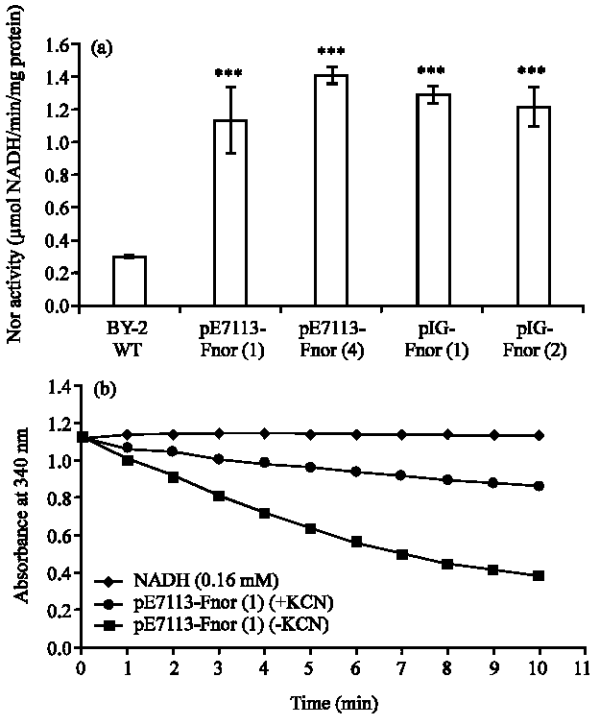

Fig. 2: Functional characterization of Fnor from transgenic BY-2 cells (a) Fnor activity measurement. The activity was assayed anaerobically in 50 mM potassium phosphate buffer (pH 7.2) with an NO donor, NOC 5, as a substrate and 0.16 mM NADH as an electron donor. The reaction was started after the addition of soluble fractions (7-23 µg protein in 1 mL assay) from wild-type or transgenic BY-2. The reaction mixture in 1 mL cuvette was anaerobically incubated at 25°C and the decrease in absorbance at 340 nm was recorded. The Fnor proteins used in the assays were referred to by their corresponding plasmids names that depicted on the graphs axis. Asterisks indicate very highly significant differences from the wild-type cells in nitric oxide reductase activity (t-test, ***$p<0.001$) and (b) cyanide inhibits Fnor activity. The assay was essentially done as described in (a) with 0.16 mM NADH as an electron donor and NOC 5 as a substrate in presence or absence of 5 mM KCN

notion that Fnor2 is not directly involved in the respiratory electron transfer system, but it requires nar and nir enzymes (Planchet *et al*., 2005) that finally 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 intact cell clones BY-2:pE7113-Fnor (1), BY-2:pE7113-Fnor (3), BY-2:pE7113-Fnor (4), BY-2:pIG-Fnor (1) and BY-2:pIG-Fnor (2) were, respectively, 1.19, 0.63, 0.57, 0.70 and 0.82% of the values estimated when cells were cultured without a cyanide supplement (Table 1). Cell-free extracts assayed with NADH in the presence of cyanide showed a major decline in Fnor activity. This result also generally agrees with the one done using the recombinant Cnor2 cell-free extract prepared from BY-2 cells (Abdel-Banat *et al*., 2008).

It is now clear that nar is a major player in the plant NO production game in addition to several other enzymes. The nar-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 strongly suggested that plant cells expressing fungal cytochrome P-450 nor generate N_2O gas during the reduction of NO_3^- in a similar manner to the denitrification pathway in fungal systems.

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