Characteristics of *Nicotiana tabacum* nitrate reductase protein produced in *Saccharomyces cerevisiae*

Hoai-Nam TRUONG,* Christian MEYER and Françoise DANIEL-VEDELE

Laboratoire de Biologie Cellulaire, I.N.R.A.–Versailles, Route de Saint-Cyr, 78026 Versailles Cedex, France

Tobacco nitrate reductase (NR) produced in yeast retains cytochrome *c* reductase activity, but not NR activity. Biochemical data suggest that the haem and FAD domains are functional, and that the molybdenum cofactor (MoCo) domain is inactive owing to the absence of MoCo in yeast. The native form of the produced NR is dimeric. Thus MoCo is not involved in NR dimerization in higher plants, contrary to current assumptions.

INTRODUCTION

Tobacco nitrate reductase (NR) (EC 1.6.6.1) is a homodimeric enzyme that reduces nitrate to nitrite in the presence of NADH (NADH:NR activity). Each of its subunits contains three domains carrying respectively the prosthetic groups FAD, haem (cytochrome *b*-557) and molybdenum cofactor (MoCo). Nitrate reduction involves electron transport successively through these three domains from NADH to nitrate (Solomonson & Barber, 1990).

In addition to its physiological NADH:NR activity, NR possesses partial catalytic activities *in vitro* such as (a) dehydrogenase activities, e.g. cytochrome *c* reductase activity, where electrons are transferred from NADH to an artificial electron acceptor, and (b) terminal activities, where an artificial electron donor (such as reduced methyl viologen) serves as reductant for nitrate.

These partial catalytic activities *in vitro* involve different domains of NR, for instance (a) haem and FAD domains for NADH:cytochrome *c* reductase activity, and (b) MoCo and eventually the haem domain for terminal activities (Kubo *et al.*, 1988; Chérel *et al.*, 1990).

The flavin domain of maize leaf NADH:NR has been expressed in *Escherichia coli* and was shown to be catalytically active (Hyde & Campbell, 1990), but to date there has been no report of plant NR produced in micro-organisms. In the present paper we report the expression of the cDNA of the *nia2* gene of tobacco (Vaucheret *et al.*, 1989) in *Saccharomyces cerevisiae*: no NADH:NR activity is recovered, but an NR-associated partial catalytic activity (NADH:cytochrome *c* reductase activity) is detected, implying that the haem and FAD domains are functional. NADH:NR activity can be restored by exogenous MoCo. It can also be restored by complementation with extracts from *Nicotiana plumbaginifolia nia* mutant D51 (Chérel *et al.*, 1990), which produces an NR-protein defective in NADH:cytochrome *c* reductase activity but not in NR-associated terminal catalytic activities. Finally it is shown that NR produced in yeast is dimeric.

MATERIALS AND METHODS

Plasmid constructs

The yeast expression vector pYoupi (Fig. 1) was derived from the *S. cerevisiae* galactose-inducible expression vector pKV482 (Delta Biotechnology, Nottingham, U.K.) by inserting a 2.8 kb *Sst*I–*Pst*I fragment of the *nia2* cDNA into the unique *Bgl*II site

of pKV482 made blunt by repair by the Klenow fragment of *E. coli* DNA polymerase I. The cDNA fragment was made blunt by T4 polymerase and includes the whole NR coding sequence as well as the 138-nucleotide-residue long 5′ untranslated region and 20 nucleotide residues of the 3′ untranslated region of the cDNA. In the resulting expression vector, expression of the cDNA is under control of a modified phosphoglycerate kinase promoter in which the normal upstream activating sequence has been replaced by the *gal4*-dependent upstream activating sequence, leading to a galactose-inducible production of NR. This nitrate reductase expression vector was maintained in yeast as a multicopy autonomously replicating plasmid.

Yeast transformation

S. cerevisiae was transformed by using the lithium acetate method as described by Ito *et al.* (1983).

Strains and culture conditions

S. cerevisiae strain (*ura3-52, tryp1-4, a*) was used as recipient strain for transformation.

Fig. 1. Expression vector pYoupi of the nitrate reductase *nia2* cDNA of *Nicotiana tabacum*

For details see the Materials and methods section. P_{PGK}, modified phosphoglycerate kinase promoter; t_{PGK}, phosphoglycerate kinase terminator; UAS_{gal}, *gal4*-dependent upstream activating sequence. The plasmid is not drawn to scale.

Abbreviations used: NR, nitrate reductase; MoCo, molybdenum cofactor.

* To whom correspondence should be addressed.

S. cerevisiae transformants were grown at 28 °C to A_{600} equal to 1–1.3 (exponential phase) in YNB glucose [7% (w/v) Difco yeast nitrogen base without amino acids plus 2% (w/v) glucose] supplemented with 50 mg of tryptophan/l.

Cells were then pelleted and stored at –80 °C when prepared for the *nit1* assay, or resuspended in YNB galactose [7% (w/v) Difco yeast nitrogen base without amino acids plus 2% (w/v) galactose] supplemented with tryptophan and cultivated further for 7–10 h when used for protein production. Yeast cells induced for NR expression were kept as frozen pellets at –80 °C until protein extraction.

For the *nit1* assay, *Hansenula wingei* wild-type strain (HS 28162) was grown in YM1 medium [0.17% (w/v) Difco yeast nitrogen base without amino acids or $(\text{NH}_4)_2\text{SO}_4$, plus 1% (w/v) glucose] supplemented with KNO_3 (4 mM) to A_{600} equal to 1, and *S. cerevisiae* transformants were grown in YM1 medium supplemented with KNO_3 (10 mM) and proline (40 mM) or in YNB glucose medium supplemented with Na_2MoO_4 (1 μM) and 50 mg of tryptophan/l.

Neurospora crassa mutant *nit1* strain (no. 54) was obtained from the Fungal Genetics Stock Center (Humboldt State University Foundation, Arcata, CA, U.S.A.) and was cultivated by the procedure of Hageman & Rajagopalan (1986).

Protein extraction

***S. cerevisiae*.** The pellet of a 50 ml culture was directly lysed in 200 μl of buffer A [0.1 M-potassium phosphate buffer, pH 7.5, containing 10% (w/v) glycerol, 1 mM-EDTA, 1 μM - Na_2MoO_4 , 0.01 mM-FAD, 2 mM-2-mercaptoethanol and 1 mM-phenylmethanesulphonyl fluoride] by vortex-mixing in the presence of glass beads. After addition of an equal volume of buffer, the cell slurry was centrifuged at 10000 *g* for 15 min and the supernatant was collected for activity or protein assays. When pellets corresponding to a smaller culture volume were used for protein extraction, the supernatant was precipitated with an equal volume of saturated $(\text{NH}_4)_2\text{SO}_4$ solution. After 1 h precipitation, tubes were centrifuged at 10000 *g* for 1 h and the pellets were resuspended in buffer A at a final protein concentration of approx. 2–5 $\mu\text{g}/\mu\text{l}$. For complementation experiments with MoCo from milk xanthine oxidase, yeast cells were lysed in buffer B (0.1 M-potassium phosphate buffer, pH 7.5, containing 0.01 mM-FAD, 20 mM- Na_2MoO_4 , 3 mM-GSH, 1 mM-EDTA, 0.001 mM-leupeptin and 1 mM-phenylmethanesulphonyl fluoride) and, for the *nit1* assay, in buffer C (50 mM-potassium phosphate buffer, pH 7.5, containing 25 mM- Na_2MoO_4 , 5 mM-GSH and 0.5 mM-EDTA) in the presence of N_2 .

Plant protein extracts. Plant protein crude extracts were prepared as described previously (Chérel *et al.*, 1986). AMP-Sepharose-purified NR from *Nicotiana plumbaginifolia* was obtained as described previously (Moureaux *et al.*, 1989).

***Neurospora crassa nit1* extracts.** *Neurospora crassa nit1* extracts were prepared by the procedure of Mendel *et al.* (1981).

Protein determination. Protein concentration was determined by the assay method of Bradford (1976).

Immunochemical tests

Two-site e.l.i.s.a. were performed as described previously (Chérel *et al.*, 1985), with the mouse monoclonal antibody ZM 96(9)25 as coating reagent and a rabbit polyclonal antiserum as second antibody. Both the monoclonal antibody and the serum were raised against maize NR. AMP-Sepharose-purified *Nicotiana plumbaginifolia* NR serves as a standard for the determination of NR protein concentration.

SDS/PAGE (Laemmli, 1970) was performed with an acrylamide concentration of 7.5% for the separating gel. Pharmacia native gradient gels (PAA 4/30) were used for analysis

of native protein. Proteins were transferred to an Immobilon-P membrane (Millipore). The blot was allowed to react successively with the rabbit polyclonal serum directed against maize NR [or with the mouse monoclonal antibody ZM 96(9)25] and a Sigma Chemical Co. goat anti-(rabbit IgG) antibody-alkaline phosphatase conjugate [or a rabbit anti-(mouse IgG) antibody-alkaline phosphatase conjugate; Sigma Chemical Co.]. Immunostaining with bromo-4-chloroindol-3-yl phosphate and Nitro Blue Tetrazolium assay was performed according to manufacturer's recommendations (Millipore).

The molecular mass of the produced NR was determined by comparison with molecular-mass standards from Bio-Rad Laboratories, Amersham International or Pharmacia.

Complementation experiments and activity assays

MoCo preparation. MoCo was prepared by using a 90 s heat treatment at 70 °C of protein samples by the procedure of Mendel (1983). Milk xanthine oxidase (grade IV; 0.1 unit/mg, 38 mg/ml) was purchased from Sigma Chemical Co.

Complementation of yeast extracts with MoCo from milk xanthine oxidase. Crude protein extracts (50–400 μl) were mixed with 0.5 vol. of MoCo (or MoCo buffer for a negative control), flushed with N_2 and incubated at room temperature for 30–60 min before assay of NADH:NR activity. Results are the means for at least three independent experiments.

***nit1* assay.** The *nit1* assay was performed as described by Mendel (1983).

Mutant complementation experiments. $(\text{NH}_4)_2\text{SO}_4$ -precipitated yeast protein extracts were mixed with 0.3–1 vol. of $(\text{NH}_4)_2\text{SO}_4$ -precipitated plant NR mutant extracts, and incubated on ice for 2 h before NR activity assay.

Assay of NR activity. Assays of NADH:NR activity and partial NR-associated catalytic activities *in vitro* were performed as previously described (Chérel *et al.*, 1990).

RESULTS

The cDNA of the *nia2* gene of *Nicotiana tabacum* was cloned in the expression vector pKV482 (Fig. 1) and introduced into a *ura⁻* *S. cerevisiae* strain. Protein production was induced by galactose.

Protein analysis

Two-site e.l.i.s.a.s were done on protein extracts from *S. cerevisiae* transformants. Cross-reacting material represents about 0.1% of $(\text{NH}_4)_2\text{SO}_4$ -precipitated soluble proteins for yeasts by comparison with standard AMP-sepharose purified NR from *Nicotiana plumbaginifolia*.

Western-blot analysis (Fig. 2) after SDS/PAGE shows that a protein from *S. cerevisiae* transformants is recognized by the polyclonal serum directed against maize NR (Fig. 2, lane 4). This protein is detected only in protein extracts of recombinant yeasts after galactose induction, and has the predicted molecular mass (110 kDa).

Enzyme activity analysis

No NADH:NR activity was found in recombinant yeast protein extracts. Extracts were analysed for the NR-associated partial activities: no terminal activities (reduced methyl viologen:NR activity or Bromophenol Blue:NR activity) were detected. NADH:cytochrome *c* reductase activity was present in yeast transformants. This activity was induced by galactose (by a factor 10–15-fold), and inhibited by the monoclonal antibody ZM 96(9)25 directed against maize NR (see Table 1). In crude extracts from induced NR transformants the NADH:cytochrome *c* reductase activity was 700 nmol of cytochrome *c* reduced/min

Fig. 2. Western-blot analysis after SDS/PAGE of *S. cerevisiae* protein extracts with a polyclonal antiserum directed against maize NR

Lanes 1 and 2, proteins from yeast transformed by pKV482 (control), before (lane 1) and after (lane 2) galactose induction; lanes 3 and 4, proteins from yeast NR transformant before (lane 3) and after (lane 4) galactose induction; lane 5, proteins from yeast NR transformant after induction mixed with AMP-Sepharose-purified NR from *Nicotiana plumbaginifolia*; lane 6, AMP-Sepharose-purified NR from *Nicotiana plumbaginifolia*.

Table 1. NADH:cytochrome *c* reductase activity in $(\text{NH}_4)_2\text{SO}_4$ -precipitated *S. cerevisiae* protein extracts

The monoclonal antibody ZM 96(9)25 directed against maize NR was used for inhibition of cytochrome *c* reductase activity. Control: yeast strain transformed by the expression vector. NR transformant: yeast strain transformed by the expression vector containing the NR cDNA insert.

Yeast strain	Conditions	ZM 96-9-25 antibody...	NADH:cytochrome <i>c</i> reductase activity (nmol/min per mg of protein)	
			–	+
<i>S. cerevisiae</i> control	Non-induced		2	2
	Galactose-induced		12	12
<i>S. cerevisiae</i> NR transformant	Non-induced		100	5
	Galactose-induced		1500	40

per mg of protein [and 1500 nmol of cytochrome *c* reduced/min per mg of protein in the case of $(\text{NH}_4)_2\text{SO}_4$ -precipitated yeast extracts]. Endogenous NADH:cytochrome *c* reductase activity, non-related to NR, is low, as estimated by the cytochrome *c* reductase activity in the extracts of control *S. cerevisiae* transformant [about 5 nmol of cytochrome *c* reduced/min per mg of protein for $(\text{NH}_4)_2\text{SO}_4$ -precipitated yeast extracts]. The NADH:cytochrome *c* reductase activity observed in the NR transformant in the absence of induction (100 nmol/min per mg of protein) is presumably due to a leaky expression of the NR cDNA from the galactose-inducible promoter of the yeast expression vector.

Complementation of NR protein with MoCo

NADH:NR activity and the terminal NR activities, in contrast with the cytochrome *c* reductase activity, require a functional MoCo domain. The absence of NADH:NR activity and of the associated terminal activities could therefore be due to an inactive MoCo domain in the produced NR.

No MoCo could be detected in *S. cerevisiae* by the *nit1* assay (Table 2), which assesses the presence of MoCo by restoration of NR activity in extracts from the *Neurospora crassa* MoCo-

Table 2. *nit1* assays performed with heat-treated protein extracts from *H. wingei* and *S. cerevisiae* or with heat-treated milk xanthine oxidase

Reconstituted NADPH:NR activity is expressed in nmol of nitrite produced/min per mg of protein from MoCo extract.

Heat-treated extract used for complementation	<i>nit1</i> extract	NADPH:NR activity (nmol of nitrite/min per mg of protein)
<i>S. cerevisiae</i>	–	0
	+	0
<i>H. wingei</i>	–	0
	+	16
Milk xanthine oxidase	–	0
	+	2000

Table 3. Restoration of NADH:NR activity by incubation of crude yeast protein extracts with MoCo of milk xanthine oxidase

Yeast strain	MoCo	NADH:NR activity (nmol of nitrite/min per mg of protein)
<i>S. cerevisiae</i> control	–	0
	+	0
<i>S. cerevisiae</i> NR transformant	–	0
	+	0.3

Table 4. Complementation of NR produced in *S. cerevisiae* by extracts of the *nia* D51 mutant of *Nicotiana plumbaginifolia*

Mutant D51 is defective in NADH:cytochrome *c* reductase activity but still retains NR partial terminal activities. The *Nicotiana plumbaginifolia nia* mutant E23, which does not produce NR enzyme, serves as a negative control (Chérel *et al.*, 1990). –, No activity detected; +, activity detected.

Yeast strain	Extract used for complementation	NADH:NR activity
<i>S. cerevisiae</i> control	E23	–
	D51	–
<i>S. cerevisiae</i> NR transformant	E23	–
	D51	+

deficient *nit1* strain. As a positive control, MoCo could be detected in similarly prepared protein extracts from the nitrate-assimilating yeast species *H. wingei*, even when starting from 20-fold less proteins than for *S. cerevisiae*. So the absence of NR activity and of NR terminal activities of the produced tobacco protein could be due simply to the absence of MoCo in *S. cerevisiae*.

In order to check whether the lack of MoCo was an explanation for the absence of NR activity, complementation experiments were performed with yeast transformant protein extracts and MoCo from milk xanthine oxidase. Nitrate reductase activity was restored by exogenous MoCo in extracts of *S. cerevisiae* NR transformants (Table 3).

Complementation of *Nicotiana plumbaginifolia nia* mutant D51 extracts

Protein extracts from *Nicotiana plumbaginifolia nia* mutants (mutated in the gene coding for the NR apoenzyme) that are

Fig. 3. Western-blot analysis after native gradient PAGE with the monoclonal antibody ZM 96(9)25 (lanes 1–5) or the polyclonal antiserum directed against maize NR (lanes 6–11)

In all cases yeast cells were collected after induction by galactose. Lanes 5 and 6, AMP-Sepharose-purified NR of *Nicotiana plumbaginifolia*; lanes 1, 2, 8, 9 and 10, respectively 40 μ g, 20 μ g, 20 μ g, 5 μ g and 2 μ g of proteins from crude extracts of yeast NR transformant; lane 7, 40 μ g of proteins from crude extracts of yeast NR transformant mixed with AMP-Sepharose-purified NR of *Nicotiana plumbaginifolia*; lanes 3, 4 and 11, respectively 40 μ g, 40 μ g and 20 μ g of proteins from crude extracts of yeast transformed by pKV482.

affected in NR terminal activities can be complemented *in vitro* by protein extracts of *nia* mutants affected in cytochrome *c* reductase activity, resulting in restoration of NADH:NR activity (Pelsy & Gonneau, 1991). Experiments were performed to test whether, in the same way, NR produced in yeast could also be complemented by extracts from the *Nicotiana plumbaginifolia nia* mutant D51 (Chérel *et al.*, 1990) lacking the cytochrome *c* reductase activity, while retaining NR terminal activities. The data (Table 4) show that, as for plant *nia* mutant extracts, NADH:NR activity can be restored by complementation in extracts of yeast NR transformants. As a control, no complementation was observed when extracts of *Nicotiana plumbaginifolia nia* mutant E23 (which has no immunodetectable NR or any NR-associated activities *in vitro*; Chérel *et al.*, 1990) were used instead of those of mutant D51, or when control yeast extracts were used to complement extracts of mutant D51.

Native form of NR produced in *S. cerevisiae*

Crude protein extracts of *S. cerevisiae* NR transformants were subjected to electrophoresis on a native polyacrylamide-gradient gel. Western-blot analysis (Fig. 3) with the polyclonal serum or the monoclonal antibody ZM 96(9)25 directed against maize NR reveals that native NR produced in yeast migrates slightly more slowly than the AMP-Sepharose-purified native dimeric NR of *Nicotiana plumbaginifolia* (lanes 2 and 5 respectively). This difference in migration could be due to salt effects or protein concentration. Alternatively, absence of MoCo in yeast could affect the NR apoenzyme conformation, leading to a different migration in native polyacrylamide-gradient gels. Thus NR in *S. cerevisiae* seems to migrate as a dimeric protein in the absence of functional MoCo.

DISCUSSION

Expression of the cDNA of the *nia2* gene of *Nicotiana tabacum* in yeast results in an inducible production of an NR-related protein, as shown by e.l.i.s.a. and Western-blot analysis. The protein produced is deficient in NR activity but retains the

catalytic NADH:cytochrome *c* reductase activity of NR, thereby proving that in yeast the prosthetic groups FAD and cytochrome *b*-557 are correctly inserted into the NR apoprotein, and that the corresponding NR domains are correctly folded.

Amounts of protein produced

The concentrations of NR produced are low as determined by e.l.i.s.a. (0.05 % of total soluble proteins from yeast). In yeast the presence of a long leader (presumably 180 nucleotide residues) and non-optimal codon usage could prevent efficient translation of the NR mRNA. Alternatively, by analogy with NR of MoCo mutants (Marion-Poll *et al.*, 1991), NR produced in yeast may not be well recognized by the monoclonal antibody 96(9)25 because of the absence of functional MoCo, which would lead to an underestimation of the concentration of produced NR.

Restoration of NADH:NR activity

NR activity could be restored in transformant yeast extracts by complementation with exogenous MoCo from milk xanthine oxidase, but not with molybdate even at high concentrations (20–25 mM). With the *nit1* assay, no MoCo was detected in *S. cerevisiae* extracts, indicating that *S. cerevisiae* lacks MoCo, a result supported by experiments performed by K. V. Rajagopalan (personal communication). Thus, although MoCo is present in a wide variety of organisms ranging from sea anemone, *Drosophila*, plants and *E. coli* to man (Coughlan, 1980), it is absent from *S. cerevisiae*. Presumably, selection of *S. cerevisiae* strains for their fermentative potential has led to the elimination of one or more of the numerous genes involved in MoCo biosynthesis.

Absence of MoCo from *S. cerevisiae* and of restoration of NADH:NR activity by high concentrations of molybdate suggest that the NR produced in yeast is inactive because of the lack of functional MoCo. However, restoration of NADH:NR activity in yeast extracts by MoCo was very inefficient (about 0.3 nmol of nitrite produced/min per mg). The NADH:cytochrome *c* reductase activity of the yeast transformant extract (700 nmol of cytochrome *c* reduced/min per mg) would lead to an expected NADH:NR activity of approx. 200 nmol/min per mg if restoration were complete, since for NR the ratio of NADH:cytochrome *c* reductase activity to NADH:NR activity is roughly equal to 3 (Chérel *et al.*, 1990). So the restoration efficiency was 0.1 %. For a comparison, complementation experiments between plant *cnx* mutants (affected in MoCo synthesis) and MoCo derived from milk xanthine oxidase (Mendel & Müller, 1985; Narayanan *et al.*, 1984) gave levels of restoration ranging between 20 % and 80 % of wild-type NR activity. In those experiments, however, NR apoenzyme was part of a plant crude extract. A factor present in plant extracts intervening in MoCo activation or insertion into NR could explain the difference in complementation efficiency. In fact Mendel & Müller (1985) reported the possible existence of a highly labile plant factor necessary for complementation between a tobacco *cnx* mutant cell line extract and MoCo from milk xanthine oxidase. More generally, different forms of MoCo (molybdopterin, molybdopterin in covalent association with GMP; see Johnson *et al.*, 1990) have been described. It may be that the difficulty in restoring NADH:NR activity efficiently was due to the inadequacy of the structure of the heterologous heat-extracted MoCo when provided without an additional modifying factor. Alternatively, the absence, in yeast, of post-translational modification of the NR apoenzyme could impair NADH:NR activity even if MoCo is correctly inserted.

Dimeric form of NR produced in *S. cerevisiae*

The form of the native enzyme produced in yeast is predominantly dimeric, as shown by native PAGE. This ob-

servation was unexpected, since it is generally assumed that MoCo is required for NR dimerization in a variety of organisms (*Neurospora crassa*, *Aspergillus nidulans*, *Nicotiana tabacum*, *Nicotiana plumbaginifolia*), on the basis of sucrose-density-gradient analysis of NR activity or of cytochrome *c* reductase activity produced in wild-type and MoCo mutants (Nason *et al.*, 1970; Ketchum *et al.*, 1970; MacDonald *et al.*, 1974; Mendel *et al.*, 1986; De Vries *et al.*, 1986). The concentration of NR in yeast crude extracts was lower than that of control AMP-Sepharose-purified plant NR (as estimated by cytochrome *c* reductase activity), so the observed dimeric form is not just an artifact due to high concentration of NR subunits in yeast protein extracts.

It should be noted that reports on MoCo requirement for dimerization of NR are somewhat contradictory, as for *A. nidulans* and *Nicotiana plumbaginifolia* a dimeric form could be detected in some of the molybdenum-non-repairable MoCo *cnx* mutants (MacDonald *et al.*, 1974; De Vries *et al.*, 1986), in which case it was concluded that the MoCo was presumably affected in its reduction function but not in its dimerization function (De Vries *et al.*, 1986). One should note that the analysis of the molecular mass of xanthine dehydrogenase I (another dimeric enzyme that possesses MoCo) in different MoCo mutants of *A. nidulans* led to the conclusion that MoCo was not necessary for the dimerization of the xanthine dehydrogenase subunits, since both monomeric and dimeric forms could be observed in almost all of the *cnx* strains tested (Cove, 1979).

In the case of *S. cerevisiae*, we observe a dimeric form in the absence of functional MoCo. If all metabolic intermediates specific to MoCo are lacking in *S. cerevisiae*, the presence of dimeric NR in the absence of MoCo rules out the necessity of MoCo for dimerization of NR in yeast and probably in plants. This is supported by recent findings in our laboratory (Marion-Poll *et al.*, 1991), as a dimeric form of NR can be detected by immunoblotting in a wide range of *Nicotiana plumbaginifolia* *cnx* mutants that are not repaired by molybdenum.

Model for MoCo involvement in stabilizing plant NR

We favour the model in which MoCo in plants would not be necessary for NR dimerization, but would nevertheless stabilize NR protein and protect it from degradation by plant proteinases. *S. cerevisiae* would lack these specific proteinases, so no degradation of NR would occur, even in the absence of MoCo, leading to a dimeric native form of NR. In fact, proteolysis of NR has been described for barley, maize and spinach (Batt & Wallace, 1983; Hamano *et al.*, 1984; Brown *et al.*, 1981; Sueyoshi *et al.*, 1989). Interestingly, in some cases the proteolysed protein retained cytochrome *c* reductase activity, indicating that the MoCo domain was affected. Moreover, in the case of barley, proteolysis yielded a 40 kDa fragment retaining cytochrome *c* reductase activity and migrating as a monomer in a sucrose density gradient (Brown *et al.*, 1981). So the interpretation of the different forms of NR in *cnx* mutants must be viewed cautiously when the results are obtained solely from an analysis of the sedimentation coefficient of the cytochrome *c* reductase activity in a sucrose density gradient.

Although NR produced in yeast is partially active, *S. cerevisiae* can be helpful for assaying NADH:cytochrome *c* reductase activity of mutated tobacco NR proteins, as the FAD and haem domains are functional. This will thus contribute to NR

structure-function studies of these domains. For expressing a fully active NR, nitrate-assimilating yeasts or fungi may turn out to be interesting alternatives as heterologous hosts.

The expression of NR in yeast has enabled us to confirm the proposed model of intragenic complementation between mutated NR proposed by Pelsy & Gonneau (1991) and extend it to complementation between defective NR from *Nicotiana tabacum* and *Nicotiana plumbaginifolia*. We confirm also the possibility of dimerization of NR subunits in the absence of functional MoCo, and propose that MoCo is involved in stabilizing NR and not in catalysing its dimerization.

This work was in part supported by a grant from Elf-Biorecherches to H.-N.T. pKV482 was kindly provided by Delta Biotechnology. We thank F. Exinger and J. R. Kinghorn for giving respectively the *S. cerevisiae* and *H. wingei* strains, and V. Cellier for processing the pictures.

REFERENCES

- Batt, R. G. & Wallace, W. (1983) Biochim. Biophys. Acta **744**, 205–211
- Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
- Brown, J., Small, I. S. & Wray, J. L. (1981) Phytochemistry **20**, 389–398
- Chérel, I., Grosclaude, J. & Rouzé, P. (1985) Biochem. Biophys. Res. Commun. **129**, 686–693
- Chérel, I., Marion-Poll, A., Meyer, C. & Rouzé, P. (1986) Plant Physiol. **81**, 376–378
- Chérel, I., Gonneau, M., Meyer, C., Pelsy, F. & Caboche, M. (1990) Plant Physiol. **92**, 659–665
- Coughlan, M. P. (1980) in Molybdenum and Molybdenum-Containing Enzymes (Coughlan, M. P., ed.), pp. 121–185, Pergamon Press, Oxford
- Cove, D. J. (1979) Biol. Rev. Cambridge Philos. Soc. **54**, 291–327
- De Vries, S. E., Dirks, R., Mendel, R. R., Schaart, J. G. & Feenstra, W. J. (1986) Plant Sci. **44**, 105–110
- Hageman, R. V. & Rajagopalan, K. V. (1986) Methods Enzymol. **122**, 399–412
- Hamano, T., Oji, Y., Mitsuhashi, Y., Matsuki, Y. & Okamoto, S. (1984) Plant Cell Physiol. **25**, 1469–1475
- Hyde, G. E. & Campbell, W. H. (1990) Biochem. Biophys. Res. Commun. **168**, 1285–1291
- Ito, H., Fukuda, V., Murata, K. & Kimura, A. (1983) J. Bacteriol. **153**, 163–168
- Johnson, J. L., Bastian, N. R. & Rajagopalan, K. V. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3190–3194
- Ketchum, P. A., Cambier, H. Y., Frazier, W. A., III, Madansky, C. H. & Nason, A. (1970) Proc. Natl. Acad. Sci. U.S.A. **66**, 1016–1023
- Kubo, Y., Ogura, N. & Nakagawa, H. (1988) J. Biol. Chem. **263**, 19684–19689
- Laemmli, U. K. (1970) Nature (London) **227**, 680–685
- MacDonald, D. W., Cove, D. J. & Coddington, A. (1974) Mol. Gen. Genet. **128**, 187–199
- Marion-Poll, A., Chérel, I., Gonneau, M. & Leydecker, M.-T. (1991) Plant Sci., in the press
- Mendel, R. R. (1983) Phytochemistry **22**, 817–819
- Mendel, R. R., Müller, A. J. (1985) Planta **163**, 370–375
- Mendel, R. R., Zerekbai, A. A., Lvov, N. P. & Müller, A. J. (1981) Mol. Gen. Genet. **181**, 395–399
- Mendel, R. R., Marton, L. & Müller, A. J. (1986) Plant Sci. **43**, 125–129
- Moureaux, T., Leydecker, M.-T. & Meyer, C. (1989) Eur. J. Biochem. **179**, 617–620
- Narayanan, K. R., Müller, A. J., Kleinhofs, A. & Warner, R. L. (1984) Mol. Gen. Genet. **197**, 358–362
- Nason, A., Antoine, A. D., Ketchum, P. A., Frazier, W. A., III, & Lee, D. K. (1970) Proc. Natl. Acad. Sci. U.S.A. **65**, 137–144
- Pelsy, F. & Gonneau, M. (1991) Genetics **127**, 199–204
- Solomonson, L. P. & Barber, M. J. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**, 225–253
- Sueyoshi, K., Ogura, N. & Nakagawa, H. (1989) Agric. Biol. **53**, 151–156
- Vaucheret, H., Kronenberger, J., Rouzé, P. & Caboche, M. (1989) Plant Mol. Biol. **12**, 597–600